Anti-Cancer Mechanism of Trastuzumab via Blocking Nuclear HER2 Function and Epigenetic Mechanism of Resistance

by

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ABSTRACT

HER2 receptor tyrosine kinase (encoded by *ERBB2* gene) is overexpressed in approximately 25% of all breast cancer tumors (known as HER2-positive breast cancers). Overexpression of HER2 causes overactivation of downstream receptor tyrosine kinase pathways including PI3K/Akt and MAPK pathways and is a poor prognosis factor in breast cancer. Trastuzumab which is a humanized monoclonal antibody designed to target HER2 receptor is approved by FDA to treat patients with early-stage and metastatic HER2-positive breast cancer as an adjuvant in combination with other chemotherapy. However, approximately 60-70% of HER2-positive breast cancer patients develop de novo resistance to trastuzumab, partially due to the loss of HER2 expression on their tumor cells during the treatment. Little is known about the exact mode of action of trastuzumab in inhibiting HER2-positive breast cancer cells and the mechanism of trastuzumab resistance. The overall aim of this thesis was to study the mechanism of action of trastuzumab in inhibiting HER2-positive breast cancer and the mechanism of resistance to trastuzumab.

We found that HER2 overexpression in Chinese hamster ovary (CHO) cells had no major effect on the activation of downstream PI3K/Akt and MAPK pathways, however, significantly increased the cell growth. These suggest a non-canonical oncogenic function of HER2. Our results showed that trastuzumab blocks proteolytic cleavage of HER2, production and nuclear localization of a C-terminal truncated HER2 protein with an approximate molecular weight of 85 kDa (p85HER2). Trastuzumab showed a synergic effect with a proteinase inhibitor in blocking HER2 cleavage and production of p85HER2 that led to cell growth inhibition. This is a new molecular anti-cancer mechanism of trastuzumab. Immunoprecipitation of nuclear p85HER2 followed by mass spectrometry analysis showed that p85HER2 directly interacts with the spliceosome protein complex and transcription factors and mediates in RNA processing, splicing, and gene expression regulation. These results demonstrate that nuclear p85HER2 mediates in the regulation of RNA processing and gene expression. Gene set enrichment analysis of the mass spectrometry results also revealed that most of the nuclear p85HER2 client proteins are downstream targets for oncogenic/stemness transcription factors which are master regulators of breast cancer stemness and epithelial-mesenchymal transition (EMT). These results

demonstrate a novel mechanism of action of trastuzumab in blocking a non-canonical pathway of HER2 via nuclear function of p85HER2.

In this study, we also hypothesized that EMT abrogates HER2 expression by chromatin-based epigenetic silencing of *ERBB2* gene as a mechanism of development of trastuzumab resistance. we found positive and negative correlation of HER2 expression levels with epithelial and mesenchymal phenotypes respectively. This indicates that epithelial-like cells are HER2-high, while mesenchymal-like cells are HER2-low. We found that the correlation is due to active and inactive chromatin dynamics of *ERBB2* gene in epithelial-like and mesenchymal-like cells respectively. HER2-low mesenchymal-like breast cancer cell lines revealed less promoter-enhancer interaction and larger chromatin loops compared to the HER2-high epithelial-like breast cancer cell lines. Further, the cell line with higher expression levels of HER2 showed higher numbers of chromatin-chromatin interaction, super-enhancers and topologically associated domains (TADs) at the chromatin of *ERBB2* gene and flanking regions. The lower HER2 expression, the higher EMT phenotype, and inactivated chromatin all were found correlated with a lower response to lapatinib. We also demonstrated that inducing EMT of HER2-positive cancer cells results in the downregulation of HER2 expression and lower binding rate of trastuzumab. These results show that the downregulation of HER2 expression in mesenchymal-like cells derived from HER2-positive breast cancer cell lines is due to *ERBB2* gene silencing by global epigenetic reprogramming during EMT.

We strongly suggest further studying the oncogenic function of p85HER2 through regulation of coding and non-coding RNA processing as well as transcription co-factor function of p85HER2 in breast cancers. We also suggest testing proteinase inhibitors in combination with trastuzumab and lapatinib to prevent the non-canonical pathways of HER2 and development of de novo trastuzumab resistance in HER2-positive breast cancers. We here strongly recommend developing and testing HER2-targeting small molecules inhibitors to inhibit HER2 cleavage as an alternative therapy for trastuzumab to use in combination with lapatinib. Further, we propose targeting EMT and cancer stem cells as an effective approach to inhibit tumor growth and overcome drug resistance in breast cancer.

PREFACE

This thesis is an original works carried out primarily by Babak Nami Mollalou under supervision and at the laboratory of Dr. Zhixiang Wang of the Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta.

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Chapter 1 contains some parts of two independent review articles published in the journal "Cancers" (2017 Apr 26;9(5), and 2018 Sep 20;10(10)).

Chapter 3 contains original work carried out equally by Hamid Maadi and Babak Nami Molallou (co-first authors), published in the journal "BMC Cancer" (2018 Mar 1;18(1):238))

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Chapter 5 contains unpublished original work carried out primarily by Babak Nami Mollalou.

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Babak Nami Mollalou
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LIST OF ABBREVIATIONS

Symbol

°C:	Degree Celsius
µg:	Microgram
µl:	Microlitre
µm:	Micrometer
µM:	Micromolar

Number

2D	Two-dimension
3D	Tree-dimension
3DIV	3D-Genome Interaction Viewer
4D	Four-dimension

A

A	Adenine
Abl	Abelson murine leukemia
ACN	Acetonitrile
ActRII	Activin type II receptor
ActRIIB	Activin type II receptor B
ADAM	A disintegrin and metalloproteinase
ADCC	Antibody-dependent cellular cytotoxicity
Akt	Protein kinase B
ALDH	Aldehyde dehydrogenases
ALK	Activing receptor-like kinases
AMHR	Anti-Müllerian hormone receptor
ANOVA	Analysis of variance
AP1	Activator protein 1
APC	Adenomatosis polyposis coli
APE1	Apurinic/apyrimidinic endonuclease 1
AR	Amphiregulin
ATAC-seq	Assay for transposase-accessible chromatin with high throughput sequencing
ATCC	American Type Culture Collection
ATF1	Activating transcription factor 1
ATP	Adenosine triphosphate

B

BAD	Bcl-2-associated agonist of cell death

Bcl	B-cell lymphoma
BCLAF1	Bcl-2-associated transcription factor 1
BCSC	Breast cancer stem cell
BET	Bromodomain and extra terminal domain
BMPRII	BMP type II receptor
bp	Base-pair
BRD4	Bromodomain-containing protein 4
BS^3	Bissulfosuccinimidyl suberate
BSA	Bovine serum albumin
BTC	Betacellulin

C

C	Cytosine
CA	California
cat#	Catalog number
CBP	CREB-binding protein
CD	Cluster of differentiation
Cdc42	Cell division control protein 42 homolog
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CDX2	Caudal type homeobox protein 2
CEB	Cytosol extraction buffer
CHD7	Chromodomain-helicase-DNA-binding protein 7
ChEA	ChIP enrichment analysis
ChIA-PET	Analysis by paired-end tag sequencing
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovary
CK1α	Casein kinase 1 alpha
cm	Centimetre
CO	Colorado
COX2	Cyclooxygenase 2
CP	CP-724714
CpG	Cytosine-pair-guanine
cRNA	Complementary RNA
CSL	CBF1, Suppressor of Hairless, Lag1
CTCF	CCCTC-binding factor
ctHER2	C-terminal truncated HER2
CXCR4	C-X-C motif receptor 4

D

Da	Dalton
DAB2	Disabled homolog 2
DAPI	4',6-Diamidino-2-Phenylindole
DCIS	Ductal carcinoma in situ
DEPC	Diethyl pyrocarbonate
DFS	Disease-free survival
dH_2O	Distilled H_2O
Dhh	Desert Hedgehog
Dkk1	Dickkopf Wnt signaling pathway inhibitor 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DSL	Delta, Serrate, and Lag2
DTT	Dithiothreitol

E

E2F1	E2F transcription factor 1
ECD	Extracellular domain
ecHER2	Extracellular part of HER2
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EGR1	Early growth response 1
EJM	Extracellular juxtamembrane
ELF2	E74-like ETS transcription factor 2
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-mesenchymal transition
ENCODE	Encyclopedia of DNA Elements
EpCAM	Epithelial cell adhesion molecule
EPG	Epigen
EPR	Epiregulin
ER	Estrogen receptor
ErbB	Erythroblastic oncogene B
Erk	Extracellular signal-regulated kinase
eRNA	Enhancer RNA

ESC Embryonic stem cells
Est1 Ever shorter telomere 1
ETS1 V-Ets avian erythroblastosis virus E2 oncogene homolog 1
ETV1 ETS variant transcription factor 1

F

FACS Fluorescence activated cell sorting
FBS Fetal bovine serum
FGF Fibroblast growth factor
FITC Fluorescein isothiocyanate
FKBP FK506 binding protein
FL Full-length
FOS FBJ murine osteosarcoma viral (V-Fos) oncogene homolog
FOXA1 Forkhead box A1
FOXC1 Forkhead box protein C1
FOXM1 Forkhead box M1
FOXO1 Forkhead box O1
FOXP1 Forkhead box P1
FUS Fused in sarcoma
Fz Frizzled

G

g Gravity
G Guanine
GATA1 Globin transcription factor 1
GEO Gene Expression Omnibus
GFP Green fluorescence protein
Gli Glioma-associated oncogene
GPCR G protein-coupled receptors
Grb2 Growth factor receptor-bound protein 2
GSC Goosecoid
GSEA Gene list enrichment analysis
GSI Gamma-secretase inhibitor
GSK3 Glycogen synthase kinase 3
GTP Guanosine triphosphate

H

H2BK120ub Histone 3B lysine 120 ubiquitination
H3K27ac Histone 3 lysine 27 acetylation
H3K27me3 Histone 3 lysine 27 trimethylation

H3K39me3 Histone 3 lysine 39 trimethylation
H3K4me1 Histone 3 lysine 4 monomethylation
H3K4me2 Histone 3 lysine 4 dimethylation
H3K4me3 Histone 3 lysine 4 trimethylation
H3K79me2 Histone 3 lysine 79 dimethylation
H3K9ac Histone 3 lysine 9 acetylation
H3K9me Histone 3 lysine 9 methylation
H4K8ac Histone 3 lysine 8 acetylation
HA Hyaluronic acid
HBS HEPES-buffered saline
HER Human epidermal growth factor receptor
Hh Hedgehog
HiC High-throughput sequencing of chromosome conformation capture
His Histidine
HMGA2 High-mobility group A protein 2
hnRNPE1 Heterogeneous nuclear ribonucleoprotein E1
HOXC9 Homeobox C9
HPLC High Performance Liquid Chromatography
HRG Heregulin
HRP Horseradish peroxidase
HSP90 Heat shock protein 90

I

IC Intracellular
icHER2 Intracellular part of HER2
Id Inhibitor of differentiation
IGF1 Insulin-like growth factor 1
IgG Immunoglobulin G
IHC Immunohistochemistry
FDA Food and Drug Administration
Ihh Indian Hedgehog
IJM Intracellular juxtamembrane
IL Interleukin
IM-PET Integrated methods for predicting enhancer targets
IP Immunopercipitation

J

JAK Janus kinase
JNK c-JUN N-terminal kinase
JUNB JunB proto-oncogene

JUND	JunD proto-oncogene

K

kbp	Kilobase-pair
kDa	Kilodalton
KDM5A	Lysine demethylase 5A
KEGG	Kyoto Encyclopedia of Genes and Genomes
KHDRBS1	KH RNA binding domain containing, signal transduction associated 1
KLF9	Kruppel-like factor 9

L

L	Litre
LB	Lysogeny broth
LEF/TCF	T-cell factor/lymphoid enhancing factor
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LIMA1	LIM domain and actin binding 1
Lin	Lineage
LOX	Lysyl oxidase
LRP	Lipoprotein receptor-related protein
Lsd1	Lysine-specific demethylase 1A

M

M	Molar
MA	Massachusetts
MAPK	Mitogen-activated protein kinase
MAX	MYC associated factor X
MAZ	MYC associated zinc finger protein
MBD3	Methyl-CpG binding domain protein 3
MEB	Membrane extraction buffer
MED	Mediator
Mek	MAPK/ERK kinase
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
Mib1	Mindbomb1
miR	MicroRNA
ml	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
MMR	DNA mismatch repair
MN	Minnesota
MO	Missouri

mRNA	Messenger RNA
MSFE	Mammosphere-formation efficiency
MSH2	MutS homolog 2
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex C1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
MXI1	MAX interactor 1
MYBBP1A	MYB binding protein 1a
MZF1	Myeloid zinc finger 1

N

NBCn1	Sodium bicarbonate cotransporter 3
NCBI	National Center for Biotechnology Information
NE	Nebraska
NEB	Nuclear extraction buffer
NF-kB	Nuclear factor-kappa B
ng	Nanogram
NGR	Neuroligin
NGS	Next-generation sequencing
NICD	Notch intracellular domain
Nipbl	Nipped-B-like protein
NK	Natural killer
nl	Nanolitre
NLS	Nuclear localization signal
NOD	Non-obese diabetic
NRF2	Nuclear factor erythroid 2-related factor 2
NuRD	Nucleosome remodeling deacetylase

O

Oct4	Octamer-binding transcription factor 4
ON	Ontario
ORF	Open reading frame
OS	Overall survival
OSM	Oncostatin M

P

P	Pertuzumab
P	Probability
p100HER2	100 kDa HER2
p185HER2	185 kDa HER2

p27	27 kDa cyclin-dependent kinase inhibitor
p300	300 kDa histone acetyltransferase
p38	38 kDa MAP Kinase
p53	53 kDa tumor protein
p85HER2	85 kDa HER2
p95HER2	95 kDa HER2
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDGF	Platelet-derived growth factor
PDK1	Pyruvate dehydrogenase kinase 1
PH	Pleckstrin homology
pH	Power of hydrogen
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKCα	Protein kinase C alpha
PLC-γ	Phosphoinositide phospholipase C gamma
Pol I	RNA polymerase I
Pol II	RNA polymerase II
PP2A	Protein phosphatase 2A
ppm	Parts per million
PR	Progestin receptor
Ptch1	Patched1
PTEN	Phosphatase and tensin homolog
Ptp-2c	protein-tyrosine phosphatase 2C
pY	Phospho-tyrosine

R

R-Smads	Receptor-activated Smads
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RE	Restriction enzyme
RhoA	Ras homolog family member A
RNA-seq	RNA sequencing
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RSEM	RNA-seq by expectation-maximization
RTK	Receptor tyrosine kinase

RUNX3 Runt-related transcription factor 3

S

S Serine
SCID Severe combined immunodeficiency
SD Standard deviation
SDF1α Stromal cell-derived factor 1 alpha
SDS Sodium dodecyl sulfate
sFRP1 Secreted frizzled related protein 1
SHC1 Src homology 2 domain-containing-transforming protein C1
SHP1 Src homology region 2 domain-containing phosphatase 1
shRNA Short hairpin RNA
SIF-seq Site-specific integration fluorescence-activated cell sorting followed by sequencing
SIRT2 Sirtuin 2
Smad Mothers against decapentaplegic
Smo Smoothened
SN Supernatant
SND1 Staphylococcal nuclease domain-containing protein 1
Sox2 SRY-related HMG-box 2
SP1 Specificity protein 1
Src Sarcoma
SRP Signal recognition particle
SS Sense-strand
STAT Signal transducer and activator of transcription
SV40 Simian vacuolating virus 40
SWI/SNF SWItch/sucrose non-fermentable

T

T (DNA) Threonine
T (Protein) Thymine
T (Drug) Trastuzumab
TAC Transcriptome Analysis Console
TAD Topologically associating domain
TAE Tris base-acetic acid-EDTA
TAF15 TATA-box binding protein associated factor 15
TBS Tris-buffered saline
TBST Tris-buffered saline-Tween®
TCF3 Transcription factor 3
TCR T cell receptor

TEAD6	TEA domain transcription factor 6
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta
THOC2	THO complex 2
THRAP3	Thyroid hormone receptor associated protein 3
TK	Tyrosine kinase
TM	Transmembrane
TN	Tennessee
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TRITC	Tetramethylrhodamine
TRPM7	Transient receptor potential melastatin-like 7
TSS	Transcription start site
TX	Texas
TβRII	TGF-β type II receptor

U

U (Volume)	Unit
U (RNA base)	Uracil
UDG	Uracil DNA glycosylase
USA	United States of America
UT	Untreated
UTR	Untranslated region
UV	Ultraviolet

V

V	Volt
VA	Virginia
VR	Vinorelbine

W

WI	Wisconsin

Y

Y	Tyrosine
YY1	Yin And Yang 1 Protein

Z

ZBTB7A	Zinc finger and BTB domain containing 7A
ZEB1	Zinc finger E-box-binding homeobox protein 1
ZF	Zinc finger

Chapter 1. General introduction

1.1. HER2 RECEPTOR-MEDIATED SIGNALING

The HER (ErbB) receptor tyrosine kinase (RTK) family consists of four cell surface receptors: EGFR (HER1/ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Each human epidermal growth factor receptor (HER) possesses an extracellular domain that binds ligands, a transmembrane region, and an intracellular protein kinase domain with tyrosine kinase activity [1]. The extracellular domain is subdivided into four further domains including domains I and III the ligand-binding site; domains II and IV the dimerization site. Ligand binding promotes conformational rearrangements of the receptors that trigger the association of both homodimers and heterodimers (Figure 1.1). HER receptor dimerization leads to the activation of a bunch of cellular signalling like PI3K/Akt, MAPK, and many other pathways to regulate cell division, proliferation, survival, migration, differentiation, apoptosis, and cell motility [1] (Figure 1.1). Like other RTKs, HER receptors are single transmembrane proteins that have an N-terminal extracellular domain, a transmembrane helix, and a cytoplasmic domain [2]. The extracellular domain contains four subdomains, including the ligand-binding subdomains (domains I and III), and receptor dimerization subdomains (domains II and IV). The intracellular domain is composed of a tyrosine kinase domain and a C-terminal regulatory domain [3].

EGFR (epidermal growth factor receptor), a 170 kDa single polypeptide chain, is the prototype of the HER family receptor [4,5]. While EGFR and HER4 are fully functional RTKs capable of signaling both as homo- and heterodimers following the binding to various ligands, the other two members, including HER2 and HER3, are different. HER2 is an orphan receptor without a ligand and HER3 is a lack of kinase activity. However, through ligand-induced heterodimerization, all HER receptors could be fully activated to mediate cell signaling [6,7].

Besides EGF (epidermal growth factor), another ten ligands have been identified to bind to and stimulate HER receptors. These ligands form the EGF family of peptide growth factors and are subdivided into three groups based on their binding partners (Figure 1.1). EGF, epigen

(EPG), amphiregulin (AR), and transforming growth factor (TGF) form one group that specifically binds to EGFR. HB-EGF, epiregulin (EPR), and betacellulin (BTC) form the second group that neuregulins to both EGFR and HER4. The four neuregulin, including Neuroligin 1 (NRG1), NRG2, NRG3, and NRG4, form the third group that binds to HER4. However, NRG1 and NRG2 also bind to HER3 (Figure 1.1) [7,8]. Through the distinctive binding specificity and affinity, each ligand contributes in a unique manner to regulate the activation and signaling of the four HER receptors [6].

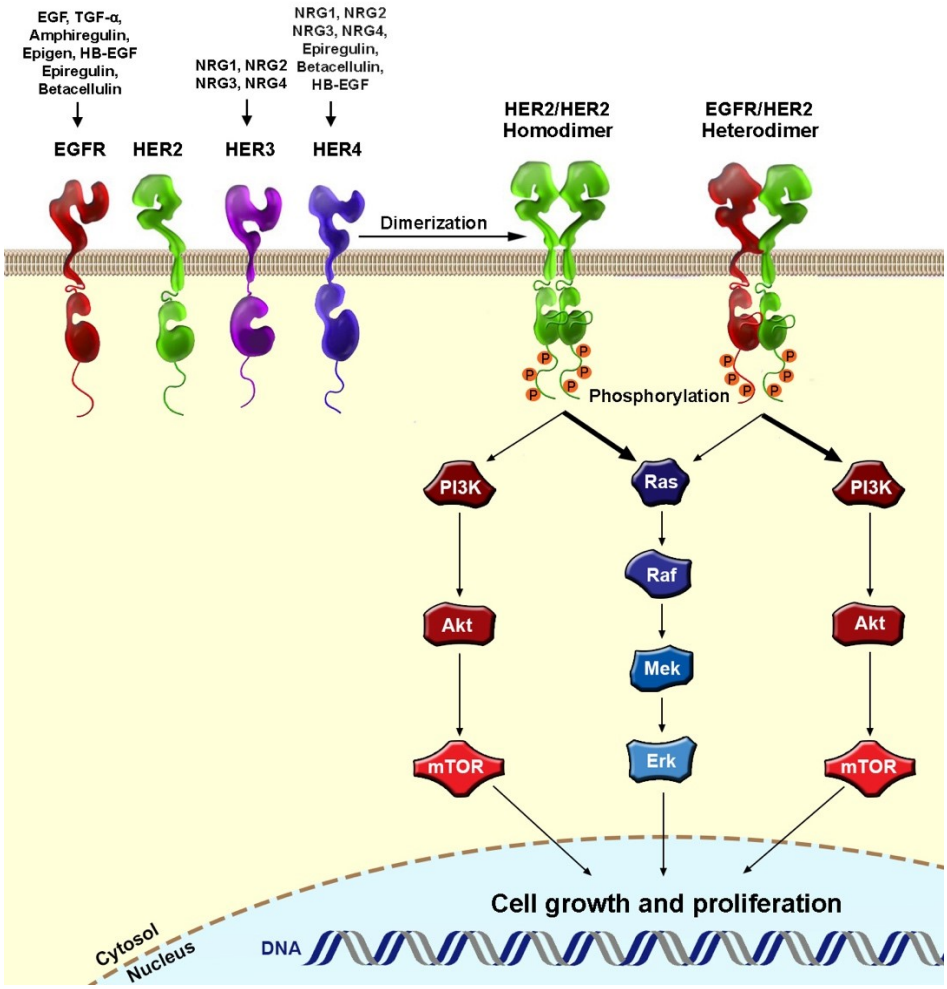

Figure 1.1. HER receptors and downstream signaling cascades. Four members of HER receptors (EGFR, HER2, HER3, and HER4) interact with 11 ligands which results in the formation of HER receptor homodimers (as for example HER2/HER2 indicated) and heterodimers (as for example EGFR/HER2 indicated). After dimerization, the kinase domain of each receptor phosphorylates and activates the couple receptor which results in the

activation of PI3K/Akt (mainly by heterodimers) and MAPK (mainly by homodimers) signaling pathways leading to cell growth and proliferation. Some graphic elements are adapted from [9].

Our understanding of HER receptor dimerization has been greatly enhanced due to the determination of the structures of the HER receptor extracellular domains. So far, the structures of all HER receptors without ligand have been determined. In addition, the structures of ligand-bound EGFR and HER4 have also been determined. Many structures of HER receptors binding to antibodies or antibody mimics have also been revealed [2,10]. With the support of other evidence, a comprehensive picture regarding ligand-receptor interaction and HER receptor dimerization has emerged. In total, ten different homo- and heterodimers are formed by four HER receptors [11].

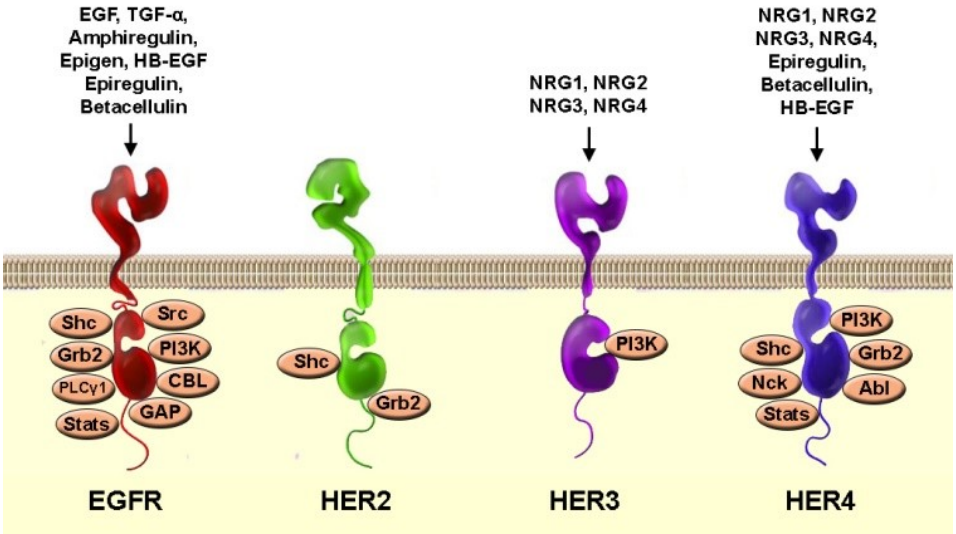

Figure 1.2. HER receptor ligands and some downstream client proteins. Figure is adapted from [9].

Structure studies indicate that the conformations of the receptors can only exist in two forms: a tethered form and an extended form. In the tethered form the receptor is unable to dimerize due to the buried dimerization element. However, in the extended form the dimerization elements of the receptor are fully exposed to allow the receptor dimerization. It has been

demonstrated that the rigid nature of the receptor extracellular domains restricted or "clicked" the receptors only to these two forms [2,12].

It is significant and interesting to find that HER2 extracellular domains are already in extended form in the absence of ligands. The subdomains I and III of HER2 extracellular domain interact directly to stabilize HER2 to the extended form. The close interaction between subdomain I and III leaves no space for a ligand in between, thus, HER2 is an orphan receptor by nature [13]. Thus, HER2 maintains a ligand-independent and constitutively activated conformation. Indeed, HER2 spontaneously forms homodimers when overexpressed in cells, and all the other HER receptors dimerize preferably with HER2 [8,11]. Moreover, the overexpression of HER2 (but not the other HER receptors) transforms cells, and HER2 overexpression is associated with poor prognosis in breast cancer [13]. On the other hand, HER3 homodimer is generally believed as non-functional due to a lack of kinase activity. However, HER3 possesses very low kinase activity (1/1000th kinase activity of EGFR) and thus it is still possible that HER3 homodimers may be functional [8].

Through homo- or heterodimerization, all HER receptors are activated, which induces the phosphorylation of multiple tyrosine residues in the C-terminal regulatory region. Various studies including large-scale phosphoproteomic screening have identified more than 100 proteins that potentially bind to HER receptors (Figure 1.2) [14–16]. Several interesting features are revealed through the mapping of these tyrosine phosphorylation residues. Both EGFR and HER4 bind to many different downstream proteins. EGFR binds to Grb2, Shc, Src, PLC-γ1, Crk, STAT5, Ptp-2c, and SHP1. HER4 binds to Syk, RasA1, Abl, Crk and Vav2, and Grb2. However, the signaling pathways linked to HER2 and HER3 are very specific and limited. HER3 contains multiple phosphor tyrosine residues that bind to p85, and as such, HER3 strongly activates the PI3K/Akt pathway. On the other hand, HER2 is mostly engaged in Shc/Grb2-mediated activation of MAPK pathway (Ras/Raf/Mek/Erk). Due to the distinctive binding to various downstream signaling proteins, the heterodimerization of HER receptors allows the activation of more signaling cascades than the homodimer of HER receptors. Based on the binding specificity, it is likely that the HER2 homodimer will stimulate the activation of the Ras/Raf/Mek/Erk pathway, and the HER2-EGFR heterodimer may function similarly to EGFR homodimer. However, the HER2-HER3 heterodimer could be much more powerful than either the HER2 homodimer or

HER3 homodimer because the HER2-HER3 heterodimer could fully activate all available HER2 and HER3 receptors, and the HER2-HER3 heterodimer could strongly activate the PI3K/Akt pathway in addition to the Ras/Raf/Mek/Erk pathway. Indeed, much research has linked the PI3K/Akt pathway to HER2-HER3 signaling and to HER2-positive breast cancer [14–16].

PI3K could be activated by HER receptors either directly through the interaction between its p85α subunit and HER receptor or indirectly through the activated Ras [17]. A negative regulator of PI3K is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN). The function of PI3K in cell survival is mediated by Akt, a serine-threonine (S-T) kinase [18,19]. Akt contains an N-terminal Pleckstrin homology (PH) domain, a C-terminal regulatory domain, and a central kinase domain. Akt is recruited to the plasma membrane by the interaction of its SH3 domain with PIP3 (generated by PI3K), which induces the conformational change of Akt to allow the phosphorylation of its T308 by membrane-localized 3-phosphoinositide-dependent kinase 1 (PDK1). Following the additional phosphorylation of S473 by mammalian target of rapamycin complex 2 (mTORC2), Akt is fully activated. Akt controls various cellular functions by phosphorylating several intracellular proteins, including the glycogen synthase kinase 3 (GSK3), the Bcl-2-associated agonist of cell death (BAD), and forkhead box O (FOXO) transcription factors. Akt also activates mTORC1, which protects the cell from undergoing apoptosis [18–20].

1.2. BREAST CANCER

Breast cancer is the most common and deadliest cancer type in women worldwide. It affects about 12% of women causes over half a million deaths each year around the world. Breast cancer arises from the epithelial compartment of the breast that consists of epithelial cells lining lobules and ducts of mammary glands. A breast tumor is a complex tissue containing cancerous cells, and various other cell types with different morphological and phenotypic characteristics, including genetics, epigenetics, gene expression, metabolism, motility and “stemness” properties.

Among all subtypes of breast cancers two are more common; invasive ductal carcinoma that starts in a milk duct of the breast and invasive lobular carcinoma that starts in the lobules. These subtypes are able to undergo metastasis. Breast cancer cells are also classified into several

conceptual subtypes based on three individual hormone receptors including estrogen receptor (ER), progestin receptor (PR), and HER2. These classes include (i) basal-like tumors that are often called triple-negative (ER-, PR- and HER2-) breast cancers (TNBC), (ii) luminal A and B breast cancers that are ER+, (iii) HER2-positive breast cancers that express very high HER2 receptors and are responsive to HER2 targeting adjuvant therapies and (iv) claudin-low breast cancers that are often triple-negative showing weak cell-cell adhesion [21]. Triple-negative type of tumors is associated with a poor patient prognosis because of the lack of the triple receptors as exquisite targets for therapeutic adjuvants.

1.3. HER2 IN BREAST CANCER

Breast cancers are classified as five intrinsic subtypes based on their gene expression profiles revealed by microarray: luminal-like subtypes A and B (expression of hormone receptors and luminal cytokeratins 8 and 18), basal-like (also called triple-negative breast cancer (TNBC), typically with no expression of estrogen receptors (ER), progestin receptors, and HER2), HER2-positive (HER2+), and normal-like [22,23]. HER receptors have been implicated in the development of many types of human cancers, especially breast cancer. Overactivation of HER receptors is mostly due to overexpression driven by gene amplification, but also could be due to the truncation of the extracellular domain, a mutation in the kinase domain, or co-expression of HER receptor ligands. The overactivation of HER receptors drives cancer development [7,8,24].

Overexpression of EGFR is observed in 20–30% of breast carcinoma. While a high percentage of HER2-positive breast cancer cells also overexpress EGFR, approximately 50% of TNBC cells overexpress EGFR. Overexpression of EGFR has been frequently associated with large tumor size and poor clinical outcomes [8,15,25,26].

Approximately 20-30% of all diagnosed breast cancers are characterized as “HER2-positive breast cancer” [27–29] (Figure 1.3). HER2 genetic mutations are observed in approximately 1.6% of breast cancer patients [30]. Full-length HER2 is a 1255 amino acid, 185 kDa transmembrane epidermal growth factor receptor tyrosine kinase protein encoded by *ERBB2* gene which is located on chromosomal location 17q2 [27,31]. HER2 receptor consists four

extracellular domains (ECD; domains I-IV; amino acids 1-641), an extracellular juxtamembrane region (EJM; amino acids 642-652) a transmembrane domain (TM; amino acids 653-675), an intracellular juxtamembrane region (IJM; amino acids 676-730), an intracellular tyrosine kinase domain (TK; amino acids 731-906), and a C-terminal tail (amino acids 907-1255) [32–35]. The ECD of HER2, by turn, consists of four subdomains including two leucine-rich domains (domains I and III), which are responsible for ligand binding, and two cysteine-rich domains (domains II and IV) with disulfide bridges, which are responsible for receptor dimerization [32–35]. HER2 does not need ligand to be activated and can make dimer with another HER2 (homo-dimerization) or other HER family receptors (HER1/EGFR and HER3; hetero-dimerization). HER2 dimerization activates HER2 kinase domain that, in turn, promotes downstream signaling cascades specially PI3K/Akt, PLC-ɣ and MAPK pathways (Figure 1.1). Upregulation of these signaling pathways in breast cancer induces tumor cell growth, survival, motility, and invasion [11]. Therefore, HER2 overexpression is a poor prognostic factor in breast cancer. HER2 positive breast cancers either make too many copies of HER2 gene (gene amplification) or too many HER2 receptors on the cell surface (protein overexpression), therefore, grow faster and spread more aggressively when compared to HER2-negative breast tumors [27–29,36].

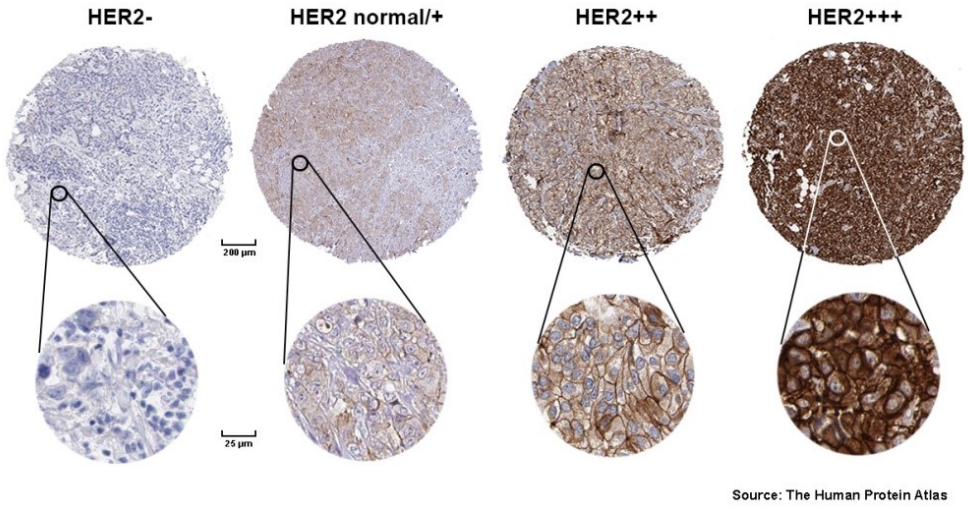

Figure 1.3. Immunohistochemistry (IHC) staining of HER2 (brown color) in a HER2-negative (HER2-), a HER2-normal/+, and two HER2-positive (HER2++ and HER2+++) breast tumors. Image source: The Human Protein Atlas.

1.4. TRASTUZUMAB AND PERTUZUMAB AS HER2-TARGETING THERAPIES

In recent years, HER2 has been an important biomarker and target of therapy for breast cancer. The main goal of the HER2-targeting approach is to inhibit HER2 receptor activation in order to block the HER2-dependent signaling and consequently tumor cell suppression [37,38]. Since the 1980s so far, scientists have published data linking the over-activity of HER2 with the aggressive and metastatic form of breast cancer. In the past 25 years, HER2 has been exploited as a promising target to arrest breast cancers for several reasons. (i) HER2 level is in a correlation directly with the breast cancer invasion and prognosis [36,39]. (ii) HER2 is a receptor tyrosine kinase with high potency to activate downstream signaling pathways involving tumor growth, in particular, PI3K/Akt and MAPK pathways [1,40]. (iii) HER2-positive tumors exhibit much more HER2 receptor on the surface that serves a remarkable hallmark useful to differentiate from normal cells in pathological characterizing [39,41]. (iv) The extracellular domain of HER2 provides very stable epitopes and putative targets to design and test tumor cell-targeting neoadjuvant [37,42].

1.4.1. Trastuzumab

Trastuzumab (originally known as 4D5 and commercially known as Herceptin®) is an anti-HER2 fully-humanized monoclonal antibody approved by Food and Drug Administration (FDA) for the treatment of HER2-positive breast cancer [43,44]. Trastuzumab binds to HER2 domain IV and is thought to block binding pocket for receptor homo-dimerization, thereby blocking HER2 homo-dimerization, phosphorylation and consequently inhibition of downstream signaling pathways [43,44]. It is believed that; (i) binding of trastuzumab to HER2 suppresses MAPK and PI3K/Akt pathways by inhibition of HER2 activation [45]. In this model, trastuzumab binding to HER2 may prevent tyrosine kinase Sarcoma (Src) signaling and upregulates PTEN activity [46,47]. This inhibition also leads to suppression of PI3K/Akt signaling, activation of p27 and suppressing cyclin-dependent kinase 2 (CDK2) thus arresting cell cycle and growth in breast cancer cells [48–50]. (ii) Trastuzumab causes endocytosis and degradation of HER2 through promoting the activity of tyrosine kinases [51]. (iii) Preclinical and clinical studies revealed that

coating HER2 over-expressed tumor cells by trastuzumab is summons more immune cells especially natural killer cells to attack the tumor by antibody-dependent cellular cytotoxicity (ADCC) mechanism [52,53] (Figure 1.4). Many clinical trial studies have demonstrated the effectiveness of trastuzumab in combination with docetaxel in HER2-positive metastatic breast cancers [54–57]. However, reports of studies on the molecular mechanism of trastuzumab in HER2-positive breast cancer have been conflicting, accordingly, the exact mode of action and resistance still remains ambiguous. Clinical trials have shown that trastuzumab ameliorates disease-free survival (DFS) and overall survival (OS) in early-stage breast cancers [57–59] and also in metastatic breast cancers [54–56,60–62]. Unfortunately, only 30-40% of all HER2-positive breast cancer patients respond to trastuzumab. This figure is more significant in the case of metastatic cancers. Approximately 66–88% of patients with metastatic breast cancer develop de novo (primary) resistance against trastuzumab therapy. The response rate for treatment with trastuzumab alone is only 10-30% [61,63–66]. However, the exact mechanisms underlying resistance to trastuzumab remain obscure.

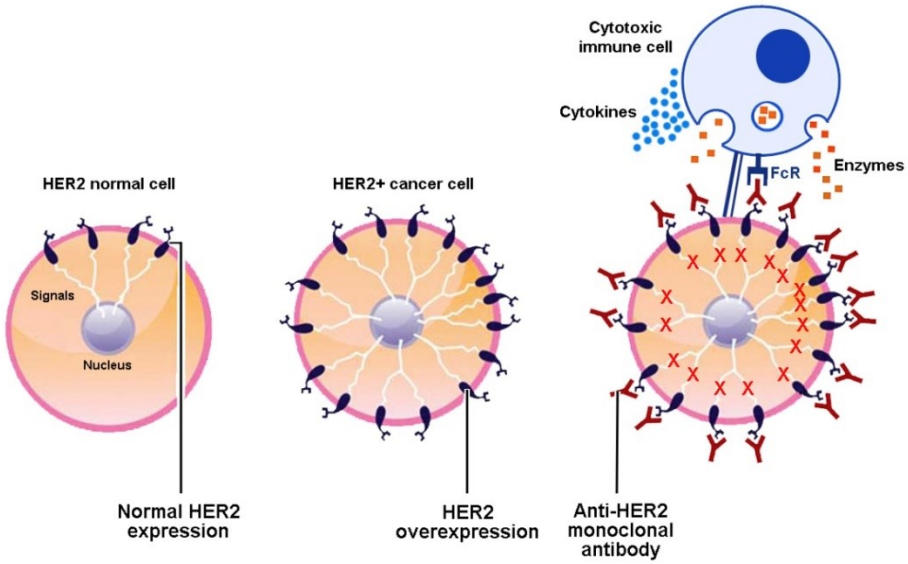

Figure 1.4. Overview of the mode of action of anti-HER2 monoclonal antibodies (trastuzumab and pertuzumab) in blocking HER2-positive breast tumors according to so far literature. Some graphic elements were adapted from www.drbhnglatest.com.

1.4.2. Pertuzumab

Pertuzumab (originally known as 2C4 and commercially known as Perjeta®) is another neoadjuvant which was recently approved by FDA, marketed by Genentech and Roche to be used in combination with trastuzumab and docetaxel to treat HER2-positive breast cancer due to a better outcome than the combined treatment with trastuzumab and docetaxel alone [44,67,68]. Pertuzumab, a fully humanized recombinant monoclonal antibody, represents a new class of agents that target HER2 dimerization. Pertuzumab binds to HER2 near the center of domain II, sterically blocking a binding pocket necessary for receptor dimerization, thereby blocking both the homodimerization of HER2 and the heterodimerization of HER2 with other HER receptors [69,70]. Since trastuzumab is only able to block the homodimerization of HER2 there is an upregulation of other members of the HER family in order to compensate for the lack of HER2 ligand-independent signaling [71]. This mechanism generally causes the development of resistance against the action of trastuzumab in the majority of patients. Pertuzumab binds to the dimerization domain of HER2; therefore, blocking the ability of HER2 to heterodimerize with other HER receptors (EGFR and HER3) and initiate HER2 ligand-dependent signaling. It is generally believed that the inhibition of dimerization will lead to the inhibition of HER2-mediated signaling [69,70]. Many clinical trial studies such as CLEOPATRA revealed prognosis in the patients who were treated with trastuzumab and pertuzumab combination [67,72–74]. Another major mechanism of pertuzumab is provoking the patient's immunity against the tumor. Previous studies have demonstrated immune system-mediated discrimination of HER2-positive cells when trastuzumab and pertuzumab bind to the cell providing evidence for the ADCC effects of pertuzumab [75–77].

1.5. BREAST CANCER STEMNESS

A normal mammary tissue contains mammary stem cells that can self-renew and can differentiate into luminal and basal epithelial cell layers including ductal, alveolar and myoepithelial cells of the mammary gland. Like basal-like and claudin-low subtypes breast cancer, normal mammary stem cells are defined as triple-negative, active stemness signaling

such as Notch and Wnt/β-catenin pathways and also a high expression of the epithelial-mesenchymal transition (EMT) elements warranting that the mammary stem cells may be the origin of normal and cancerous breast cells [78,79]. Therefore, transforming normal breast stem cells to breast cancer stem cells (BCSCs) with the properties of stem and cancer cells is a major factor in breast tumorigenesis. BCSCs synchronously are able to self-renew and differentiate to epithelial cancer cells with various gene regulatory networks. BCSCs definition emerged shortly after the discovery of only a small fraction of mammary tumor cells being able to form colonies or new tumors. In 2003, All-Hajj et al. [80] reported a small fraction of mammary tumor cells with self-renewal potency and expressing certain surface markers being able to form colonies or new tumors. They found that a small fraction of cells exhibiting CD44+/CD24−/Lineage (Lin)-phenotype on the surface had a higher tumor-forming ability in immunocompromised mice and self-renewal property in the reiterated passage than CD44+/CD24+/Lin- cells.

CD44 is a multifunctional transmembrane glycoprotein involved in binding of the cell to extracellular matrix hyaluronic acid (HA) and plays role in cell-cell interactions, cell adhesion, growth, proliferation, survival, motility, migration, angiogenesis, and differentiation [81,82]. CD44 can interact with other ligands, including Osteopontin, Collagens, Fibronectin, Integrin, Laminin, matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) and is associated with many malignancies, chronic inflammatory, and autoimmune dysfunctions [83]. CD44 is encoded by a highly conserved gene located on chromosomal location 11p13 and consists of 20 exons and 19 introns [84]. CD44 gene generates more than 20 isoforms (known as "variant") from extensive RNA alternative splicing of the ten central exons. The CD44v isoforms mediate and promote the activation of many signal transduction pathways initiated by HER2, T cell receptor (TCR), integrin and other receptor tyrosine kinases [85]. For example, the CD44/HER2 signaling increases the activation of Wnt/β-catenin signaling while CD44/EGFR signaling leads to the activation of TGF-β [86,87]. It is also well-documented that the cleavage of CD44 following ligand binding induces stemness and EMT [88,89].

CD24 is a heavily glycosylated small transmembrane glycoprotein anchored in the cell membrane by glycosyl-phosphatidyl-inositol. Like CD44, in cancer, CD24 is involved in cell-cell and cell-matrix junction and in cell migration and is a significant marker for tumor prognosis as well as diagnosis. CD24 acts as a ligand for P-selectin that is an abundant protein in thrombin-

activated platelets and endothelial cells [90]. Therefore, CD24 can promote cancer cell migration and metastasis by facilitating the attachment of cancer cells to activated platelets and endothelial cells [91,92]. CD24 also interacts with chemokine (C-X-C motif) receptor 4 (CXCR4) and the stromal cell-derived factor 1α (SDF1α) receptor [93]. It is a negative regulator of nuclear factor-kappa B (NF-kB) and MAPK signaling pathways in CD44-positive tumor cells [94], and a positive regulator of Src and STAT3 [95].

In 2007, Ginestier et al. [96] introduced aldehyde dehydrogenases (ALDHs) as additional markers for the BCSCs. The ALDH enzymes are a family of conserved enzymes that oxidize aldehydes and have 19 isoforms localized to various cellular compartments including cytosol, mitochondria, endoplasmic reticulum and the nucleus [97–99]. ALDHs are responsible for the oxidation of aldehydes to their corresponding carboxylic acids and catalysis of retinaldehyde to retinoic acid. They also mediate the inactivation of alkylating agent cyclophosphamide analogous and other xenobiotics. In addition, ALDHs also play roles in detoxification pathways, cyclophosphamide metabolism, and biosynthesis of retinoic acid, folate, amino acid, and ethanol [97]. Stem cells from a variety of tissues show high levels of ALDH activity, which is a characteristic of “stemness”. Normal (hematopoietic and neural) and cancer stem cells are enriched in cells with high levels of ALDH expression. ALDH high cells with low side scatter are self-renewing and multipotent [100]. ALDH+ cells isolated from breast cancer cell lines were highly invasive, exhibited high potency of self-renewal, and resistant to hypoxia and chemotherapeutic drugs when compared with ALDH- cells [101].

In tumors, CD24 is expressed on more differentiated cells while CD44 is expressed on more progenitor-like cells. Normally, CD44+/CD24- phenotype represents undifferentiated basal/mesenchymal-like cells whereas the cells with CD44-/CD24+ phenotype are luminal/ epithelial-like cells [80,102]. CD44+/CD24- breast cancer cells are highly tumorigenic and are correlated with the presence of distant metastases compared to the cells carrying other markers [80,103,104]. The majority of tumor cells derived from metastatic sites of breast cancer patients were highly enriched for a CD44+/CD24- subpopulation [105–107]. While CD44+/CD24- cells may be sensitive to some inhibitors [108,109], they often drive tumor resistance to traditional therapies [110]. Studies with tumor tissues from breast cancer patients who had already received chemotherapy revealed an increased percentage of mammosphere-forming cells with

CD44+/CD24- phenotype subpopulation [111]. In breast cancer cells, the gene expression profile following chemotherapy is very similar to that of CD44+/CD24- cells. This suggests that the remaining breast cancer cells could be CD44+/CD24- cells [112]. In addition to these markers, high expression of CD133 was also found in BCSCs. Breast cancer cells with phenotypes CD44+/CD24−/ALDH+ and CD44+/CD133+/ALDH+ showed increased tumorigenicity and metastases when compared with the non-stem cancer cells [113].

In the study by All-Hajj et al. [80] only cells with CD44+/CD24-/Lin- phenotype (approximately 100 cells) were able to grow to form tumors in the animals, whereas tens of thousands of cells with other phenotypes failed to form tumors [80]. Hence, there is a positive relationship between the size of the subpopulation of BCSCs and tumorigenesis, tumor invasion and refractory [80,110,114]. In recent years, many reports have been published focusing on BCSCs in different types of breast tumors. Among them, some results suggest a positive role of HER2 receptor in the emergence of BCSCs inside breast tumors [80,110,114]. In this paper, we review the interaction of HER2 with stemness signaling pathways, which mostly causes HER2-positive breast cancer cells to attain stem cell properties and trastuzumab resistance.

1.6. EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

EMT is a complex biologic process by which epithelial cells normally lied on the basement membrane undergoes several epigenetic reprogramming and gene expression regulation, leading to the loss of cell polarity and cell-cell adherent junctions, and the gain of mesenchymal stem cells properties with an ability to migrate (Figure 1.5). EMT is necessary for three physiological and pathological processes including (i) embryogenesis, and organ developmental processes (ii) tissue regeneration and organ fibrosis, (iii) cancer migration and metastasis [115]. EMT requires cooperation of a complex cellular possesses including certain transcriptional regulatory factors including Snail1, Snail2 (Slug), Zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, Forkhead box protein C1 (FOXC1), FOXC2, Transcription factor 3 (TCF3) and homeobox protein Goosecoid (GSC), activity of tyrosine kinase receptors, a network of several stemness signaling pathways such as TGF-β, Wnt/β-catenin, Notch, JAK/STAT, Hedgehog and also

inflammatory pathways such as NF-κB, extracellular and intracellular growth factors such as EGFs, Insulin-like growth factor 1 (IGF1), Fibroblast growth factors (FGFs), Platelet-derived growth factor (PDGF) and interleukin 6 (IL6) and IL8, cell adhesion transmembrane proteins such as E and N-cadherins and filament protein Vimentin. These processes reprogram epithelial cells to transition to the cells with the more mesenchymal phenotype (Figure 1.5) [116].

CD44+/CD24- cells are BCSCs with mesenchymal properties that localized at the tumor invasive margins and are migration engines of breast tumors, while ALDH+ cells are defined as epithelial-like BCSCs located in deeper sites of the tumors and have more differentiation and proliferative properties. CD44+/CD24- cells were isolated by fluorescence activated cell sorting (FACS) from non-tumorigenic human mammary epithelial cells that have undergone an induced EMT, exhibited many properties of BCSCs including mammosphere-formation ability [117]. CD44+/CD24- BCSCs isolated from breast tumors show a low level of E-cadherin, but high levels of EMT markers including N-cadherin, Vimentin, Fibronectin, ZEB1/2, FOXC2, Snail, Slug, and Twist1/2 compared to ALDH+ cells [117].

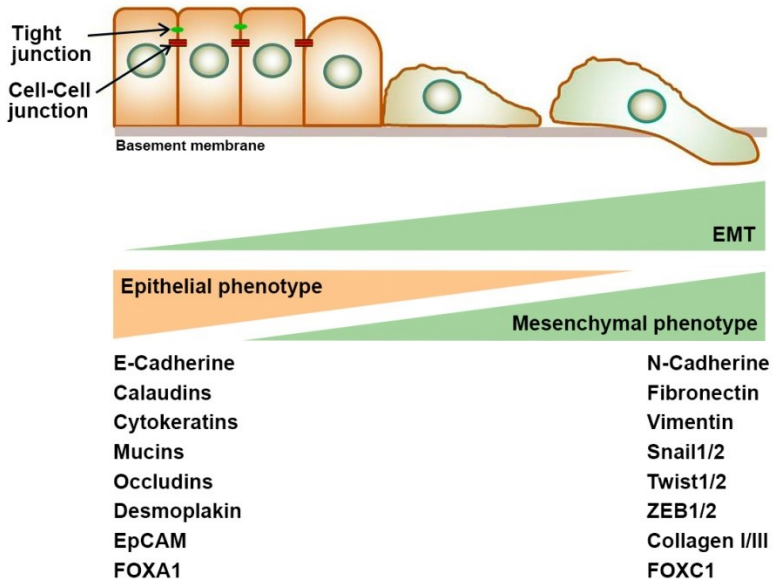

Figure 1.5. Epithelial-mesenchymal transition (EMT) in breast cancer. EMT of epithelial breast cancer cells starts by intensive epigenetic regulation that causes loss of cell-cell and cell-matrix junctions. During EMT epithelial cells that lay on the basement membrane also lose their epithelial phenotype including shape and expression of epithelial marker proteins

(such as E-cadherin, Claudins, Cytokeratins, Mucins, Occludins, Despoplaktin, EpCAM and FOXA1), and gain mesenchyme phenotype including round shape and expression of mesenchymal marker proteins (such as N-cadherin, Fibronectin, Vimentin, Snail1, Snail2 (slug), Twist1, Twist2, ZEB1, ZEB2, Collagen I, Collagen III and FOXC1). EMT is a natural process that allows cells to migrate. The figure partially adapted from [118].

1.7. BREAST CANCER STEMNESS SIGNALING PATHWAYS

1.7.1. TGF-β/Smad signaling pathway

Transforming growth factor-β (TGF-β) superfamily signaling plays critical roles in embryo development, adult tissue regeneration, and tumorigenesis by regulating cell growth, differentiation, apoptosis, cellular homeostasis and other cellular functions [119]. TGF-β proteins are encoded by 33 genes that produce different structurally related polypeptides correspond to pleiotropic cytokine ligands [120,121]. Signaling by TGF-β is transduced through binding TGF-β ligands to type II cell surface receptors that are serine/threonine receptor kinase that catalyzes the phosphorylation of type I receptors [122,123]. A mammalian cell utilizes seven known type I receptors that called activing receptor-like kinases (ALK)1 to 7 and five type II receptors called as TGF-β type II receptor (TβRII), Activin type II receptor (ActRII), Activin type II receptor B (ActRIIB), BMP type II receptor (BMPRII), and Anti-Müllerian hormone receptor (AMHR). Among the ligands, TGF-β activates ALK5, Activin activates ALK4, Nodal activates ALK4 and ALK7 and BMPs activate ALK1, ALK2, ALK3 and ALK6 [124]. Activation of type I receptors induces phosphorylation of downstream signal transducer receptor-activated Smads (R-Smads). Phosphorylated R-Smads form a heteroligomeric complexes with Smad4 (Co-Smad). The Smad complexes translocate into the nucleus and regulate the expression of target genes by direct binding to the target gene promoter and/or via interaction with various transcriptional cofactors depending on the status of the cell [124]. TGF-β can also drive several non-Smad signaling pathways including Erk, p38 kinase, c-JUN N-terminal kinase (JNK), PI3K/Akt, RhoA, Rac1, and Cdc42 GTPases [125].

The role of the TGF-β signaling pathway in growth, apoptosis, self-renewal, and differentiation of stem cells/progenitor cells has been strongly demonstrated [119]. It is well known that Smad and non-Smad pathways play a critical role in stemness and acts as an inducer of EMT of normal mammary epithelial cells [126–128]. TGF-β signaling promotes tumor cell proliferation, survival, motility, invasion, metastatic colonization and acquisition of mesenchymal markers, such as increased Fibronectin and Vimentin expression, increased invasiveness, and exhibiting CD44+/CD24- phenotype toward BCSC progression straits through induction of EMT [129–131]. TGF-β/Smad signaling increases the expression of transcription factors and transcription regulators involved in EMT, including Snail [132], Slug [133], ZEB1 [131] ZEB2 [134], High-mobility group A2 (HMGA2) [135] and Ets1 [136]. TGF-β/Smads signaling also suppresses E-cadherin by upregulating mesenchymal phenotype and also by downregulating the Inhibitor of differentiation (Id)1, 2, and 3 proteins that are the negative regulators of the TGF-β-induced ZEB1 and ZEB2 [137,138]. Smad2 can suppress epithelial markers E-cadherin, Claudin 4, Kallikrein 10, and Cingulin by activation of DNA methyltransferase 1 (DNMT1)-mediated epigenetic silencing of the corresponding genes [139]. There is more evidence supporting the epigenetic modification of EMT by TGF-β. The microRNA-200 (miR-200) family members have been shown to increase E-cadherin expression and to alter the cancer cell morphology to an epithelial phenotype by predominantly downregulating TGF-β and ZEBs. In continue, TGF-β-mediated ZEB1 activation inhibits transcription of miR-200 family members resulting in suppressed E-cadherin and increased Vimentin [140–143]. Additionally, Smad3 can form a complex with Myocardin-related transcription factors (MRTFs), which lead to nuclear translocation of MRTFs promoting expression of Slug [144].

In addition to Smads-dependent pathways of TGF-β signaling, non-Smad pathways induced by TGF-β such as PI3K/Akt, MAPK [125,145], RhoA, and Cofilin are also involved in promoting EMT [146]. TGF-β activates mTORC1 and mTORC2 through PI3K/Akt pathway [147–149]. Akt increases the expression level of Snail and MMP9. Akt also upregulates Snail via the phosphorylation and inactivation of GSK3, a serine-threonine kinase [150,151]. TGF-β-induced Akt phosphorylation releases Heterogeneous nuclear ribonucleoprotein E1 (hnRNPE1) from the 3′ untranslated regions of Disabled 2 (DAB2) and IL-like EMT inducer mRNA and

allows progression of EMT [152]. TGF-β ligands also promote the p38, JNK and MAPK signaling pathways [153]. TGF-β-induced MAPK signaling inhibits GSK3, therefore stabilizes the activity of Snail [154]. Besides, active oncogenic Ras signaling positively regulates the induction of Snail by TGF-β [155]. Cooperation between the TGF-β and MAPK pathways also causes emerging CD24- stem cell-like cells from CD24+ differentiated cells, which suggests b a role for TGF-β in EMT and the exhibition of CD24- phenotype [156]. In addition, TGF-β signaling retains the mesenchymal state of CD44+/CD24-/ALDH+ cells and their tumorigenicity after TGF-β-induced EMT [157,158]. Moreover, TGF-β induces JNK phosphorylation, transactivation of c-JUN and Activator protein 1 (AP1) complex, which leads to EMT [159]. Other studies showed that TGF-β-mediated EMT requires the activation of RhoA, a positive regulator of the actin cytoskeleton and cadherin junctions in cell-cell contact [160,161].

1.7.2. Notch signaling pathway

Notch signaling is an evolutionarily conserved pathway that acts as a mediator of short-range cell-cell communication and is present in most multicellular organisms. Notch signaling regulates multiple aspects of invertebrate and vertebrate cell fate determination during development and maintains adult tissue homeostasis. Like TGF-β signaling, Notch signaling is an essential process for self-renewal, differentiation and is critical in multiple stages of development, in lineage-specific differentiation of pluripotent embryonic stem cells, and in controlling stem cell population and activity in the context of tissue degeneration, regeneration, and malignancy [162]. Notch proteins are cell surface transmembrane-spanning receptors which normally activates by ligand binding during direct cell-to-cell contact [163,164]. The extracellular domain of all Notch proteins contains 29–36 tandem EGF-like repeats that interact with the Delta, Serrate, and Lag2 (DSL) domain of ligands from the neighboring cell [164–166]. In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands [162]. Interaction between Notch receptor and Notch ligands initiates proteolytic cleavage of the receptor by metalloproteinases. The cleavage of the Notch receptor by γ-secretase causes the release of the Notch intracellular domain (NICD) [167–169]. Upon intracellular cleavage, the NICD translocates to the nucleus and interacts with the CSL (CBF1, Suppressor of Hairless, Lag1) family of DNA-binding proteins to form a transcriptional activator complex, which regulates the expression of target genes [167–169].

Dontu and colleagues [170] have demonstrated the critical role of Notch signaling in maintaining normal human mammary stemness by increasing self-renewal efficiency. Upregulated Notch signaling increases the self-renewal and transformation of luminal mammary stem cells, leading to hyperplasia and tumorigenesis [171]. It is well-demonstrated that the Notch signaling has a regulatory role in breast tumorigenesis, metastasis, and resistance. Notch signaling maintains breast cancer stemness by promoting the BCSC phenotype and EMT. Inhibition of Notch signaling by Notch4 neutralizing antibody or γ-secretase inhibitors (GSIs), suppresses BCSC subpopulation and blocks mammosphere-formation effectively [170,172–174]. Notch3 has also been found as a positive factor in the self-renewal of BCSC mammospheres [175,176]. Activated Notch signaling increases ALDH1 activity and promotes breast cancer stemness through induction of deacetylase Sirtuin 2 (SIRT2), an enzyme that deacetylates and activates ALDH1 [177]. Whereas, inhibiting Notch activity in the cells by glucose functionalized nanoparticles containing GSIs reduced pool of ALDH1+ BCSCs [178]. Notch signaling in epithelial breast cancer cell line MCF7 reduces the expression of estrogen receptors and increases CD44 expression in vitro and in vivo models. Moreover, Notch1 blockade with a GSI, DAPT, and shRNA reduces the expression of CD44+/CD24- phenotype, matrigel invasion and micro- and macrometastases [179]. [180] Radioresistance of CD44+/CD24- cells derived from MCF7 and MDA-MB-231 breast cancer monolayer cultures was correlated with high expression of Notch1 in the BCSCs implying association of Notch pathway with stemness-related resistance [180]. Recently, it is found that Notch signaling is a critical regulator of breast tumor EMT by ionizing radiation. During radiation, induced Notch2 accelerates tumor malignancy by increasing mesenchymal markers through IL6/JAK/STAT3 signaling axis [181]. However, Azzam et al. [182] reported that CD44+/CD24+ but not CD44+/CD24- cells in TNBC cell lines express activated Notch1 intracellular domain (NICD1) and its target genes, GSI reduces mammosphere-formation and tumor growth of CD44+/CD24+ cells, but not CD44+/CD24-cells [183].

As pointed out above, the Notch signaling pathway has been known as an important regulator of EMT induction. Timmerman et al. [184] demonstrated that Notch signaling activity can promote EMT during both cardiac development and oncogenic transformation by transcriptional induction of the Snail and repression of E-cadherin expression. Notch signaling-mediated EMT takes place by downregulation of endothelial markers and upregulation of

mesenchymal markers [183]. Slug has been reported as a direct target for the Notch pathway. Notch signaling can upregulate the expression of Slug and Snail either directly or indirectly through interaction with TGF-β signaling [185–187]. Moreover, during hypoxia-induced EMT, NICD1 can activate the expression of Snail directly by regulating Snail mRNA and indirectly via the upregulation of lysyl oxidase (LOX) that stabilizes the Snail protein [188].

1.7.3. Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is a conserved pathway that regulates crucial aspects of cell fate decisions, cell migration, cell polarity, stem cell pluripotency, and neural patterning. Wnt/β-catenin signaling initiates through binding Wnt ligands to two distinct receptor families; Frizzled (Fz) family of transmembrane receptor proteins and lipoprotein receptor-related proteins 5 and 6 (LRP5/6) [189,190]. In humans, there are seven Fz receptors and nineteen cysteine-rich Wnt glycoprotein ligands with highly conserved approximately 350-400 amino acids [191]. Wnt receptor activation initiates canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) pathways. In canonical signaling pathway formation of ligand-receptor complex activates kinase domain of the receptor that causes phosphorylation of serine residues in the cytoplasmic tail of LRP5/6. Phosphorylated LRP5/6 recruits scaffolding protein Axin. Axin is a necessary component of a multi-protein complex that degrades β-catenin [192]. This β-catenin destruction complex also includes Adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), GSK3 and Casein kinase 1α (CK1α) [193–195]. In the absence of Wnt ligand, Axin contributes to the formation of the β-catenin destruction complex, which leads to phosphorylation of β-catenin on serine and threonine residues near its N-terminus providing β-catenin a target for ubiquitination and rendering it to ubiquitin-dependent proteosome-mediated degradation [193–195]. With active signal, restraining Axin by LRP5/6 prohibits the formation of the β-catenin destruction complex and causes an accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus [192,196]. In the nucleus, β-catenin acts as a transcriptional coactivator and forms a complex with members of the T-cell factor/lymphoid enhancing factor (LEF/TCF) family of DNA binding proteins that regulates transcription of target genes [197,198]. β-catenin is also involved in the regulation and coordination of cell-cell adhesion by contributing to the adherens junction complex in association with E-cadherin, an essential process for the maintenance of the epithelial cell layer [192].

The driving role of the Wnt/β-catenin signaling pathway has been well-defined in the development of many human cancers including breast cancer and appears to be associated with cancer stem cell biology. Several studies in mice have revealed that Wnt/β-catenin signaling controls mammary gland development and differentiation during embryogenesis and is critical for stem cell maintenance inside mammary tissue [199,200]. Wnt/β-catenin signaling determines the developmental fate of mammary gland stem cells by regulating mammary epithelium [201]. Studies in both mouse models and human breast cancers have shown that active Wnt/β-catenin signaling is essential for breast tumorigenesis. Wnt/β-catenin signaling is higher in BCSCs than in normal stem cells [202]. Inhibition of β-catenin suppresses stemness activity in patient-derived metastatic breast cancer, implicating an important role of the Wnt/β-catenin signaling in BCSCs [202,203]. Whereas, activated Wnt/β-catenin signaling is associated with increased stemness activity and radiation resistance of BCSCs [204].

Expression of Wnt3 in ER- breast cancers increases the mammosphere-forming ability [202]. Expression of Wnt3 in trastuzumab-resistant cells also increases the expression of EMT markers including N-cadherin, Twist1, Slug, and decreases E-cadherin [205]. In normal mammary epithelial cells c-Fos oncogene decreases E-cadherin and induces EMT through Wnt/β-catenin signaling [206]. β-catenin itself has been shown to induce EMT via induction of LEF1 expression [207]. Wnt receptor LGR5 has been shown to be a stemness marker for the mammary gland and essential for postnatal mammary gland organogenesis [208]. BCSCs with high-level LGR5 expression form more mammospheres and are more potent to drive breast cancer progression and metastasis [209]. Yang et al. [210] reported that LGR5 expression in breast cancer increases cell mobility, tumor growth, pulmonary metastasis, mammosphere-formation and stemness properties of breast cancer cells through Wnt/β-catenin-induced EMT. LGR5 potentiates Wnt/β-catenin pathway in BCSCs and is required for the maintenance of spheroid-derived CD44+/CD24- BCSCs [210]. During EMT, β-catenin binds to Twist1 to increases the transcriptional activity of the β-catenin/TCF4 complex by binding to the promoter DNA of ABCG2, a cancer stemness marker [211].

1.7.4. JAK/STAT signaling pathway

In human mammary tissue, JAK/STAT signaling pathway transmits signals to the nucleus, which leads to the transcription of a wide range of genes involved in cell proliferation, differentiation, migration, and apoptosis. JAK/STAK signaling controls immunity, spermatogenesis, hematopoiesis, and development of the mammary gland and breast tumor. JAK/STAT signaling is initiated by binding of various ligands (mostly cytokines) to a Janus kinase (JAK) receptor, which induces JAK dimerization and phosphorylation of its tyrosine residues [212,213]. This event provides a binding site for the SH2 domain of signal transducer and activator of transcription (STAT). The binding of STATs to the JAK receptors results in the phosphorylation of STATs by JAKs. Phosphorylated STATs dimerize with each other and migrate to the nucleus where the dimer regulates the transcription of target genes [214–216]

Activation of JAK/STAT signaling pathway is necessary for growth, proliferation, survival and chemo-resistance of CD44+/CD24- BCSCs [89,217,218]. Targeting JAK2 and/or STAT3 results in a reduction of the CD44+/CD24- subpopulation and in vivo tumorigenicity of breast cancer cells, which suggests that JAK/STAT signaling plays important role in BCSC maintenance in basal-like tumors [89]. In patient-derived Claudin-low breast cancer cells, STAT3 activity is associated with increased mammosphere-forming efficiency and tumorigenicity [218]. High STAT proteins level is also found in CD44+/CD24- and ALDH+ BCSCs. The inhibition of STAT3 by shRNA reduces the viability and mammosphere-forming ability of breast cancer cells [219]. In addition, selective inhibition of STAT3 by small molecule inhibitors suppressed CD44+/CD24-/ALDH+ BCSCs, mammosphere-forming efficiency and tumor growth in human breast tumor xenograft rodents [220]. Moreover, targeting CD44 in basal-like breast cancer cells leads to repression of JAK/STAT signaling as well as invasive markers MMPs [221]. In addition, epigenomics analysis of BCSCs derived from mammospheres revealed that JAK/STAT signaling is associated with the exhibition of CD44+/CD24- cancer stemness phenotype [222].

JAK/STAT signaling has been shown to play an important role in breast cancer EMT induction. Sullivan et al. [223] showed that IL6 induces the expression of Twist1 via activating STAT3 in the MCF7 cell line. Additionally, exposure of JAK to IL6 increases the population of CD44+ BCSC through inducing STAT3-mediated EMT of epithelial-like breast cancer cells [224]. Oncostatin M (OSM), another inducer of JAK/STAT signaling, is expressed in an

autocrine/paracrine fashion during EMT of breast tumor cells. OSM has been shown to enhance cell migration and to upregulate the expression of EMT inducers including extracellular matrix (ECM) protein and Fibronectin in mammary epithelial cells through STAT3 [225,226]. OSM-mediated activation of STAT3 is also able to upregulate EMT by downregulating miR-200. During breast cancer EMT, STAT3 also promotes the transcription of Lin28, resulting in the downregulation of Let7 therefor, upregulation of HMGA2 [227]. EGF-mediated induction of JAK/STAT3 signaling is also able to induce breast cancer EMT via upregulating Twist1 [266]. Moreover, Transient receptor potential-melastatin-like 7 (TRPM7) channel upregulates the expression of Vimentin through increasing EGF-induced STAT3 activation, which suggests the importance of EGF-STAT3-TRPM7 in the regulation of calcium-dependent EMT in breast cancer [228].

1.7.5. Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway regulates embryogenesis, organogenesis, and adult tissue maintenance by controlling cell proliferation, renewal, differentiation, cell motility and adhesion as well as EMT. Aberrant activity of Hh signaling is directly linked to many human diseases including cancers. It has been reported that Hh signaling plays a key role in the development of breast cancer through the transformation of adult stem cells into cancer stem cells [229]. In mammals, the canonical Hh signaling pathway can be initiated by binding of three Hh ligands [230][231] to the twelve-pass transmembrane protein receptors Patched1 (Ptch1) and Patched2 (Ptch2) [230–232]. The three Hh ligands include Sonic Hedgehog (Shh, the most broadly expressed and best-studied Hh molecule) [230], Indian Hedgehog (Ihh, primarily involved in bone differentiation) [276] and Desert Hedgehog (Dhh, involved in gonad differentiation) [232]. In the absence of Hh, Ptch1 constitutively represses GPCR-like protein Smoothened (Smo), a seven-transmembrane domain receptor [233]. Hh binding to Patch1 relieves the inhibition of Smo, which results in Smo accumulation in cilia and the phosphorylation of its cytoplasmic tail [234]. This signal facilitates the release of the Glioma-associated oncogene (Gli) family of latent zinc-finger (ZF) transcriptional mediators from kinesin-family proteins Kif7 and Sufu, leading to the activation and nuclear translocation of the Gli transcription factors. Gli transcription factors then regulate the transcription of the target genes [235–238].

Growing evidence suggests an important role of Hh signaling pathway in maintain breast cancer stemness [239]. Recent clinical studies indicate that high expression of Ptch1 and Gli1 is associated with larger tumors, metastasis, pathological progression and with significantly shorter OS and DFS in breast cancer patients with CD44+/CD24-/BCSC-enriched tumors [240]. High RNA expression levels of Ptch1, Gli1 and Gli2 have been reported in CD44+/CD24-/Lin- BCSCs [241,242]. Activation of Hh signaling increases CD44+/CD24- cell population and mammosphere size. However, inhibition of Hh signaling pathway suppresses CD44+/CD24- BCSC subpopulation, mammosphere-forming and abrogates drug resistance of BCSCs [242–245]. Inhibition of Hh signaling also suppresses EMT by inhibiting Snail, Slug and ZEB2 [246]. It is recently found that salinomycin that shows selective toxicity in BCSCs, inhibits Shh-mediated Hh signaling activation through downregulating the expression of Ptch1, Smo, Gli1, and Gli2 as well as stemness markers Snail, Nanog, Oct4 and Sox2 [245,247,248]. Therefore, Hh signaling induces self-renewal and EMT of breast cancer cells [246,249,250].

1.8. ENHANCER REGULATORY ELEMENTS

In pro- and eukaryotes, enhancers are short (200-1500 bp) *cis*-regulatory region of DNA located in upstream or downstream of the gene from the transcription start site (TSS) [251]. The term enhancer was first used when SV40 DNA increased ectopic expression of a cloned rabbit β-globin gene increased [252]. Enhancers harbor recognition elements with multiple binding sites for a variety of transcription factors. Binding transcription factors to enhancer activates transcription at a distance target gene independently of enhancer sequence orientation by formation an intervening chromatin loop [251]. In eukaryotes, Chromatin loop formation brings the enhancer to target gene promoter at distance into physical proximity with direct interaction which leads to increase the local concentration of chromatin-modifying factors, assembly of transcription coactivators including p300, Mediator (MED) complex, Bromodomain-containing protein 4 (BRD4), and recruitment of RNA polymerase II (Pol II). This modification of chromatin is associated with increased DNA accessibility by enrichment of histone modification including Histone 3 lysine 4 mono and tri-methylation (H3K4me1 and H3K4me3) and H3K27 acetylation (H3K27ac) and other modifications [253]. About 400,000 putative enhancers have

been found in the human genome based on analysis of histone modifications and proximity to transcription start sites annotated by the ENCODE (Encyclopedia of DNA Elements) project [254]. Enhancer activity is largely cell-type specific and is critical for forming cell-type-specific gene expression patterns, mentioning cell identity and determining cell fate during development [251].

The structure of the eukaryote gene enhancer is shown in Figure 1.6. Enhancers show high enrichment of a broad spectrum of cofactors and chromatin regulators including RNA polymerase II, MED1, Nipped-B-like protein (NIPBL), Cohesin, p300, CREB-binding protein (CBP), Chromodomain-helicase-DNA-binding protein 7 (CHD7), BRD4, BRG1, LSD1-NuRD complexes and long non-coding enhancer RNAs (eRNAs) [255–257]. MED1 is a subunit of the Mediator coactivator complex which regulates transcriptional initiation by the RNA polymerase II [258]. MED1 facilitates chromatin loop formation by binding to NIPBL which is a Cohesin protein and facilitates enhancer-promoter interaction by large spinning [258]. The Cohesin protein complex colocalizes with CCCTC-binding factor (CTCF) a transcription factor that regulates euchromatic and heterochromatic DNA and loop formation. Colocalization of cohesion proteins with CTCF regulates the spatial clustering of enhancer elements and chromatin looping [259]. The coactivators p300 and CBP are histone acetyltransferases and facilitate RNA polymerase II binding to the promoter [260]. CHD7 is a chromodomain helicase DNA-binding domain family of ATP-dependent chromatin remodeling enzymes mediates in enhancer chromatin remodeling by interaction with p300 and binding to methylated histones [261]. BRD4 a member of the bromodomain and extra terminal domain (BET) family binds to MED1, acetylates histones and promotes RNA polymerase II by stimulating phosphorylation of its carboxy-terminal domain [262]. ATP-dependent chromatin remodeler BRG1 is a member of the mammalian SWI/SNF family that modulates the chromatin structure in many pluripotent cells, specifically during development [263]. Lysine-specific demethylase 1A (Lsd1) is a subunit of the Nucleosome remodeling deacetylase (NuRD) complex which mediates in histone modification and occupies enhancers of genes that are critical for maintaining embryonic stem cells (ESC) [264]. Enrichment of eRNAs is a mark of highly enhancer-specific activity and has specific roles in the expression of genes near the enhancers including stabilizing enhancer-promoter looping by cohesin and Mediator complexes [265].

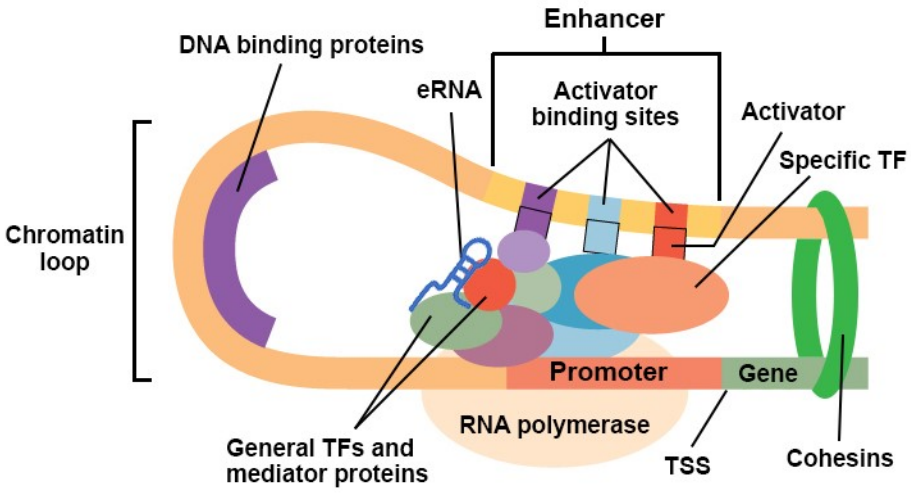

Figure 1.6. Structure of promoter-enhancer interaction and transcriptional regulation complex. eRNA: enhancer RNA. TF: transcription factor. TSS: transcription start site. The figure partially adapted from www.courses.lumenlearning.com

Enhancers can be characterized by enhancer trap techniques using a reporter gene such as LacZ or by comparative sequence analysis and computational genomics to genome-wide *cis*-regulatory elements [266]. In traditional method expression level of a reporter gene which is integrated into a genomic region near a putative enhancer reflects the activity of the enhancer. Thus, cloning of the neighboring regions and subsequent further experiments such as chromatin immunoprecipitation (ChIP) and DNA sequencing can characterize the enhancer circumstance in terms of epigenetic modification, bound transcription factor complexes, DNA sequences and genomic map [251]. Densely positioned nucleosomes (closed chromatin) restrict access of transcription complex to enhancer while open (nucleosome-free) chromatin of enhancer region makes enhancer accessible for the transcription factors and co-activators [251]. Chromatin accessibility is controlled by certain chromatin modification signature which is crucial for packaging and interpreting the genome that governs the gene expression profile of each cell. This signature contains a wide board of processes that exert histone modifications, DNA methylation, non-coding RNA-based modifications, and chromatin architecture and acts as "gatekeeper" of *cis*-regulatory elements across the genome. Open chromatin is associated with nucleosomes containing histone variants H3.3 and H2A.Z. H3.3/H2A.Z histones co-occurrence relaxes nucleosome and unstrings DNA twist [267]. These nucleosomes that flank TSS and active

enhancers and other transcription factor binding regions are often marked by specific histone modifications including, but not limited to, H3K4me1 and H3K27ac [268,269]. In addition, chromatin regions with these two modifications are highly sensitive to DNA nucleases such as DNase I (DNase I hypersensitivity) due to the high accessibility of the DNA to the enzymes [270]. Taking together, enhancers are distinguished from non-regulatory genomic DNA by (1) transcription factor binding signature (2) chromatin modification signature and (3) level of sensitivity to DNase I. These three criteria allow discovering novel enhancer as tree principle of enhancer discovery platforms

Development of genome and epigenome-wide analyzing technologies such as NGS (next-generation sequencing), ChIP-seq (chromatin immunoprecipitation followed by NGS), DNase-seq, ATAC-seq (assay for transposase-accessible chromatin with high throughput sequencing) [271], IM-PET (integrated methods for predicting enhancer targets), SIF-seq (site-specific integration fluorescence-activated cell sorting followed by sequencing) [272], HiC (high-throughput sequencing of chromosome conformation capture), and ChIA-PET (analysis by paired-end tag sequencing) which is combination of HiC and ChIP-seq methods allowed researcher identify wide-scale putative enhancers based the three criteria. ChIP-seq is a powerful tool to determine the 2D genome structure and for enhancer discovery based on histone modifications (methylation, acetylation, and ubiquitination) also based on binding of general and specific transcription factors as well as co-activators [255,256,273–275]. Typically, approximately 10,000 to 150,000 putative enhancer elements can be identified in a specific cell by using ChIP-seq [268,276]. HiC is used to determine 3D chromatin interactions that can reveal *trans*-acting regions in addition to *cis*-acting regions. ChIA-PET which is a combination of HiC and ChIP-seq methods is another useful method to determine chromatin looping and chromatin-chromatin interaction sites based on a cross-linked transcription factor [277,278].

In 2013, Whyte *et al*. [256] described clusters of enhancer elements called “super-enhancer” in murine ESC that are exclusively occupied by three master pluripotency transcription factors Oct4, Sox2 and Nanog. The super-enhancer regulated expression of genes, which control cell identity of ESCs including *Oct4*, *Sox2* and *Nanog* themselves. Super-enhancers increase expression of linked gene with higher efficiency than typical enhancers. A super-enhancer differs from a typical enhancer by higher enrichment by specific and general transcription factors,

mediators (such as MED1 and BRD4), eRNAs, active chromatin histone marks (such as H3K4me and H3K27ac) and forming larger chromatin loop (> 50 kbp) [255,256,279,280]. super-enhancers are highly associated with topologically associating domains (TADs) which are clusters of self-interacting chromatin regions and a part of forming 3D chromosome structure and chromosome packaging in the nucleus [281]. TADs are found associated with several diseases by altering 3D organization of the chromosome that disrupts gene regulation [281].

Using high-throughput chromatin ChIP-Seq for histone marks and/or transcription factors, over 75,000 super-enhancers have been found in human genome that make up to about 15% of the genomic DNA [282]. Super-enhancers can be located upstream or downstream of the TSS of the corresponding gene and are essential for maintaining cell identity and specificity [256,264]. Investigation of super-enhancers in cancer led to identify many genes which play important role in cancer biology. Association of super-enhancers with oncogenes was found in several cancer types including colon [255,283], breast [255,283], cervical [282], lung [255,279,284], prostate [255], pancreas [255], glioblastoma [279], medulloblastoma [285], neuroblastoma [284], and various leukemias [255,274,285,286]. About 20,000 super-enhancers are identified in different human cancer cell types [282].

1.9. REFERENCES

1. Lemmon MA, Schlessinger J. Cell signaling by receptor-tyrosine kinases. Cell. 2010. pp. 1117–1134. doi:10.1016/j.cell.2010.06.011
2. Kovacs E, Zorn JA, Huang Y, Barros T, Kuriyan J. A structural perspective on the regulation of the epidermal growth factor receptor. Annu Rev Biochem. 2015;84: 739–764. doi:10.1146/annurev-biochem-060614-034402
3. Ward CW, Hoyne PA, Flegg RH. Insulin and epidermal growth factor receptors contain the cysteine repeat motif found in the tumor necrosis factor receptor. Proteins. 1995;22: 141–153. doi:10.1002/prot.340220207
4. Carpenter G, Lembach KJ, Morrison MM, Cohen S. Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. J Biol Chem. 1975;250:

4297–4304.

5. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature. 1984;309: 418–425. doi:10.1038/309418a0

6. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7: 505–516. doi:10.1038/nrm1962

7. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5: 341–354. doi:10.1038/nrc1609

8. Roskoski RJ. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res. 2014;79: 34–74. doi:10.1016/j.phrs.2013.11.002

9. Zhang H, Berezov A, Wang Q, Zhang G, Drebin J, Murali R, et al. ErbB receptors: from oncogenes to targeted cancer therapies. J Clin Invest. 2007;117: 2051–2058. doi:10.1172/JCI32278

10. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5: 317–328.

11. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2: 127–137. doi:10.1038/35052073

12. Lemmon MA. Ligand-induced ErbB receptor dimerization. Exp Cell Res. 2009;315: 638–648. doi:10.1016/j.yexcr.2008.10.024

13. Ferguson KM. Structure-based view of epidermal growth factor receptor regulation. Annu Rev Biophys. 2008;37: 353–373. doi:10.1146/annurev.biophys.37.032807.125829

14. Rotin D, Honegger AM, Margolis BL, Ullrich A, Schlessinger J. Presence of SH2 domains of phospholipase C gamma 1 enhances substrate phosphorylation by increasing the affinity toward the epidermal growth factor receptor. J Biol Chem. 1992;267: 9678–9683.

15. Wang Z. ErbB Receptors and Cancer. Methods Mol Biol. 2017;1652: 3–35. doi:10.1007/978-1-4939-7219-7_1

16. Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ 2nd. Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol Ther. 2009;122: 1–8. doi:10.1016/j.pharmthera.2008.11.008

17. Castellano E, Downward J. RAS Interaction with PI3K: More Than Just Another Effector Pathway. Genes Cancer. 2011;2: 261–274. doi:10.1177/1947601911408079

18. Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK, Nakagawa H. Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. J Biol Chem. 2000;275: 30934–30942. doi:10.1074/jbc.M004112200

19. Burgering BM, Coffer PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature. 1995;376: 599–602. doi:10.1038/376599a0

20. Skeen JE, Bhaskar PT, Chen C-C, Chen WS, Peng X, Nogueira V, et al. Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell. 2006;10: 269–280. doi:10.1016/j.ccr.2006.08.022

21. Weigelt B, Horlings HM, Kreike B, Hayes MM, Hauptmann M, Wessels LFA, et al. Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol. 2008;216: 141–150. doi:10.1002/path.2407

22. Yardley DA. Drug resistance and the role of combination chemotherapy in improving patient outcomes. Int J Breast Cancer. 2013;2013: 137414. doi:10.1155/2013/137414

23. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406: 747–752. doi:10.1038/35021093

24. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. Cancer Cell. 2014;25: 282–303. doi:10.1016/j.ccr.2014.02.025

25. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat. 2012;136: 331–345. doi:10.1007/s10549-012-2289-9

26. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for

selection of targeted therapies. J Clin Invest. 2011;121: 2750–2767. doi:10.1172/JCI45014

27. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989;244: 707–712.

28. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235: 177–182.

29. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/neu oncogene protein and prognosis in breast cancer. J Clin Oncol. 1989;7: 1120–1128. doi:10.1200/jco.1989.7.8.1120

30. Bose R, Kavuri SM, Searleman AC, Shen W, Shen D, Koboldt DC, et al. Activating HER2 mutations in HER2 gene amplification negative breast cancer. Cancer Discov. 2013;3: 224–237. doi:10.1158/2159-8290.CD-12-0349

31. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, et al. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature. 1984;312: 513–516.

32. Carpenter G. Receptors for epidermal growth factor and other polypeptide mitogens. Annu Rev Biochem. 1987;56: 881–914. doi:10.1146/annurev.bi.56.070187.004313

33. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DWJ, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421: 756–760. doi:10.1038/nature01392

34. Aertgeerts K, Skene R, Yano J, Sang B-C, Zou H, Snell G, et al. Structural analysis of the mechanism of inhibition and allosteric activation of the kinase domain of HER2 protein. J Biol Chem. 2011;286: 18756–18765. doi:10.1074/jbc.M110.206193

35. Garrett TPJ, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell. 2003;11: 495–505.

36. Pegram MD, Konecny G, Slamon DJ. The molecular and cellular biology of HER2/neu gene amplification/overexpression and the clinical development of herceptin

(trastuzumab) therapy for breast cancer. Cancer Treat Res. 2000;103: 57–75.

37. Rimawi MF, Schiff R, Osborne CK. Targeting HER2 for the treatment of breast cancer. Annu Rev Med. 2015;66: 111–128. doi:10.1146/annurev-med-042513-015127

38. Davoli A, Hocevar BA, Brown TL. Progression and treatment of HER2-positive breast cancer. Cancer Chemother Pharmacol. 2010;65: 611–623. doi:10.1007/s00280-009-1208-1

39. Elias SG, Adams A, Wisner DJ, Esserman LJ, van't Veer LJ, Mali WPTM, et al. Imaging features of HER2 overexpression in breast cancer: a systematic review and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2014;23: 1464–1483. doi:10.1158/1055-9965.EPI-13-1170

40. Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: Biology driving targeted therapeutics. Cellular and molecular life sciences : CMLS. 2008. pp. 1566–1584. doi:10.1007/s00018-008-7440-8

41. Hanna W, Barnes P, Berendt R, Chang M, Magliocco A, Mulligan AM, et al. Testing for her2 in breast cancer: current pathology challenges faced in Canada. Current Oncology. 66 Martin St. Milton, ON, Canada L9T 2R2; 2012. pp. 315–323. doi:10.3747/co.19.1173

42. Li JY, Perry SR, Muniz-Medina V, Wang X, Wetzel LK, Rebelatto MC, et al. A Biparatopic HER2-Targeting Antibody-Drug Conjugate Induces Tumor Regression in Primary Models Refractory to or Ineligible for HER2-Targeted Therapy. Cancer Cell. 2016;29: 117–129. doi:10.1016/j.ccell.2015.12.008

43. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A. 1992;89: 4285–4289.

44. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 1990;50: 1550–1558.

45. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell. 2009;15: 429–440.

doi:10.1016/j.ccr.2009.03.020

46. Nagata Y, Lan K-H, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004;6: 117–127. doi:10.1016/j.ccr.2004.06.022

47. Zhang S, Huang W-C, Li P, Guo H, Poh S-B, Brady SW, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat Med. 2011;17: 461–469. doi:10.1038/nm.2309

48. Nahta R, Takahashi T, Ueno NT, Hung M-C, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res. 2004;64: 3981–3986. doi:10.1158/0008-5472.CAN-03-3900

49. Le X-F, Bedrosian I, Mao W, Murray M, Lu Z, Keyomarsi K, et al. Anti-HER2 antibody trastuzumab inhibits CDK2-mediated NPAT and histone H4 expression via the PI3K pathway. Cell Cycle. 2006;5: 1654–1661.

50. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, et al. Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A. 2011;108: 3761–3766. doi:10.1073/pnas.1014835108

51. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. Cancer Res. 2000;60: 3384–3388.

52. Clynes RA, Towers TL, Presta LG, Ravetch J V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med. 2000;6: 443–446. doi:10.1038/74704

53. Raab S, Steinbacher J, Schmiedel BJ, Kousis PC, Steinle A, Jung G, et al. Fc-optimized NKG2D-Fc constructs induce NK cell antibody-dependent cellular cytotoxicity against breast cancer cells independently of HER2/neu expression status. J Immunol. 2014;193: 4261–4272. doi:10.4049/jimmunol.1400872

54. Esteva FJ, Valero V, Booser D, Guerra LT, Murray JL, Pusztai L, et al. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20: 1800–1808. doi:10.1200/JCO.2002.07.058

55. Sato N, Sano M, Tabei T, Asaga T, Ando J, Fujii H, et al. Combination docetaxel and trastuzumab treatment for patients with HER-2-overexpressing metastatic breast cancer: a multicenter, phase-II study. Breast Cancer. 2006;13: 166–171.

56. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol. 2005;23: 4265–4274. doi:10.1200/JCO.2005.04.173

57. Gianni L, Pienkowski T, Im Y-H, Roman L, Tseng L-M, Liu M-C, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012;13: 25–32. doi:10.1016/S1470-2045(11)70336-9

58. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353: 1659–1672. doi:10.1056/NEJMoa052306

59. Dahabreh IJ, Linardou H, Siannis F, Fountzilas G, Murray S. Trastuzumab in the adjuvant treatment of early-stage breast cancer: a systematic review and meta-analysis of randomized controlled trials. Oncologist. 2008;13: 620–630. doi:10.1634/theoncologist.2008-0001

60. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344: 783–792. doi:10.1056/NEJM200103153441101

61. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20: 719–726.

62. Eiermann W. Trastuzumab combined with chemotherapy for the treatment of HER2-positive metastatic breast cancer: pivotal trial data. Ann Oncol Off J Eur Soc Med Oncol.

2001;12 Suppl 1: S57-62.

63. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol. 1996;14: 737–744.

64. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol. 1999;17: 2639–2648.

65. Wong H, Leung R, Kwong A, Chiu J, Liang R, Swanton C, et al. Integrating Molecular Mechanisms and Clinical Evidence in the Management of Trastuzumab Resistant or Refractory HER-2(+) Metastatic Breast Cancer. The Oncologist. Durham, NC, USA; 2011. pp. 1535–1546. doi:10.1634/theoncologist.2011-0165

66. Wilken JA, Maihle NJ. Primary trastuzumab resistance: new tricks for an old drug. Annals of the New York Academy of Sciences. 2010. pp. 53–65. doi:10.1111/j.1749-6632.2010.05782.x

67. Swain SM, Baselga J, Kim S-B, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015;372: 724–734. doi:10.1056/NEJMoa1413513

68. Fleeman N, Bagust A, Beale S, Dwan K, Dickson R, Proudlove C, et al. Pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2-positive metastatic or locally recurrent unresectable breast cancer. Pharmacoeconomics. 2015;33: 13–23. doi:10.1007/s40273-014-0206-2

69. Sakai K, Yokote H, Murakami-Murofushi K, Tamura T, Saijo N, Nishio K. Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway. Cancer Sci. 2007;98: 1498–1503. doi:10.1111/j.1349-7006.2007.00553.x

70. Harbeck N, Beckmann MW, Rody A, Schneeweiss A, Muller V, Fehm T, et al. HER2 Dimerization Inhibitor Pertuzumab - Mode of Action and Clinical Data in Breast Cancer.

Breast Care (Basel). 2013;8: 49–55. doi:10.1159/000346837

71. Vu T, Claret FX. Trastuzumab: Updated Mechanisms of Action and Resistance in Breast Cancer. Frontiers in Oncology. 2012. doi:10.3389/fonc.2012.00062

72. Swain SM, Kim S-B, Cortes J, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. Lancet Oncol. 2013;14: 461–471. doi:10.1016/S1470-2045(13)70130-X

73. Kawajiri H, Takashima T, Kashiwagi S, Noda S, Onoda N, Hirakawa K. Pertuzumab in combination with trastuzumab and docetaxel for HER2-positive metastatic breast cancer. Expert Rev Anticancer Ther. 2015;15: 17–26. doi:10.1586/14737140.2015.992418

74. Baselga J, Cortes J, Kim S-B, Im S-A, Hegg R, Im Y-H, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med. 2012;366: 109–119. doi:10.1056/NEJMoa1113216

75. Diessner J, Bruttel V, Becker K, Pawlik M, Stein R, Hausler S, et al. Targeting breast cancer stem cells with HER2-specific antibodies and natural killer cells. Am J Cancer Res. 2013;3: 211–220.

76. Yamashita-Kashima Y, Iijima S, Yorozu K, Furugaki K, Kurasawa M, Ohta M, et al. Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models. Clin Cancer Res. 2011;17: 5060–5070. doi:10.1158/1078-0432.CCR-10-2927

77. Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, Hasmann M. Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. Cancer Res. 2009;69: 9330–9336. doi:10.1158/0008-5472.CAN-08-4597

78. Soady KJ, Kendrick H, Gao Q, Tutt A, Zvelebil M, Ordonez LD, et al. Mouse mammary stem cells express prognostic markers for triple-negative breast cancer. Breast Cancer Res. 2015;17: 31. doi:10.1186/s13058-015-0539-6

79. Tiede B, Kang Y. From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. Cell Research. 2011. pp. 245–257.

doi:10.1038/cr.2011.11

80. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100: 3983–3988. doi:10.1073/pnas.0530291100

81. Draffin JE, McFarlane S, Hill A, Johnston PG, Waugh DJJ. CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. Cancer Res. 2004;64: 5702–5711. doi:10.1158/0008-5472.CAN-04-0389

82. Pham P V, Phan NLC, Nguyen NT, Truong NH, Duong TT, Le D V, et al. Differentiation of breast cancer stem cells by knockdown of CD44: promising differentiation therapy. Journal of Translational Medicine. 2011. p. 209. doi:10.1186/1479-5876-9-209

83. Naor D, Sionov R V, Ish-Shalom D. CD44: structure, function, and association with the malignant process. Adv Cancer Res. 1997;71: 241–319.

84. Gao AC, Lou W, Dong JT, Isaacs JT. CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. Cancer Res. 1997;57: 846–849.

85. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol. 2003;4: 33–45. doi:10.1038/nrm1004

86. Bourguignon LY, Zhu H, Chu A, Iida N, Zhang L, Hung MC. Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. J Biol Chem. 1997;272: 27913–27918.

87. Meran S, Luo DD, Simpson R, Martin J, Wells A, Steadman R, et al. Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. J Biol Chem. 2011;286: 17618–17630. doi:10.1074/jbc.M111.226563

88. Cho SH, Park YS, Kim HJ, Kim CH, Lim SW, Huh JW, et al. CD44 enhances the epithelial-mesenchymal transition in association with colon cancer invasion. Int J Oncol. 2012;41: 211–218. doi:10.3892/ijo.2012.1453

89. Marotta LLC, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR, et al. The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(-) stem cell-like breast cancer cells in human tumors. J Clin Invest. 2011;121: 2723–2735.

doi:10.1172/JCI44745

90. Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, Uede T, et al. CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res. 2005;65: 10783–10793. doi:10.1158/0008-5472.CAN-05-0619

91. Aigner S, Sthoeger ZM, Fogel M, Weber E, Zarn J, Ruppert M, et al. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood. 1997;89: 3385–3395.

92. Friederichs J, Zeller Y, Hafezi-Moghadam A, Grone HJ, Ley K, Altevogt P. The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res. 2000;60: 6714–6722.

93. Schabath H, Runz S, Joumaa S, Altevogt P. CD24 affects CXCR4 function in pre-B lymphocytes and breast carcinoma cells. J Cell Sci. 2006;119: 314–325. doi:10.1242/jcs.02741

94. Ju J, Jang K, Lee K-M, Kim M, Kim J, Yi JY, et al. CD24 enhances DNA damage-induced apoptosis by modulating NF-kappaB signaling in CD44-expressing breast cancer cells. Carcinogenesis. 2011;32: 1474–1483. doi:10.1093/carcin/bgr173

95. Bretz N, Noske A, Keller S, Erbe-Hofmann N, Schlange T, Salnikov A V, et al. CD24 promotes tumor cell invasion by suppressing tissue factor pathway inhibitor-2 (TFPI-2) in a c-Src-dependent fashion. Clin Exp Metastasis. 2012;29: 27–38. doi:10.1007/s10585-011-9426-4

96. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell stem cell. 2007. pp. 555–567. doi:10.1016/j.stem.2007.08.014

97. Jackson B, Brocker C, Thompson DC, Black W, Vasiliou K, Nebert DW, et al. Update on the aldehyde dehydrogenase gene (ALDH) superfamily. Human Genomics. 2011. pp. 283–303. doi:10.1186/1479-7364-5-4-283

98. Hsu LC, Chang WC, Yoshida A. Genomic structure of the human cytosolic aldehyde dehydrogenase gene. Genomics. 1989;5: 857–865.

99. Thomas M, Halsall S, Peters TJ. Role of hepatic acetaldehyde dehydrogenase in

alcoholism: demonstration of persistent reduction of cytosolic activity in abstaining patients. Lancet (London, England). 1982;2: 1057–1058.

100. Tomita H, Tanaka K, Tanaka T, Hara A. Aldehyde dehydrogenase 1A1 in stem cells and cancer. Oncotarget. 2016. pp. 11018–11032. doi:10.18632/oncotarget.6920

101. Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev. 2011;7: 292–306. doi:10.1007/s12015-010-9208-4

102. Honeth G, Bendahl P-O, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res. 2008;10: R53. doi:10.1186/bcr2108

103. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin Cancer Res. 2005;11: 1154–1159.

104. Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, et al. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. Proc Natl Acad Sci U S A. 2010;107: 18115–18120. doi:10.1073/pnas.1006732107

105. Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. Breast Cancer Res. 2009;11: R46. doi:10.1186/bcr2333

106. Balic M, Lin H, Young L, Hawes D, Giuliano A, McNamara G, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. Clin Cancer Res. 2006;12: 5615–5621. doi:10.1158/1078-0432.CCR-06-0169

107. Theodoropoulos PA, Polioudaki H, Agelaki S, Kallergi G, Saridaki Z, Mavroudis D, et al. Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer. Cancer Lett. 2010;288: 99–106. doi:10.1016/j.canlet.2009.06.027

108. Nami B, Donmez H, Kocak N. Tunicamycin-induced endoplasmic reticulum stress reduces in vitro subpopulation and invasion of CD44+/CD24- phenotype breast cancer stem cells. Exp Toxicol Pathol. 2016. doi:10.1016/j.etp.2016.06.004

109. Opyrchal M, Salisbury JL, Iankov I, Goetz MP, McCubrey J, Gambino MW, et al. Inhibition of Cdk2 kinase activity selectively targets the CD44(+)/CD24(-)/Low stem-like subpopulation and restores chemosensitivity of SUM149PT triple-negative breast cancer cells. Int J Oncol. 2014;45: 1193–1199. doi:10.3892/ijo.2014.2523

110. Doherty MR, Smigiel JM, Junk DJ, Jackson MW. Cancer Stem Cell Plasticity Drives Therapeutic Resistance. Cancers (Basel). 2016;8. doi:10.3390/cancers8010008

111. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu M-F, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst. 2008;100: 672–679. doi:10.1093/jnci/djn123

112. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci U S A. 2009;106: 13820–13825. doi:10.1073/pnas.0905718106

113. Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med. 2009;13: 2236–2252. doi:10.1111/j.1582-4934.2008.00455.x

114. Nami B, Ghasemi-Dizgah A, Vaseghi A. Overexpression of molecular chaperons GRP78 and GRP94 in CD44hi/CD24lo breast cancer stem cells. Bioimpacts. 2016;6: 105–110. doi:10.15171/bi.2016.15

115. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of Clinical Investigation. 2009. pp. 1420–1428. doi:10.1172/JCI39104

116. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nature reviews. Molecular cell biology. 2014. pp. 178–196. doi:10.1038/nrm3758

117. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008. pp. 704–715. doi:10.1016/j.cell.2008.03.027

118. Bartis D, Mise N, Mahida RY, Eickelberg O, Thickett DR. Epithelial-mesenchymal

transition in lung development and disease: does it exist and is it important? Thorax. 2014;69: 760–765. doi:10.1136/thoraxjnl-2013-204608

119. Watabe T, Miyazono K. Roles of TGF-beta family signaling in stem cell renewal and differentiation. Cell Res. 2009;19: 103–115. doi:10.1038/cr.2008.323

120. Derynck R, Rhee L, Chen EY, Van Tilburg A. Intron-exon structure of the human transforming growth factor-beta precursor gene. Nucleic Acids Res. 1987;15: 3188–3189.

121. Dickinson ME, Kobrin MS, Silan CM, Kingsley DM, Justice MJ, Miller DA, et al. Chromosomal localization of seven members of the murine TGF-beta superfamily suggests close linkage to several morphogenetic mutant loci. Genomics. 1990;6: 505–520.

122. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, et al. TGF beta signals through a heteromeric protein kinase receptor complex. Cell. 1992;71: 1003–1014.

123. Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J Biol Chem. 1994;269: 20172–20178.

124. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol. 2012;13: 616–630. doi:10.1038/nrm3434

125. Zhang YE. Non-Smad pathways in TGF-beta signaling. Cell Res. 2009;19: 128–139. doi:10.1038/cr.2008.328

126. Kim H, Choi J-A, Kim J-H. Ras promotes transforming growth factor-beta (TGF-beta)-induced epithelial-mesenchymal transition via a leukotriene B4 receptor-2-linked cascade in mammary epithelial cells. J Biol Chem. 2014;289: 22151–22160. doi:10.1074/jbc.M114.556126

127. Mori S, Kodaira M, Ito A, Okazaki M, Kawaguchi N, Hamada Y, et al. Enhanced Expression of Integrin alphavbeta3 Induced by TGF-beta Is Required for the Enhancing Effect of Fibroblast Growth Factor 1 (FGF1) in TGF-beta-Induced Epithelial-Mesenchymal Transition (EMT) in Mammary Epithelial Cells. PLoS One. 2015;10: e0137486. doi:10.1371/journal.pone.0137486

128. Pang M-F, Georgoudaki A-M, Lambut L, Johansson J, Tabor V, Hagikura K, et al. TGF-beta1-induced EMT promotes targeted migration of breast cancer cells through the

lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis. Oncogene. 2016;35: 748–760. doi:10.1038/onc.2015.133

129. Asiedu MK, Ingle JN, Behrens MD, Radisky DC, Knutson KL. TGFβ/TNFα-Mediated Epithelial-Mesenchymal Transition Generates Breast Cancer Stem Cells with a Claudin-Low Phenotype. Cancer research. 2011. pp. 4707–4719. doi:10.1158/0008-5472.CAN-10-4554

130. Stankic M, Pavlovic S, Chin Y, Brogi E, Padua D, Norton L, et al. TGF-beta-Id1 signaling opposes Twist1 and promotes metastatic colonization via a mesenchymal-to-epithelial transition. Cell Rep. 2013;5: 1228–1242. doi:10.1016/j.celrep.2013.11.014

131. Shirakihara T, Saitoh M, Miyazono K. Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. Mol Biol Cell. 2007;18: 3533–3544. doi:10.1091/mbc.E07-03-0249

132. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000;2: 76–83. doi:10.1038/35000025

133. Savagner P, Yamada KM, Thiery JP. The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J Cell Biol. 1997;137: 1403–1419.

134. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell. 2001;7: 1267–1278.

135. Thuault S, Valcourt U, Petersen M, Manfioletti G, Heldin C-H, Moustakas A. Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol. 2006;174: 175–183. doi:10.1083/jcb.200512110

136. Koinuma D, Tsutsumi S, Kamimura N, Taniguchi H, Miyazawa K, Sunamura M, et al. Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor beta signaling. Mol Cell Biol. 2009;29: 172–186. doi:10.1128/MCB.01038-08

137. Kowanetz M, Valcourt U, Bergstrom R, Heldin C-H, Moustakas A. Id2 and Id3 define the

potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. Mol Cell Biol. 2004;24: 4241–4254.

138. Kondo M, Cubillo E, Tobiume K, Shirakihara T, Fukuda N, Suzuki H, et al. A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal transdifferentiation. Cell Death Differ. 2004;11: 1092–1101. doi:10.1038/sj.cdd.4401467

139. Papageorgis P, Lambert AW, Ozturk S, Gao F, Pan H, Manne U, et al. Smad signaling is required to maintain epigenetic silencing during breast cancer progression. Cancer Res. 2010;70: 968–978. doi:10.1158/0008-5472.CAN-09-1872

140. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10: 593–601. doi:10.1038/ncb1722

141. Park S-M, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes & Development. 2008. pp. 894–907. doi:10.1101/gad.1640608

142. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 Family Inhibits Epithelial-Mesenchymal Transition and Cancer Cell Migration by Direct Targeting of E-cadherin Transcriptional Repressors ZEB1 and ZEB2. The Journal of Biological Chemistry. 2008. pp. 14910–14914. doi:10.1074/jbc.C800074200

143. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Reports. 2008. pp. 582–589. doi:10.1038/embor.2008.74

144. Morita T, Mayanagi T, Sobue K. Dual roles of myocardin-related transcription factors in epithelial mesenchymal transition via slug induction and actin remodeling. J Cell Biol. 2007;179: 1027–1042. doi:10.1083/jcb.200708174

145. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. 2002;21: 3749–3759. doi:10.1093/emboj/cdf366

146. Papageorgis P. TGFβ Signaling in Tumor Initiation, Epithelial-to-Mesenchymal Transition, and Metastasis. Journal of Oncology. 2015. doi:10.1155/2015/587193

147. Lamouille S, Derynck R. Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J Cell Biol. 2007;178: 437–451. doi:10.1083/jcb.200611146

148. Lamouille S, Connolly E, Smyth JW, Akhurst RJ, Derynck R. TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. J Cell Sci. 2012;125: 1259–1273. doi:10.1242/jcs.095299

149. Bakin A V, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000;275: 36803–36810. doi:10.1074/jbc.M005912200

150. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol. 2004;6: 931–940. doi:10.1038/ncb1173

151. Bachelder RE, Yoon S-O, Franci C, de Herreros AG, Mercurio AM. Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. J Cell Biol. 2005;168: 29–33. doi:10.1083/jcb.200409067

152. Chaudhury A, Hussey GS, Ray PS, Jin G, Fox PL, Howe PH. TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. Nat Cell Biol. 2010;12: 286–293. doi:10.1038/ncb2029

153. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425: 577–584. doi:10.1038/nature02006

154. Marchetti A, Colletti M, Cozzolino AM, Steindler C, Lunadei M, Mancone C, et al. ERK5/MAPK is activated by TGFbeta in hepatocytes and required for the GSK-3beta-mediated Snail protein stabilization. Cell Signal. 2008;20: 2113–2118. doi:10.1016/j.cellsig.2008.08.002

155. Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M. Role of Ras signaling in the induction of snail by transforming growth factor-beta. J Biol Chem. 2009;284: 245–253. doi:10.1074/jbc.M804777200

156. Morel A-P, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast

cancer stem cells through epithelial-mesenchymal transition. PLoS One. 2008;3: e2888. doi:10.1371/journal.pone.0002888

157. Scheel C, Eaton EN, Li SH-J, Chaffer CL, Reinhardt F, Kah K-J, et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell. 2011;145: 926–940. doi:10.1016/j.cell.2011.04.029

158. Buijs JT, van der Horst G, van den Hoogen C, Cheung H, de Rooij B, Kroon J, et al. The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. Oncogene. 2012;31: 2164–2174. doi:10.1038/onc.2011.400

159. Santibanez JF. JNK mediates TGF-beta1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes. FEBS Lett. 2006;580: 5385–5391. doi:10.1016/j.febslet.2006.09.003

160. Tavares ALP, Mercado-Pimentel ME, Runyan RB, Kitten GT. TGF beta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. Dev Dyn. 2006;235: 1589–1598. doi:10.1002/dvdy.20771

161. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell. 2001;12: 27–36.

162. Baker AT, Zlobin A, Osipo C. Notch-EGFR/HER2 Bidirectional Crosstalk in Breast Cancer. Front Oncol. 2014;4: 360. doi:10.3389/fonc.2014.00360

163. Rebay I, Fehon RG, Artavanis-Tsakonas S. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell. 1993;74: 319–329.

164. Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell. 1991;67: 687–699.

165. Fitzgerald K, Greenwald I. Interchangeability of Caenorhabditis elegans DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. Development. 1995;121: 4275–4282.

166. Henderson ST, Gao D, Christensen S, Kimble J. Functional domains of LAG-2, a putative

signaling ligand for LIN-12 and GLP-1 receptors in Caenorhabditis elegans. Mol Biol Cell. 1997;8: 1751–1762.

167. Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature. 1998;393: 382–386. doi:10.1038/30756

168. Mumm JS, Schroeter EH, Saxena MT, Griesemer A, Tian X, Pan DJ, et al. A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol Cell. 2000;5: 197–206.

169. Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, et al. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell. 2000;5: 207–216.

170. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Research. London; 2004. pp. R605-15. doi:10.1186/bcr920

171. Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat M-L, Oakes SR, et al. Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. Cell Stem Cell. 2008;3: 429–441. doi:10.1016/j.stem.2008.08.001

172. Grudzien P, Lo S, Albain KS, Robinson P, Rajan P, Strack PR, et al. Inhibition of Notch signaling reduces the stem-like population of breast cancer cells and prevents mammosphere formation. Anticancer Res. 2010;30: 3853–3867.

173. Farnie G, Clarke RB, Spence K, Pinnock N, Brennan K, Anderson NG, et al. Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways. J Natl Cancer Inst. 2007;99: 616–627. doi:10.1093/jnci/djk133

174. Harrison H, Farnie G, Howell SJ, Rock RE, Stylianou S, Brennan KR, et al. Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. Cancer Res. 2010;70: 709–718. doi:10.1158/0008-5472.CAN-09-1681

175. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and

normal mammary gland. J Clin Invest. 2007;117: 3988–4002. doi:10.1172/JCI32533

176. Sansone P, Storci G, Giovannini C, Pandolfi S, Pianetti S, Taffurelli M, et al. p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland expanded in vitro as mammospheres. Stem Cells. 2007;25: 807–815. doi:10.1634/stemcells.2006-0442

177. Zhao D, Mo Y, Li M-T, Zou S-W, Cheng Z-L, Sun Y-P, et al. NOTCH-induced aldehyde dehydrogenase 1A1 deacetylation promotes breast cancer stem cells. J Clin Invest. 2014;124: 5453–5465. doi:10.1172/JCI76611

178. Mamaeva V, Niemi R, Beck M, Ozliseli E, Desai D, Landor S, et al. Inhibiting Notch Activity in Breast Cancer Stem Cells by Glucose Functionalized Nanoparticles Carrying gamma-secretase Inhibitors. Mol Ther. 2016;24: 926–936. doi:10.1038/mt.2016.42

179. McGowan PM, Simedrea C, Ribot EJ, Foster PJ, Palmieri D, Steeg PS, et al. Notch1 inhibition alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer. Mol Cancer Res. 2011;9: 834–844. doi:10.1158/1541-7786.MCR-10-0457

180. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst. 2006;98: 1777–1785. doi:10.1093/jnci/djj495

181. Kim R-K, Kaushik N, Suh Y, Yoo K-C, Cui Y-H, Kim M-J, et al. Radiation driven epithelial-mesenchymal transition is mediated by Notch signaling in breast cancer. Oncotarget. 2016. doi:10.18632/oncotarget.10802

182. Azzam DJ, Zhao D, Sun J, Minn AJ, Ranganathan P, Drews-Elger K, et al. Triple negative breast cancer initiating cell subsets differ in functional and molecular characteristics and in γ-secretase inhibitor drug responses. EMBO Molecular Medicine. 2013. pp. 1502–1522. doi:10.1002/emmm.201302558

183. Noseda M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, et al. Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res. 2004;94: 910–917. doi:10.1161/01.RES.0000124300.76171.C9

184. Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, et al.

Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev. 2004;18: 99–115. doi:10.1101/gad.276304

185. Niessen K, Fu Y, Chang L, Hoodless PA, McFadden D, Karsan A. Slug is a direct Notch target required for initiation of cardiac cushion cellularization. J Cell Biol. 2008;182: 315–325. doi:10.1083/jcb.200710067

186. Leong KG, Niessen K, Kulic I, Raouf A, Eaves C, Pollet I, et al. Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. J Exp Med. 2007;204: 2935–2948. doi:10.1084/jem.20071082

187. Shao S, Zhao X, Zhang X, Luo M, Zuo X, Huang S, et al. Notch1 signaling regulates the epithelial-mesenchymal transition and invasion of breast cancer in a Slug-dependent manner. Mol Cancer. 2015;14: 28. doi:10.1186/s12943-015-0295-3

188. Sahlgren C, Gustafsson M V, Jin S, Poellinger L, Lendahl U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. Proceedings of the National Academy of Sciences of the United States of America. 2008. pp. 6392–6397. doi:10.1073/pnas.0802047105

189. Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, et al. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc Natl Acad Sci U S A. 1997;94: 2859–2863.

190. Hsieh J-C, Lee L, Zhang L, Wefer S, Brown K, DeRossi C, et al. Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell. 2003;112: 355–367.

191. Mikels AJ, Nusse R. Wnts as ligands: processing, secretion and reception. Oncogene. 2006;25: 7461–7468. doi:10.1038/sj.onc.1210053

192. Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z, et al. A mechanism for Wnt coreceptor activation. Mol Cell. 2004;13: 149–156.

193. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, et al. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science. 1998;280: 596–599.

194. Seeling JM, Miller JR, Gil R, Moon RT, White R, Virshup DM. Regulation of beta-

catenin signaling by the B56 subunit of protein phosphatase 2A. Science. 1999;283: 2089–2091.

195. Gao Z-H, Seeling JM, Hill V, Yochum A, Virshup DM. Casein kinase I phosphorylates and destabilizes the beta-catenin degradation complex. Proc Natl Acad Sci U S A. 2002;99: 1182–1187. doi:10.1073/pnas.032468199

196. Mao J, Wang J, Liu B, Pan W, Farr GH 3rd, Flynn C, et al. Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol Cell. 2001;7: 801–809.

197. Brannon M, Gomperts M, Sumoy L, Moon RT, Kimelman D. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. 1997;11: 2359–2370.

198. Brunner E, Peter O, Schweizer L, Basler K. pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature. 1997;385: 829–833. doi:10.1038/385829a0

199. Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, Brisken C, et al. Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development. 2004;131: 4819–4829. doi:10.1242/dev.01347

200. Veltmaat JM, Van Veelen W, Thiery JP, Bellusci S. Identification of the mammary line in mouse by Wnt10b expression. Dev Dyn. 2004;229: 349–356. doi:10.1002/dvdy.10441

201. van Amerongen R, Bowman AN, Nusse R. Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell. 2012;11: 387–400. doi:10.1016/j.stem.2012.05.023

202. Lamb R, Ablett MP, Spence K, Landberg G, Sims AH, Clarke RB. Wnt pathway activity in breast cancer sub-types and stem-like cells. PLoS One. 2013;8: e67811. doi:10.1371/journal.pone.0067811

203. Khalil S, Tan GA, Giri DD, Zhou XK, Howe LR. Activation status of Wnt/ss-catenin signaling in normal and neoplastic breast tissues: relationship to HER2/neu expression in human and mouse. PLoS One. 2012;7: e33421. doi:10.1371/journal.pone.0033421

204. Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM. WNT/β-catenin mediates radiation resistance of mouse mammary progenitor cells. Proceedings of the National Academy of Sciences of the United States of America. 2007. pp. 618–623. doi:10.1073/pnas.0606599104

205. Wu Y, Ginther C, Kim J, Mosher N, Chung S, Slamon D, et al. Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. Mol Cancer Res. 2012;10: 1597–1606. doi:10.1158/1541-7786.MCR-12-0155-T

206. Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J Cell Biol. 2000;148: 173–188.

207. Kim K, Lu Z, Hay ED. Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. Cell Biol Int. 2002;26: 463–476.

208. Plaks V, Brenot A, Lawson DA, Linneman J, Van Kappel EC, Wong K, et al. Lgr5 expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell reports. 2013. pp. 70–78. doi:10.1016/j.celrep.2012.12.017

209. Oskarsson T, Acharyya S, Zhang XH-F, Vanharanta S, Tavazoie SF, Morris PG, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. Nature medicine. 2011. pp. 867–874. doi:10.1038/nm.2379

210. Yang L, Tang H, Kong Y, Xie X, Chen J, Song C, et al. LGR5 Promotes Breast Cancer Progression and Maintains Stem-Like Cells Through Activation of Wnt/beta-Catenin Signaling. Stem Cells. 2015;33: 2913–2924. doi:10.1002/stem.2083

211. Chang Y-W, Su Y-J, Hsiao M, Wei K-C, Lin W-H, Liang C-L, et al. Diverse Targets of beta-Catenin during the Epithelial-Mesenchymal Transition Define Cancer Stem Cells and Predict Disease Relapse. Cancer Res. 2015;75: 3398–3410. doi:10.1158/0008-5472.CAN-14-3265

212. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, et al. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components.

Science. 1994;263: 92–95.

213. Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell. 1993;74: 237–244.

214. Heim MH, Kerr IM, Stark GR, Darnell JEJ. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. Science. 1995;267: 1347–1349.

215. Shuai K, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ, et al. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature. 1993;366: 580–583. doi:10.1038/366580a0

216. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JEJ. DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. EMBO J. 1996;15: 5616–5626.

217. Wang X, Wang G, Zhao Y, Liu X, Ding Q, Shi J, et al. STAT3 mediates resistance of CD44(+)CD24(-/low) breast cancer stem cells to tamoxifen in vitro. J Biomed Res. 2012;26: 325–335. doi:10.7555/JBR.26.20110050

218. Wei W, Tweardy DJ, Zhang M, Zhang X, Landua J, Petrovic I, et al. STAT3 signaling is activated preferentially in tumor-initiating cells in claudin-low models of human breast cancer. Stem Cells. 2014;32: 2571–2582. doi:10.1002/stem.1752

219. Lin L, Hutzen B, Lee H-F, Peng Z, Wang W, Zhao C, et al. Evaluation of STAT3 signaling in ALDH+ and ALDH+/CD44+/CD24- subpopulations of breast cancer cells. PLoS One. 2013;8: e82821. doi:10.1371/journal.pone.0082821

220. Dave B, Landis MD, Tweardy DJ, Chang JC, Dobrolecki LE, Wu M-F, et al. Selective small molecule Stat3 inhibitor reduces breast cancer tumor-initiating cells and improves recurrence free survival in a human-xenograft model. PLoS One. 2012;7: e30207. doi:10.1371/journal.pone.0030207

221. So JY, Smolarek AK, Salerno DM, Maehr H, Uskokovic M, Liu F, et al. Targeting CD44-STAT3 signaling by Gemini vitamin D analog leads to inhibition of invasion in basal-like breast cancer. PLoS One. 2013;8: e54020. doi:10.1371/journal.pone.0054020

222. Hernandez-Vargas H, Ouzounova M, Le Calvez-Kelm F, Lambert M-P, McKay-Chopin S, Tavtigian S V, et al. Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. Epigenetics. 2011;6: 428–439.

223. Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N, et al. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene. 2009;28: 2940–2947. doi:10.1038/onc.2009.180

224. XIE G, YAO Q, LIU Y, DU S, LIU A, GUO Z, et al. IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. International Journal of Oncology. 2012. pp. 1171–1179. doi:10.3892/ijo.2011.1275

225. Zhang F, Li C, Halfter H, Liu J. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene. 2003;22: 894–905. doi:10.1038/sj.onc.1206158

226. Park J, Schwarzbauer JE. Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition. Oncogene. 2014;33: 1649–1657. doi:10.1038/onc.2013.118

227. Guo L, Chen C, Shi M, Wang F, Chen X, Diao D, et al. Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition. Oncogene. 2013;32: 5272–5282. doi:10.1038/onc.2012.573

228. Davis FM, Azimi I, Faville RA, Peters AA, Jalink K, Putney JWJ, et al. Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. Oncogene. 2014;33: 2307–2316. doi:10.1038/onc.2013.187

229. Espinoza I, Pochampally R, Xing F, Watabe K, Miele L. Notch signaling: targeting cancer stem cells and epithelial-to-mesenchymal transition. OncoTargets and therapy. 2013. pp. 1249–1259. doi:10.2147/OTT.S36162

230. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, et al. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 1993;75: 1417–1430.

231. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, et al. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science. 1996;273: 663–666.

232. Parmantier E, Lynn B, Lawson D, Turmaine M, Namini SS, Chakrabarti L, et al. Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. Neuron. 1999;23: 713–724.

233. Alcedo J, Ayzenzon M, Von Ohlen T, Noll M, Hooper JE. The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell. 1996;86: 221–232.

234. Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DYR, Reiter JF. Vertebrate Smoothened functions at the primary cilium. Nature. 2005;437: 1018–1021. doi:10.1038/nature04117

235. Kinzler KW, Vogelstein B. The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol Cell Biol. 1990;10: 634–642.

236. Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B. The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature. 1988;332: 371–374. doi:10.1038/332371a0

237. Chen M-H, Wilson CW, Li Y-J, Law KK Lo, Lu C-S, Gacayan R, et al. Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes Dev. 2009;23: 1910–1928. doi:10.1101/gad.1794109

238. Cheung HO-L, Zhang X, Ribeiro A, Mo R, Makino S, Puviindran V, et al. The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. Sci Signal. 2009;2: ra29. doi:10.1126/scisignal.2000405

239. Cochrane CR, Szczepny A, Watkins DN, Cain JE. Hedgehog Signaling in the Maintenance of Cancer Stem Cells. Lo H-W, editor. Cancers. 2015. pp. 1554–1585. doi:10.3390/cancers7030851

240. Zhao H, Tang H, Xiao Q, He M, Zhao L, Fu Y, et al. The Hedgehog signaling pathway is associated with poor prognosis in breast cancer patients with the CD44+/CD24 phenotype. Mol Med Rep. 2016;14: 5261–5270. doi:10.3892/mmr.2016.5856

241. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al.

Molecular definition of breast tumor heterogeneity. Cancer Cell. 2007;11: 259–273. doi:10.1016/j.ccr.2007.01.013

242. Liu S, Dontu G, Mantle ID, Patel S, Ahn N, Jackson KW, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res. 2006;66: 6063–6071. doi:10.1158/0008-5472.CAN-06-0054

243. Tanaka H, Nakamura M, Kameda C, Kubo M, Sato N, Kuroki S, et al. The Hedgehog signaling pathway plays an essential role in maintaining the CD44+CD24-/low subpopulation and the side population of breast cancer cells. Anticancer Res. 2009;29: 2147–2157.

244. Yang N, Zhou T-C, Lei X, Wang C, Yan M, Wang Z-F, et al. Inhibition of Sonic Hedgehog Signaling Pathway by Thiazole Antibiotic Thiostrepton Attenuates the CD44+/CD24-Stem-Like Population and Sphere-Forming Capacity in Triple-Negative Breast Cancer. Cell Physiol Biochem. 2016;38: 1157–1170. doi:10.1159/000443066

245. He M, Fu Y, Yan Y, Xiao Q, Wu H, Yao W, et al. The Hedgehog signalling pathway mediates drug response of MCF-7 mammosphere cells in breast cancer patients. Clin Sci (Lond). 2015;129: 809–822. doi:10.1042/CS20140592

246. Sun M, Zhang N, Wang X, Li Y, Qi W, Zhang H, et al. Hedgehog pathway is involved in nitidine chloride induced inhibition of epithelial-mesenchymal transition and cancer stem cells-like properties in breast cancer cells. Cell Biosci. 2016;6: 44. doi:10.1186/s13578-016-0104-8

247. Lu Y, Ma W, Mao J, Yu X, Hou Z, Fan S, et al. Salinomycin exerts anticancer effects on human breast carcinoma MCF-7 cancer stem cells via modulation of Hedgehog signaling. Chem Biol Interact. 2015;228: 100–107. doi:10.1016/j.cbi.2014.12.002

248. Fu Y-Z, Yan Y-Y, He M, Xiao Q-H, Yao W-F, Zhao L, et al. Salinomycin induces selective cytotoxicity to MCF-7 mammosphere cells through targeting the Hedgehog signaling pathway. Oncol Rep. 2016;35: 912–922. doi:10.3892/or.2015.4434

249. Memmi EM, Sanarico AG, Giacobbe A, Peschiaroli A, Frezza V, Cicalese A, et al. p63 Sustains self-renewal of mammary cancer stem cells through regulation of Sonic Hedgehog signaling. Proc Natl Acad Sci U S A. 2015;112: 3499–3504.

doi:10.1073/pnas.1500762112

250. Han B, Qu Y, Yu-Rice Y, Johnson J, Cui X. FOXC1-induced Gli2 activation: A non-canonical pathway contributing to stemness and anti-Hedgehog resistance in basal-like breast cancer. Mol Cell Oncol. 2016;3: e1131668. doi:10.1080/23723556.2015.1131668

251. Shlyueva D, Stampfel G, Stark A. Transcriptional enhancers: from properties to genome-wide predictions. Nat Rev Genet. 2014;15: 272–286. doi:10.1038/nrg3682

252. Banerji J, Rusconi S, Schaffner W. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. Cell. 1981;27: 299–308.

253. Calo E, Wysocka J. Modification of enhancer chromatin: what, how, and why? Mol Cell. 2013;49: 825–837. doi:10.1016/j.molcel.2013.01.038

254. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489: 57–74. doi:10.1038/nature11247

255. Hnisz D, Abraham BJ, Lee TI, Lau A, Saint-André V, Sigova AA, et al. Transcriptional super-enhancers connected to cell identity and disease. Cell. 2013. doi:10.1016/j.cell.2013.09.053

256. Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, et al. Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes. Cell. 2013. pp. 307–319. doi:10.1016/j.cell.2013.03.035

257. Ounzain S, Pedrazzini T. Super-enhancer lncs to cardiovascular development and disease. Biochim Biophys Acta. 2016;1863: 1953–1960. doi:10.1016/j.bbamcr.2015.11.026

258. Allen BL, Taatjes DJ. The Mediator complex: a central integrator of transcription. Nat Rev Mol Cell Biol. 2015;16: 155–166. doi:10.1038/nrm3951

259. Zuin J, Dixon JR, van der Reijden MIJA, Ye Z, Kolovos P, Brouwer RWW, et al. Cohesin and CTCF differentially affect chromatin architecture and gene expression in human cells. Proc Natl Acad Sci U S A. 2014;111: 996–1001. doi:10.1073/pnas.1317788111

260. von Mikecz A, Zhang S, Montminy M, Tan EM, Hemmerich P. CREB-binding protein (CBP)/p300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. J Cell Biol. 2000;150: 265–273.

261. Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. Genome Res. 2009;19: 590–601. doi:10.1101/gr.086983.108

262. Fujisawa T, Filippakopoulos P. Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. Nat Rev Mol Cell Biol. 2017;18: 246–262. doi:10.1038/nrm.2016.143

263. Seo S, Richardson GA, Kroll KL. The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. Development. 2005;132: 105–115. doi:10.1242/dev.01548

264. Whyte WA, Bilodeau S, Orlando DA, Hoke HA, Frampton GM, Foster CT, et al. Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature. 2012;482: 221–225. doi:10.1038/nature10805

265. Kim T-K, Hemberg M, Gray JM. Enhancer RNAs: a class of long noncoding RNAs synthesized at enhancers. Cold Spring Harb Perspect Biol. 2015;7: a018622. doi:10.1101/cshperspect.a018622

266. Weber F, de Villiers J, Schaffner W. An SV40 “enhancer trap” incorporates exogenous enhancers or generates enhancers from its own sequences. Cell. 1984;36: 983–992.

267. Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, et al. H3.3/H2A.Z double variant-containing nucleosomes mark “nucleosome-free regions” of active promoters and other regulatory regions. Nat Genet. 2009;41: 941–945. doi:10.1038/ng.409

268. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature. 2009;459: 108–112. doi:10.1038/nature07829

269. Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J. A unique chromatin signature uncovers early developmental enhancers in humans. Nature. 2011;470: 279–283. doi:10.1038/nature09692

270. Gross DS, Garrard WT. Nuclease hypersensitive sites in chromatin. Annu Rev Biochem. 1988;57: 159–197. doi:10.1146/annurev.bi.57.070188.001111

271. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native

chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013;10: 1213–1218. doi:10.1038/nmeth.2688

272. Dickel DE. Function-based Identification of Mammalian Enhancers Using Site-Specific Integration. 2014. pp. 566–571. doi:10.1038/nmeth.2886

273. Visel A, Blow MJ, Li Z, Zhang T, Akiyama JA, Holt A, et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature. 2009;457: 854–858. doi:10.1038/nature07730

274. Mansour MR, Abraham BJ, Anders L, Berezovskaya A, Gutierrez A, Durbin AD, et al. Oncogene regulation. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. Science. 2014;346: 1373–1377. doi:10.1126/science.1259037

275. Parker SCJ, Stitzel ML, Taylor DL, Orozco JM, Erdos MR, Akiyama JA, et al. Chromatin stretch enhancer states drive cell-specific gene regulation and harbor human disease risk variants. Proceedings of the National Academy of Sciences of the United States of America. 2013. pp. 17921–17926. doi:10.1073/pnas.1317023110

276. Nord AS, Blow MJ, Attanasio C, Akiyama JA, Holt A, Hosseini R, et al. Rapid and pervasive changes in genome-wide enhancer usage during mammalian development. Cell. 2013;155: 1521–1531. doi:10.1016/j.cell.2013.11.033

277. Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation. Science. 2002;295: 1306–1311. doi:10.1126/science.1067799

278. Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed Y Bin, et al. An oestrogen-receptor-alpha-bound human chromatin interactome. Nature. 2009;462: 58–64. doi:10.1038/nature08497

279. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell. 2013;153: 320–334. doi:10.1016/j.cell.2013.03.036

280. Jung YH, Sauria MEG, Lyu X, Cheema MS, Ausio J, Taylor J, et al. Chromatin States in Mouse Sperm Correlate with Embryonic and Adult Regulatory Landscapes. Cell Rep.

2017;18: 1366–1382. doi:10.1016/j.celrep.2017.01.034

281. Gong Y, Lazaris C, Sakellaropoulos T, Lozano A, Kambadur P, Ntziachristos P, et al. Stratification of TAD boundaries reveals preferential insulation of super-enhancers by strong boundaries. Nat Commun. 2018;9: 542. doi:10.1038/s41467-018-03017-1

282. Wei Y, Zhang S, Shang S, Zhang B, Li S, Wang X, et al. SEA: a super-enhancer archive. Nucleic Acids Res. 2016;44: D172-9. doi:10.1093/nar/gkv1243

283. Hnisz D, Schuijers J, Lin CY, Weintraub AS, Abraham BJ, Lee TI, et al. Convergence of developmental and oncogenic signaling pathways at transcriptional super-enhancers. Mol Cell. 2015;58: 362–370. doi:10.1016/j.molcel.2015.02.014

284. Chipumuro E, Marco E, Christensen CL, Kwiatkowski N, Zhang T, Hatheway CM, et al. CDK7 inhibition suppresses super-enhancer-linked oncogenic transcription in MYCN-driven cancer. Cell. 2014;159: 1126–1139. doi:10.1016/j.cell.2014.10.024

285. Herranz D, Ambesi-Impiombato A, Palomero T, Schnell SA, Belver L, Wendorff AA, et al. A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. Nat Med. 2014;20: 1130–1137. doi:10.1038/nm.3665

286. Groschel S, Sanders MA, Hoogenboezem R, de Wit E, Bouwman BAM, Erpelinck C, et al. A single oncogenic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in leukemia. Cell. 2014;157: 369–381. doi:10.1016/j.cell.2014.02.019

Chapter 2. Aims and hypothesis

Recent preclinical and clinical studies show the association of nuclear HER2 fragments with poor prognosis and increased tumor cell growth and invasion. Nuclear HER2 fragments are described as C-terminal truncated HER2 fragments possessing intracellular parts with kinase activity but without extracellular part. Nuclear HER2 usually arises by alternative splicing of ERBB2 mRNA leading to initialing of translation from the intracellular part. In addition, cleavage of full-length HER2 (p185HER2) from intracellular juxtamembrane regions, just bellow the transmembrane domain can produce free C-terminal truncated HER2 fragments with 80-90 kDa molecular weight (called p85HER2) with the ability to traffic inside the cytoplasm and translocate into the nucleus since it possesses nuclear localization signal (NLS) sequences. In this case, the extracellular part of the HER2 can still remain at the plasma membrane due to possessing the transmembrane domain. However, cleavage from the extracellular juxtamembrane region that occurs majorly by matrix metalloproteinases (MMPs), results in shedding of the extracellular part and production of 95 kDa HER2 (called p95HER2) which remains encored at the plasma membrane since it still possesses the transmembrane domain. Both the HER2 cleavage mechanisms and production of p85HER2 and p95HER2 fragments have been reported to be associated with trastuzumab resistance, higher rate of tumor growth, poor clinical outcome, and have been suggested to be used as poor prognosis and predictive trastuzumab resistance markers in clinical investigations.

2.1. TO STUDY THE EFFECTS OF TRASTUZUMAB ON HER2 RECEPTOR ACTIVATION

Despite striking clinical outcome of trastuzumab, the exact mode of action and resistance mechanisms remain ambiguous. It showed strong antitumor effects in both the mouse model and HER2-positive breast cancer patients [1,2]. While many mechanisms have been proposed for the antitumor activity of trastuzumab, including both extracellular and intracellular actions [1,2], little is known about the exact mechanisms of action. The extracellular action is through

immune-mediated response. When bound to the target cells, the Fc portion of trastuzumab will be recognized and attacked by the Fc receptorS of cytotoxic immune cells and initialing ADCC. There is solid evidence to support ADCC as a major mechanism for trastuzumab action [3–10]. On the other hand, the data regarding the intracellular mechanisms are either controversial at the beginning or challenged by the recent data [11]. initial studies report that binding trastuzumab to HER2 inhibits RTK signaling pathways bu blocking HER2 receptor activation. However, several other studies disprove this conclusion and suggest that trastuzumab has no major effect of HER2 receptor activation and subsequently its canonical downstream pathways [1,2].

The aim of this part of our study was to investigate the effects of trastuzumab on homodimerization and tyrosine residue phosphorylation of HER2 receptor as well as on the activation of Pi3K/Akt and MAPK pathways. We also studied whether treating the HER2-positive cells with trastuzumab activates the ADCC of the cells. To achieve our objective, we adopted a Chinese hamster ovary (CHO) cell model. Besides the parental CHO cell line that does not express any detectable HER receptors, three stable CHO cell lines that stably express only a single HER receptor including EGFR (CHO-EGFR), HER2 (CHO-K6), and HER3 (CHO-HER3) were employed in this research.

2.2. TO STUDY THE EFFECTS OF PERTUZUMAB AND ITS COMBINATION WITH TRASTUZUMAB ON HER2 RECEPTOR ACTIVATION AND GENE EXPRESSION

Pertuzumab another fully-humanized recombinant anti-HER2 monoclonal antibody is approved by the FDA to be used as neoadjuvant in combination with trastuzumab and docetaxel for the treatment of early-stage and metastatic HER2-positive breast cancer [12–14]. In addition to induction ADCC, pertuzumab also showed to inhibit HER2-positive cancer cell proliferation in the absence of immune cells, implicating the anti-cancer effects of the pertuzumab through alteration of HER2-mediated signaling pathways [15–17]. Pertuzumab binds to the dimerization pocket in the domain II of the extracellular part of HER2 that is believed to inhibit HER2/EGFR [18] and HER2/HER3 heterodimerizations [19–22]. Since the heterodimerization between HER2

and EGFR/HER3 is induced by ligand-binding, pertuzumab is believed to blocks ligand-dependent activation of HER2 and downstream signaling [18,21–23].

Adding pertuzumab to trastuzumab and docetaxel has produced better outcomes than treatment with trastuzumab and docetaxel alone, including a significant improvement in progression-free and overall survival rates [24–26]. The aim of this part of our study was to assess the effects of pertuzumab and its combination with trastuzumab on HER2 receptor homodimerization and activation phosphorylation using CHO cell line models described is the previous section. We also aimed to investigate the whole transcriptome profile of the CHO-K6 cells under treatment with pertuzumab, trastuzumab and their combination to examine the effects of the monoclonal antibodies on cell cycle, survival, and apoptosis. This study will allow us to understand and whether the monoclonal antibodies exert their antiproliferative effect via blocking HER2 activation or not.

2.3. TO STUDY THE EFFECT OF TRASTUZUMAB ON HER2 NON-CANONICAL PATHWAY

Recent preclinical and clinical studies show the association of nuclear HER2 fragments with poor prognosis and increased tumor cell growth and invasion. Nuclear HER2 fragments are described as C-terminal truncated HER2 fragments possessing intracellular parts with kinase activity but without extracellular part. Nuclear HER2 usually arises by alternative splicing of ERBB2 mRNA leading to initialing of translation from the intracellular part. In addition, cleavage of full-length HER2 (p185HER2) from intracellular juxtamembrane regions, just bellow the transmembrane domain can produce free C-terminal truncated HER2 (ctHER2) fragments with 80-90 kDa molecular weight (called p85HER2) with ability to traffic inside the cytoplasm and translocate into the nucleus since possesses nuclear localization signal (NLS) sequences. In this case, extracellular part of the HER2 can still remain at the plasma membrane due to possessing the transmembrane domain. However, cleavage from the extracellular juxtamembrane region that occurs majorly by matrix metalloproteinases (MMPs), results in shedding of the extracellular part and production of 95 kDa HER2 (called p95HER2) which

remains encored at the plasma membrane since it still possesses the transmembrane domain. Both the HER2 cleavage mechanisms and production of p85HER2 and p95HER2 have been reported to be associated with trastuzumab resistance, higher rate of tumor growth, poor clinical outcome, and have been suggested to be used as poor prognosis and predictive trastuzumab resistance markers in clinical investigations.

ctHER2 is a client for heat shock protein 90 (HSP90) chaperone protein which is important for HER2 trafficking [27]. HER2 is shown to translocate to the nucleus, enrich at the chromatin and regulate transcription (Figure 2.1A). It has been demonstrated that HER2 binds to a promoter chromatin of *PTGS2* gene (coding for COX2 protein) and increase the transcription of COX2 [28]. Another study showed that HER2 regulates the promoter activity of the *CCND1* gene (coding for Cyclin D1) in a complex with STAT3, the regulation which is associated with poor clinical outcome [29,30]. In addition, nuclear HER2 increases the transcription of microRNA-21 [31], ribosomal RNA [32] and RNA polymerase I [32] by enriching at the promoter chromatin of the genes. Further, HER2 stabilizes the mRNA of Na^+-HCO_3^- co-transporter SLC4A7 mRNA, an oncogenic protein in breast cancer, by direct binding to 3′UTR sequence of the mRNA that results in increased protein synthesis of SLC4A7 [33]. Cohort studies showed association of C-terminal truncated HER2 proteins with breast cancer poor prognosis, metastasis, worse therapy outcome and trastuzumab resistance [34–37]. Overexpressing ctHER2 increased tumor growth and trastuzumab resistance, however, inhibition of ctHER2 nuclear localization by blocking HSP90 and deleting HER2 NLS resulted in reduced tumor growth, invasion and overcame trastuzumab resistance [30,38].

In this part of our study, we aimed to study the effect of trastuzumab on HER2 cleavage, production and nuclear localization of ctHER2 as well as the function of nuclear HER2 in HER2-positive breast cancer cells. We hypothesize that (i) Binding trastuzumab to HER2 prevents cleavage of full-length HER2 and inhibits translocation of ctHER2 (Figure 2.1B) and (ii) nuclear HER2 mediates in the regulation of gene expression by contributing to the transcription complex.

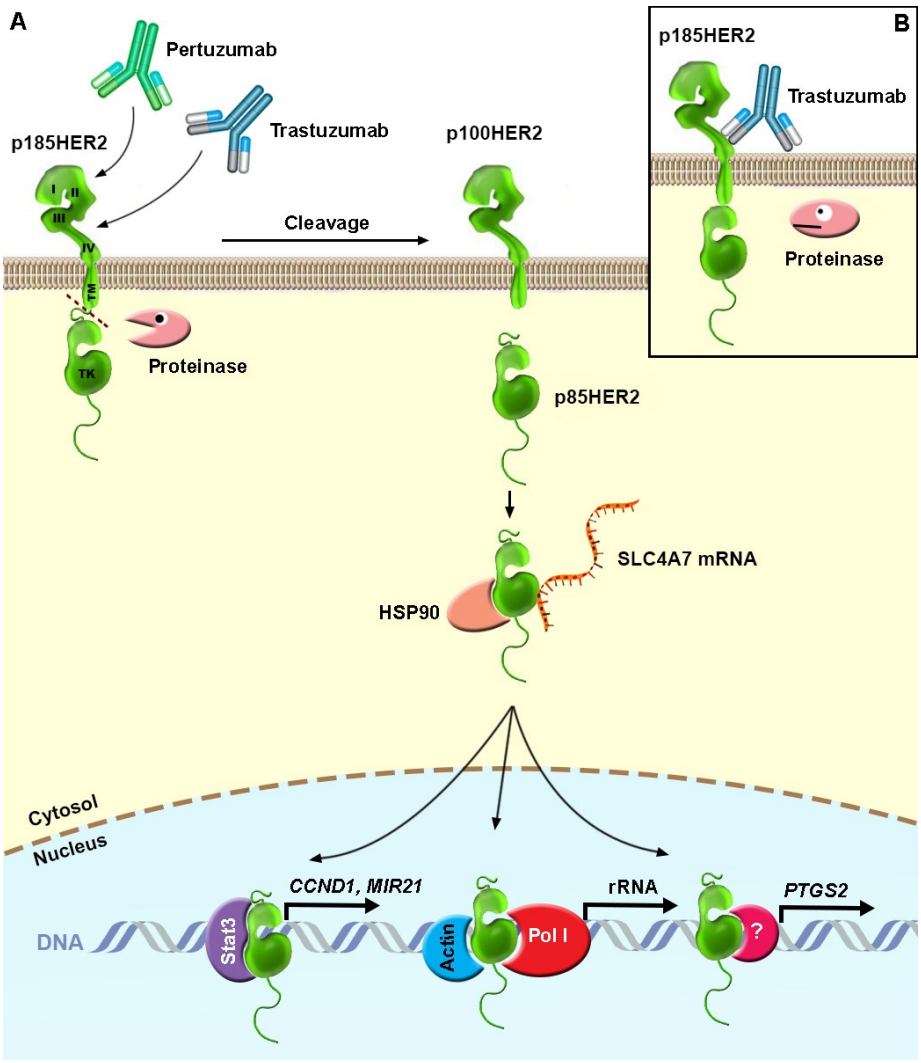

Figure 2.1. Model showing mechanism of cleavage and transcription co-factor function of p85HER2. (**A**) Full-length membranous HER2 (p185HER2) can be cleaved from the juxtamembrane region by metalloproteinases or intracellular proteinases that results in releasing p85HER2 from the membrane. p85HER2 can bind to 3'UTR of SLC4A7 mRNA and stabilize the mRNA that leads to increased expression of SLC4A7 protein. By engaging heat shock protein 90 kDa (HSP90) or some other chaperons, p85HER2 can translocate to the nucleus wherein it makes a complex with actin that binds to RNA polymerase I (Pol I) resulting in increased synthesis of ribosomal RNA (rRNA). Nuclear p85HER2 can also make a complex with STAT3 which can regulate the expression of CCND1 (coding for Cyclin D1) and MIR21 (coding for microRNA-21) genes. p85HER2 can also bind to the promoter of the PTGS2 gene (coding for COX2) and increase the expression of COX2 protein. This

regulation can lead breast tumor cells to more growth and proliferation. (**B**) Model of our hypothesis. Binding trastuzumab to domain IV of the extracellular part of p185HER2 changes its confirmation burying the juxtamembrane cleavage site that prevents cleavage of HER2 by client proteinases.

2.4. TO STUDY THE EPIGENETIC MECHANISM OF TRASTUZUMAB RESISTANCE

Over 60% of all HER2-positive breast cancers develop resistance to HER2-targeting agents lapatinib and trastuzumab. This is mainly due to the loss of expression of HER2 on the tumor cells. A potential mechanism of HER2 downregulation is epigenetic regulations including chromatin remodeling of *ERBB2* gene. A drastic global epigenetic reprogramming of epithelial cells happens during EMT which is characterized by wide-scale chromatin remodeling of genes involved in the emergence of epithelial and mesenchymal phenotypes. Many studies demonstrate that HER2-high cells mostly show epithelial phenotype, whereas HER2-low cells exhibit high mesenchymal phenotype, therefore mesenchymal-like cells are intrinsically resistant to HER2-targeting therapies. For example, JIMT-1 cell line is a HER2+ breast cancer cell line that can quickly develop resistance to trastuzumab. Studies showed that JIMT-1 was composed of approximately 10% CD44+/CD24- BCSC in initial cultures. This level rose to 85% at the late-passages [39]. Concurrently, the level of HER2 expression significantly reduced in late-passage cultures when compared to the early cultures. This regulation was associated with the development of trastuzumab-resistance. High passage JIMT-1 cells that were enriched mesenchymal CD44+/CD24- BCSCs expressing a lower level of HER2 also exhibited a highly-migratogenic phenotype and produced pro-invasive/metastatic proteins more than low-passage JIMT-1 cells culture. This phenomenon may explain the resistance of HER2-high breast tumors to trastuzumab due to an increased population of HER2-low CD44+/CD24 mesenchymal cells at the late-passages. Further, the CD44+/CD24- cells escape from trastuzumab-mediated ADCC. The cells could survive the immunoselection process in breast cancer cells co-cultured with NK cells and trastuzumab. This resistance may be attributed to the reduced HER2 expression levels on their surface [40]. Overall, this evidence suggests that EMT of HER2-high breast cancer cells

may lead to the development of trastuzumab resistance by increasing the population of HER2-low mesenchymal cells.

In this part of our study, we suggest EMT as an authentic mechanism of trastuzumab resistance in breast cancer. We hypothesize that epigenetic reprogramming during EMT of trastuzumab-responsive HER2-high epithelial-like breast cancer cells results in the inactivation of *ERBB2* chromatin, downregulation of HER2 expression and emergence of trastuzumab-refractory HER2-low mesenchymal-like breast cancer cells. To investigate this hypothesis, we aimed to study the chromatin architecture of *ERBB2* gene and its association with HER2 expression as well as epithelial and mesenchymal phenotypes in breast cancer.

2.5. REFERENCES

1. Spector NL, Blackwell KL. Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol. 2009;27: 5838–5847. doi:10.1200/JCO.2009.22.1507
2. Fiszman GL, Jasnis MA. Molecular Mechanisms of Trastuzumab Resistance in HER2 Overexpressing Breast Cancer. Int J Breast Cancer. 2011;2011: 352182. doi:10.4061/2011/352182
3. Clynes RA, Towers TL, Presta LG, Ravetch J V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med. 2000;6: 443–446. doi:10.1038/74704
4. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br J Cancer. 2006;94: 259–267. doi:10.1038/sj.bjc.6602930
5. Varchetta S, Gibelli N, Oliviero B, Nardini E, Gennari R, Gatti G, et al. Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res. 2007;67: 11991–11999. doi:10.1158/0008-5472.CAN-07-2068

6. Kute T, Stehle JRJ, Ornelles D, Walker N, Delbono O, Vaughn JP. Understanding key assay parameters that affect measurements of trastuzumab-mediated ADCC against Her2 positive breast cancer cells. Oncoimmunology. 2012;1: 810–821. doi:10.4161/onci.20447

7. Petricevic B, Laengle J, Singer J, Sachet M, Fazekas J, Steger G, et al. Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent in both adjuvant and metastatic HER2/neu breast cancer patients. J Transl Med. 2013;11: 307. doi:10.1186/1479-5876-11-307

8. Duong MN, Cleret A, Matera E-L, Chettab K, Mathe D, Valsesia-Wittmann S, et al. Adipose cells promote resistance of breast cancer cells to trastuzumab-mediated antibody-dependent cellular cytotoxicity. Breast Cancer Res. 2015;17: 57. doi:10.1186/s13058-015-0569-0

9. Shi Y, Fan X, Deng H, Brezski RJ, Rycyzyn M, Jordan RE, et al. Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcgamma receptors on macrophages. J Immunol. 2015;194: 4379–4386. doi:10.4049/jimmunol.1402891

10. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28: 803–814. doi:10.1038/onc.2008.432

11. Dokmanovic M, Wu Y, Shen Y, Chen J, Hirsch DS, Wu WJ. Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition. Cancer Biol Ther. 2014;15: 1029–1041. doi:10.4161/cbt.29171

12. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 1990;50: 1550–1558.

13. Fleeman N, Bagust A, Beale S, Dwan K, Dickson R, Proudlove C, et al. Pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2-positive metastatic or locally recurrent unresectable breast cancer. Pharmacoeconomics. 2015;33:

13–23. doi:10.1007/s40273-014-0206-2

14. Swain SM, Baselga J, Kim S-B, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015;372: 724–734. doi:10.1056/NEJMoa1413513

15. Brockhoff G, Heckel B, Schmidt-Bruecken E, Plander M, Hofstaedter F, Vollmann A, et al. Differential impact of Cetuximab, Pertuzumab and Trastuzumab on BT474 and SK-BR-3 breast cancer cell proliferation. Cell Prolif. 2007;40: 488–507. doi:10.1111/j.1365-2184.2007.00449.x

16. Nahta R, Hung M-C, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64: 2343–2346.

17. Diermeier-Daucher S, Breindl S, Buchholz S, Ortmann O, Brockhoff G. Modular anti-EGFR and anti-Her2 targeting of SK-BR-3 and BT474 breast cancer cell lines in the presence of ErbB receptor-specific growth factors. Cytometry A. 2011;79: 684–693. doi:10.1002/cyto.a.21107

18. Hughes JB, Berger C, Rodland MS, Hasmann M, Stang E, Madshus IH. Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization. Mol Cancer Ther. 2009;8: 1885–1892. doi:10.1158/1535-7163.MCT-09-0291

19. Baselga J. A new anti-ErbB2 strategy in the treatment of cancer: prevention of ligand-dependent ErbB2 receptor heterodimerization. Cancer Cell. 2002;2: 93–95.

20. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5: 317–328.

21. Sak MM, Szymanska M, Bertelsen V, Hasmann M, Madshus IH, Stang E. Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. Carcinogenesis. 2013;34: 2031–2038. doi:10.1093/carcin/bgt173

22. Sakai K, Yokote H, Murakami-Murofushi K, Tamura T, Saijo N, Nishio K. Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells

mediated by the HER3 signaling pathway. Cancer Sci. 2007;98: 1498–1503. doi:10.1111/j.1349-7006.2007.00553.x

23. King CR, Borrello I, Porter L, Comoglio P, Schlessinger J. Ligand-independent tyrosine phosphorylation of EGF receptor and the erbB-2/neu proto-oncogene product is induced by hyperosmotic shock. Oncogene. 1989;4: 13–18.

24. Baselga J, Cortes J, Kim S-B, Im S-A, Hegg R, Im Y-H, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med. 2012;366: 109–119. doi:10.1056/NEJMoa1113216

25. von Minckwitz G, Procter M, de Azambuja E, Zardavas D, Benyunes M, Viale G, et al. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer. N Engl J Med. 2017;377: 122–131. doi:10.1056/NEJMoa1703643

26. Swain SM, Kim S-B, Cortes J, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. Lancet Oncol. 2013;14: 461–471. doi:10.1016/S1470-2045(13)70130-X

27. Modi S, D'Andrea G, Norton L, Yao TJ, Caravelli J, Rosen PP, et al. A phase I study of cetuximab/paclitaxel in patients with advanced-stage breast cancer. Clin Breast Cancer. 2006;7: 270–277. doi:10.3816/CBC.2006.n.040

28. Wang S-C, Lien H-C, Xia W, Chen I-F, Lo H-W, Wang Z, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell. 2004;6: 251–261. doi:10.1016/j.ccr.2004.07.012

29. Beguelin W, Diaz Flaque MC, Proietti CJ, Cayrol F, Rivas MA, Tkach M, et al. Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. Mol Cell Biol. 2010;30: 5456–5472. doi:10.1128/MCB.00012-10

30. Cordo Russo RI, Beguelin W, Diaz Flaque MC, Proietti CJ, Venturutti L, Galigniana N, et al. Targeting ErbB-2 nuclear localization and function inhibits breast cancer growth and overcomes trastuzumab resistance. Oncogene. 2015;34: 3413–3428. doi:10.1038/onc.2014.272

31. Venturutti L, Romero L V, Urtreger AJ, Chervo MF, Cordo Russo RI, Mercogliano MF, et al. Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis. Oncogene. 2016;35: 2208–2222. doi:10.1038/onc.2015.281

32. Li L-Y, Chen H, Hsieh Y-H, Wang Y-N, Chu H-J, Chen Y-H, et al. Nuclear ErbB2 enhances translation and cell growth by activating transcription of ribosomal RNA genes. Cancer Res. 2011;71: 4269–4279. doi:10.1158/0008-5472.CAN-10-3504

33. Gorbatenko A, Olesen CW, Loebl N, Sigurdsson HH, Bianchi C, Pedraz-Cuesta E, et al. Oncogenic p95HER2 regulates Na+-HCO3- cotransporter NBCn1 mRNA stability in breast cancer cells via 3'UTR-dependent processes. Biochem J. 2016;473: 4027–4044. doi:10.1042/BCJ20160054

34. Duchnowska R, Sperinde J, Chenna A, Haddad M, Paquet A, Lie Y, et al. Quantitative measurements of tumoral p95HER2 protein expression in metastatic breast cancer patients treated with trastuzumab: independent validation of the p95HER2 clinical cutoff. Clin Cancer Res. 2014;20: 2805–2813. doi:10.1158/1078-0432.CCR-13-2782

35. Lipton A, Goodman L, Leitzel K, Cook J, Sperinde J, Haddad M, et al. HER3, p95HER2, and HER2 protein expression levels define multiple subtypes of HER2-positive metastatic breast cancer. Breast Cancer Res Treat. 2013;141: 43–53. doi:10.1007/s10549-013-2665-0

36. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, et al. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res. 2006;12: 424–431. doi:10.1158/1078-0432.CCR-05-1807

37. Sperinde J, Jin X, Banerjee J, Penuel E, Saha A, Diedrich G, et al. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin Cancer Res. 2010;16: 4226–4235. doi:10.1158/1078-0432.CCR-10-0410

38. Chandarlapaty S, Scaltriti M, Angelini P, Ye Q, Guzman M, Hudis CA, et al. Inhibitors of HSP90 block p95-HER2 signaling in Trastuzumab-resistant tumors and suppress their growth. Oncogene. 2010;29: 325–334. doi:10.1038/onc.2009.337

39. Oliveras-Ferraros C, Vazquez-Martin A, Martin-Castillo B, Cufi S, Del Barco S, Lopez-

Bonet E, et al. Dynamic emergence of the mesenchymal CD44(pos)CD24(neg/low) phenotype in HER2-gene amplified breast cancer cells with de novo resistance to trastuzumab (Herceptin). Biochem Biophys Res Commun. 2010;397: 27–33. doi:10.1016/j.bbrc.2010.05.041

40. Reim F, Dombrowski Y, Ritter C, Buttmann M, Hausler S, Ossadnik M, et al. Immunoselection of breast and ovarian cancer cells with trastuzumab and natural killer cells: selective escape of CD44high/CD24low/HER2low breast cancer stem cells. Cancer Res. 2009;69: 8058–8066. doi:10.1158/0008-5472.CAN-09-0834

Chapter 3. The effects of trastuzumab on HER2 receptor activation

3.6. SUMMARY

Targeted therapy with trastuzumab has become a mainstay for HER2-positive breast cancer without a clear understanding of the mechanism of its action. While many mechanisms have been suggested for the action of trastuzumab, most of them are not substantiated by experimental data. It has been suggested that trastuzumab functions by inhibiting intracellular signaling initiated by HER2, however, the data are very controversial. A major issue is the different cellular backgrounds of various breast cancer cell lines used in these studies. Each breast cancer cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab. To overcome this problem, in this research we adopted a cell model that allows us to specifically examine the effects of trastuzumab on a single HER receptor without the influence of other HER receptors. Three CHO cell lines stably expressing only human EGFR (CHO-EGFR), HER2 (CHO-K6), or HER3 (CHO-HER3) were used. Various methods including cytotoxicity assay, immunoblotting, indirect immunofluorescence, cross-linking, and ADCC were employed in this research. We showed that trastuzumab did not bind EGFR and HER3, and thus did not affect the homodimerization and phosphorylation of EGFR and HER3. However, overexpression of HER2 in CHO cells, in the absence of other HER receptors, resulted in the homodimerization of HER2 and the phosphorylation of HER2 at all major pY residues. Trastuzumab bound to HER2 specifically and with high affinity. Trastuzumab inhibited neither the homodimerization of HER2 nor the phosphorylation of HER2 at most phosphotyrosine residues. Moreover, trastuzumab did not inhibit the phosphorylation of Erk and Akt in CHO-K6 cells and did not inhibit the proliferation of CHO-K6 cells. However, trastuzumab induced strong ADCC in CHO-K6 cells. We concluded that, in the absence of other HER receptors, trastuzumab exerts its antitumor activity through the induction of ADCC, rather than the inhibition of HER2-homodimerization and phosphorylation.

3.7. INTRODUCTION

While many recent publications claim that early studies support the role of trastuzumab in inhibiting HER2 phosphorylation [15,16], many data indicate that trastuzumab either has no effect or stimulates HER2 phosphorylation [15–19]. The data regarding the effects of trastuzumab on the dimerization of HER2, activation of major signaling pathways including Akt and Erk [20,21], and HER2 endocytosis/downregulation [22–24] are all controversial. The data regarding the role of trastuzumab on DNA repair [25], proteolytic cleavage of HER2 extracellular domain [26], and angiogenesis [27,28] are very limited. The most controversial mechanism regarding trastuzumab function is its effect on the inhibition of HER2 activation. A major reason behind this controversy is the different cellular backgrounds of various breast cancer cell lines used in those studies. Each breast cancer cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab. To overcome this problem, in this research we adopted a cell model that allows us to specifically examine the effects of trastuzumab on a single HER receptor without the influence of other HER receptors.

The aim of this study was to investigate the effects of trastuzumab on induction of ADCC of the HER2+ cells, homodimerization and tyrosine residue phosphorylation of HER2 receptor as well as on the activation of Pi3K/AKT and MAPK pathways. In this study, we used CHO cell lines stably expressing high levels of HER2, or EGFR or HER3 that allow us to investigate HER2 receptor activity avoiding interaction of other HER receptor which functionally and structurally resemble to each other.

3.8. RESULTS

3.8.1. Stable CHO cell lines expressing EGFR, HER2, and HER3

HER2 heterodimerizes with EGFR and HER3 in response to ligand stimulation [29–31]. HER2 also homodimerizes and activates in cells with over-expressed HER2 [32–34]. Most HER2-positive breast cancer cells also express either EGFR, HER3 or both, which makes it difficult to explain the observed effects of trastuzumab. Thus, to understand the effects of trastuzumab on HER2-mediated cell signaling in breast cancer cells, we plan first to study the effects of trastuzumab in CHO cells that selectively express a single HER receptor. The results

from these CHO cells will unambiguously define the role of trastuzumab on HER2-mediated cell signaling under various expression profiles of HER receptors. Thus, these data could be used to accurately interpret observation in breast cancer cells. We have established CHO cell lines stably expressing EGFR (CHO-EGFR) [35]. CHO cells expressing HER2 (CHO-K6) or HER3 (CHO-HER3) were obtained from other labs [36,37]. Parental CHO cells were used as control. We confirmed the expression of HER receptors in these cell lines by immunoblotting and immunofluorescence. As shown in Figure 3.1, CHO-K6 cells expressed high level of HER2. CHO-EGFR cells expressed high level of EGFR. CHO-HER3 cells expressed high level of HER3 and the CHO parental cells did not express detectable HER2, EGFR and HER3.

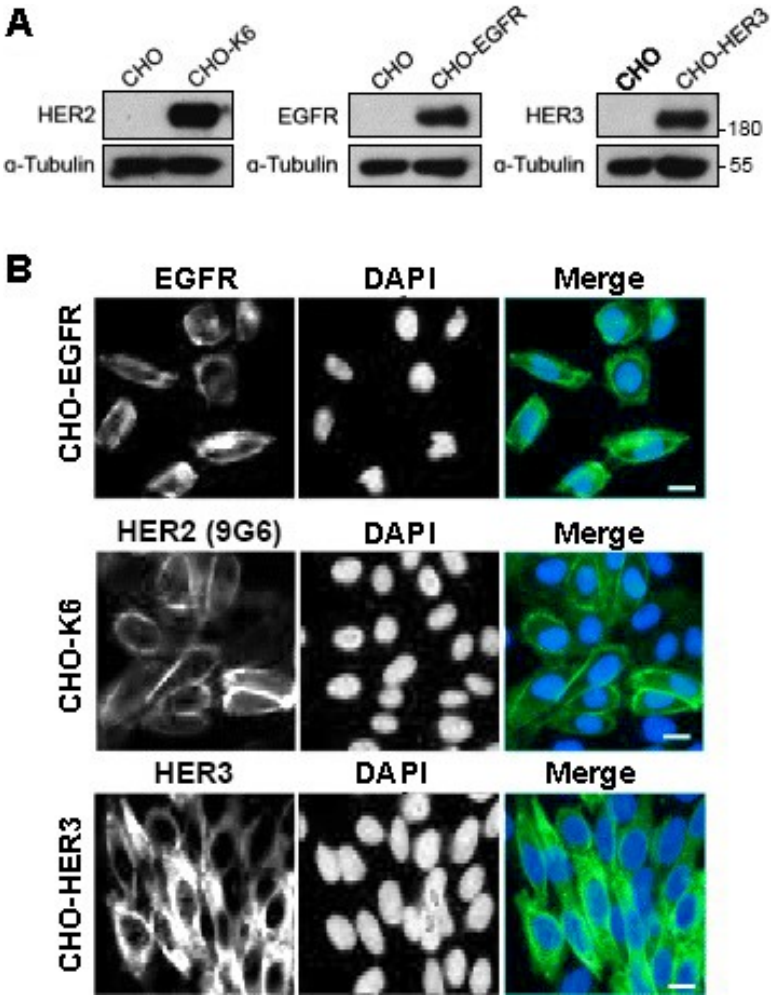

Figure 3.1. The expression of HER receptors in CHO cells stably transfected with a single HER receptor including CHO-EGFR, CHO-K6, and CHO-HER3. (**A**) Immunoblotting. The lysates of various CHO cells were separated by gel electrophoresis and immunoblotted with

antibodies to HER receptors as indicated. The parent CHO cells (CHO) were used as control. (**B**) Immunofluorescence. Various CHO cells were fixed and stained with antibodies to HER receptors as indicated. The expression of HER receptor was revealed by the FITC-conjugated secondary antibody (green). Cell nuclei were counterstained with DAPI. Scaled size of each picture width: 100 μm.

3.8.2. Binding of trastuzumab to HER receptors

While trastuzumab is an antibody to HER2, it is possible that it may weakly interact with EGFR and HER3 due to the sequence homology among these receptors. Thus, we next examined the binding of trastuzumab to HER2, EGFR, and HER3. We showed by immunofluorescence that trastuzumab only bound to HER2, but not EGFR and HER3 (Figure 3.2). As shown in Figure 3.2 at the dosage ranging from 0.1 μg/ml to 10 μg/ml, trastuzumab showed strong binding to HER2 in CHO-K6 cells. HER2 was localized to the plasma membrane in CHO-K6 cells under all conditions as expected. Trastuzumab was also located to plasma membrane, co-localizing with HER2, which indicates the binding of trastuzumab to HER2 (Figure 3.2A). Plasma membrane localization of trastuzumab was increased with the increased dosage. We also determined the time course of trastuzumab binding to HER2 in CHO-K6 cells. As shown in Figure 3.2B at 5 minutes following trastuzumab addition, trastuzumab had already been well localized to the plasma membrane, indicating a rapid binding between trastuzumab and HER2. Longer incubation only partially increased the plasma membrane localization of trastuzumab. However, even at the high dosage of 10 μg/ml, no binding of trastuzumab to EGFR and HER3 was detectable in CHO-EGFR and CHO-HER3 cells, respectively (Figures 2.2C and D). These results indicate that trastuzumab binds to HER2 specifically with high affinity.

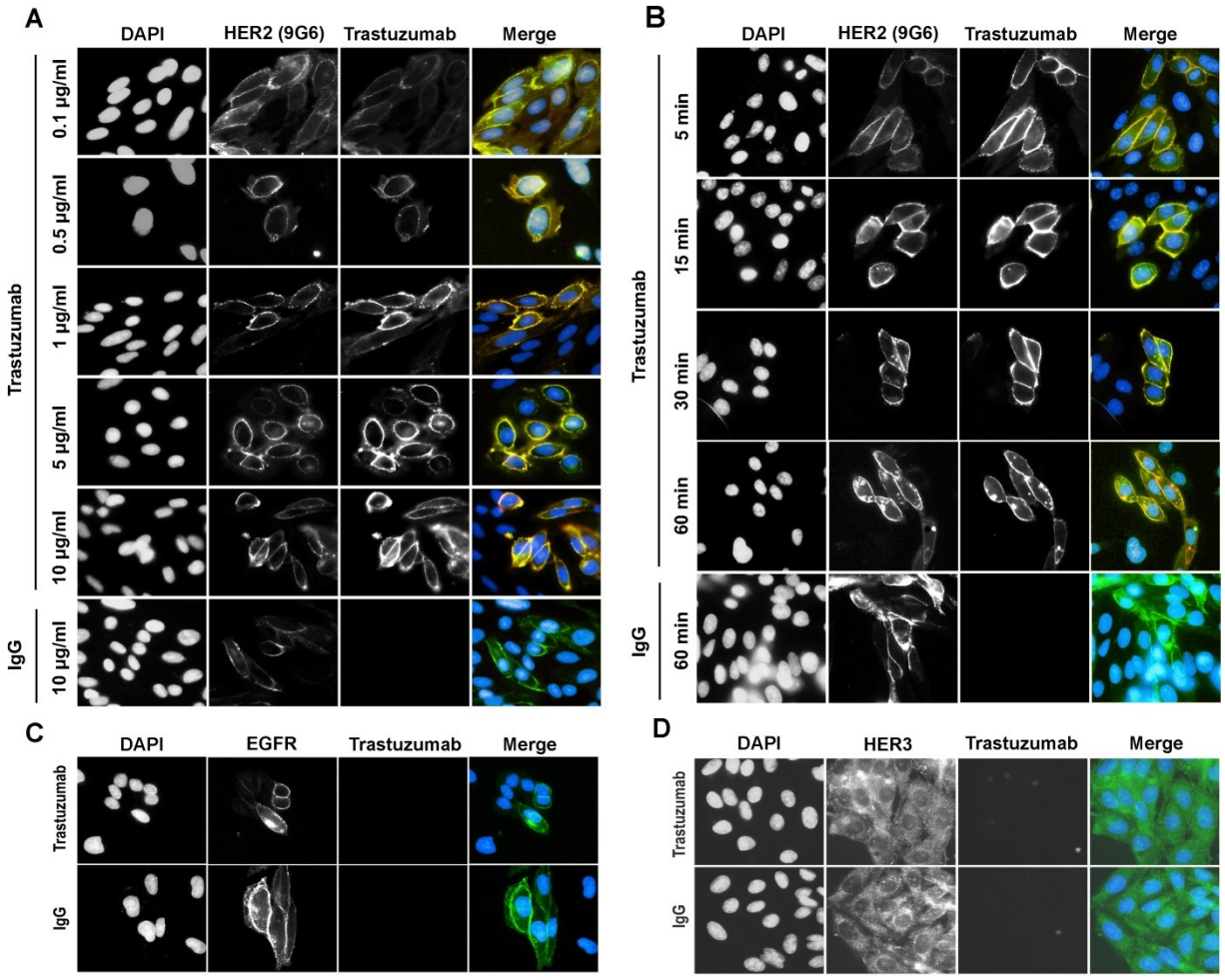

Figure 3.2. Binding of trastuzumab to HER receptors in CHO-K6, CHO-EGFR and CHO-HER3 cells as revealed by immunofluorescence. (**A** and **B**) The binding of trastuzumab to HER2 in CHO-K6 cells. CHO cells were treated with trastuzumab at various concentrations as indicated for 1 hour (**A**) or at various time periods as described at 10 µg/ml (**B**) as indicated. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER2 was revealed by the rabbit anti-HER2 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counterstained with DAPI. Yellow indicated the co-localization of trastuzumab and HER2. (**C**) The binding of trastuzumab to EGFR in CHO-EGFR cells. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of EGFR was revealed by the rabbit anti-EGFR antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counterstained with DAPI. (**D**)

The binding of trastuzumab to HER3 in CHO-HER3 cells. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER3 was revealed by the rabbit anti-HER3 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counterstained with DAPI. Scaled size of each picture width: 100 μm.

3.8.3. The effects of trastuzumab on the homodimerization of HER2

So far, the reports are controversial regarding the effects of trastuzumab on the dimerization or HER2. Here we examined the effects of trastuzumab on the homodimerization of HER2 by crosslinking and immunoblotting (Figure 3.3). As shown in Figure 3.3, the overexpression of HER2 by itself resulted in a high level of HER2 homodimerization. Clearly, trastuzumab did not block the homodimerization of HER2. Interestingly, with the increase of the dosage from 0.1 to 10 μg/ml, trastuzumab induced the dimerization of HER2 (Figure 3.3A). The induction of the homodimerization of HER2 by trastuzumab was even more visible in the time course experiments (Figure 3.3B).

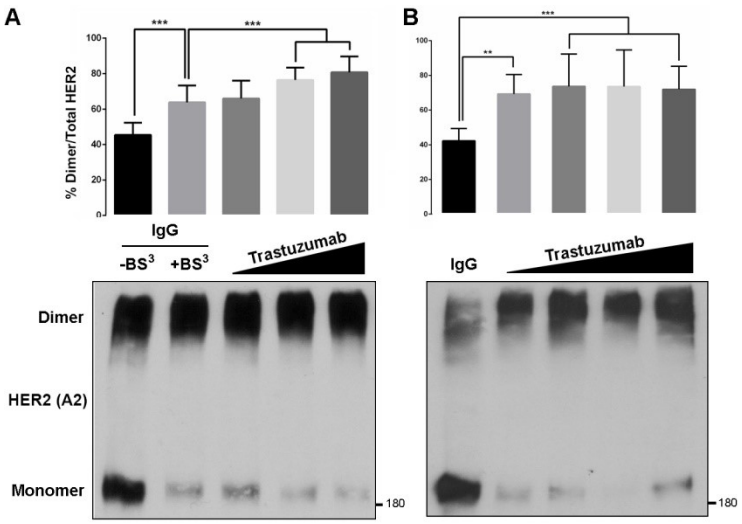

Figure 3.3. The effects of trastuzumab on HER2 homodimerization in CHO-K6 cells as revealed by crosslinking. Following trastuzumab treatment as indicated, CHO-K6 cells were treated with BS3 and the homodimerization of HER2 was revealed by immunoblotting as described in Materials and Methods. (**A**) CHO-K6 cells were treated with trastuzumab at various concentrations of 0.1, 1, and 10 μg/ml for 1 hour. (**B**) CHO-K6 cells were treated with

10 μg/ml trastuzumab for 15, 30, 60 and 120 minutes. Cells treated with normal human IgG (10 μg/ml) were used as control. The level of HER2 homodimerization was quantitated by densitometry and expressed as the ratio of dimer/total HER2. Each value is the average of at least three experiments and the error bar is the standard error. **: $P < 0.01$, ***: $P < 0.001$.

3.8.4. The effects of trastuzumab on the phosphorylation of HER2

Activated HER2 phosphorylates multiple tyrosine (Y) residues at its C-terminus. We have examined the phosphorylation of the following six tyrosine residues including Y1005, Y1112, Y1127, Y1139, Y1196, and Y1248 (Figures 2.4, 2.5 and 2.6). As shown by immunoblotting, for the control cells treated with normal IgG, HER2 was well phosphorylated in all of the pY residues examined (Figures 2.4A and B). The phosphorylation is likely due to the homodimerization induced by the overexpression of HER2. Treatment with trastuzumab at the dosage ranging from 0.1 μg/ml to 10 μg/ml did not significantly alter the phosphorylation levels of most phosphotyrosine residues including Y1005, Y1127, Y1196, and Y1248. However, trastuzumab partially inhibited the phosphorylation of Y1139. Similar to normal IgG, EGF did not have any effects on the phosphorylation of all the pY residues of HER2, which is not surprising as HER2 does not bind to EGF (Figures 2.4A and B). These results were confirmed by time-course experiments (Figure 3.4C). Treatment from 15 minutes up to 2 hours, did not change the phosphorylation levels of all pY residues except for pY1139 that is partially inhibited (Figure 3.4C).

As controls, we have examined the effects of trastuzumab on EGFR phosphorylation in CHO-EGFR cells. As shown in Figure 3.5, EGFR was not phosphorylated in CHO-EGFR cells, and the addition of EGF stimulated the phosphorylation of EGFR. Treatment with trastuzumab was not able to inhibit EGF-induced EGFR phosphorylation. Moreover, trastuzumab by itself did not have any detectable effects on EGFR phosphorylation in CHO-EGFR cells. We also examine the effects of a chemical inhibitor of HER2, CP-724714 on HER2 phosphorylation in CHO-K6 cells. As shown in Figure 3.5B, various concentrations of CP-724714 ranging from 1 - 100 μM significantly block the phosphorylation of HER2 at Y1005, which is in stark contrast from trastuzumab as shown in Figure 3.4. Furthermore, CP-724714 also significantly inhibited the phosphorylation of HER2 pY1139, however, trastuzumab only partially inhibited pY1139. These

results indicated that trastuzumab has little if any, inhibitory effects on HER2 activation/ phosphorylation.

We further examine the effects of trastuzumab on the phosphorylation of HER2 by indirect immunofluorescence (Figure 3.6). CHO-K6 cells either treated with trastuzumab or control IgG were double-stained for both trastuzumab (TRITC, red) and phospho-HER2 (FITC, green). Antibodies specific to six HER2 pY residues including Y1005, Y1112, Y1127, Y1139, Y1196, and Y1248 were used to determine the effects of trastuzumab on HER2 phosphorylation. As shown in Figure 3.6, HER2 was well phosphorylated on all of these six pY residues in the absence of trastuzumab, indicating the autophosphorylation due to overexpression. Treatment with trastuzumab at the concentration ranging from 0.1 - 10 μg/ml had no effects on the phosphorylation levels of these HER2 pY residues including Y1005 (Figure 3.6A), Y1112 (Figure 3.6B), Y1127 (Figure 3.6C), Y1196 (Figure 3.6E), and Y1248 (Figure 3.6F). However, for pY1139, trastuzumab at 1 - 10 μg/ml showed some inhibitory effect (Figure 3.6D).

Together, our results indicated that overexpression of HER2 resulted in strong HER2 phosphorylation in all its pY residues studied here. The addition of trastuzumab, in general, did not inhibit the phosphorylation of HER2. The only possible exception is that trastuzumab at higher dosage (1 - 10 μg/ml) partially reduced the phosphorylation of HER2 at pY1139.

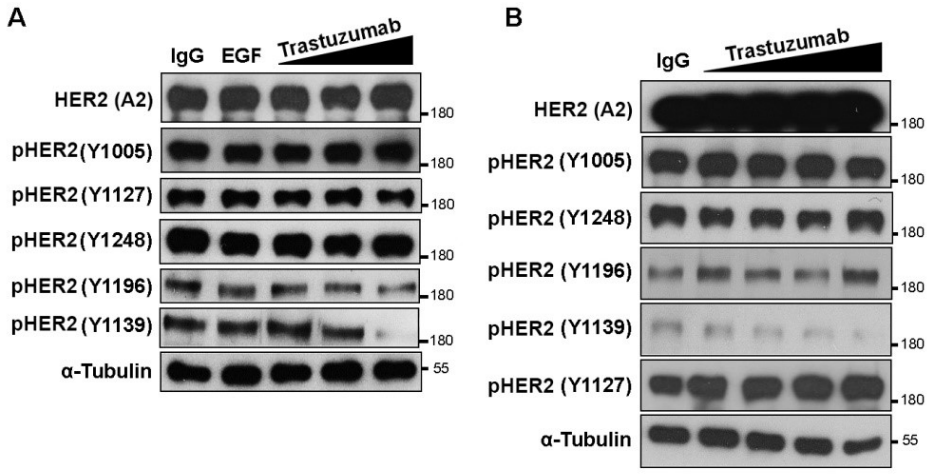

Figure 3.4. The effects of trastuzumab on HER2 phosphorylation in CHO-K6 cells by immunoblotting. (**A**) CHO-K6 cells were treated with trastuzumab at concentrations of 0.1, 1 and 10 μg/ml for 1 hour. The phosphorylation of HER2 at Y1005, Y1127, Y1139, Y1196, and

Y1248 were then examined by immunoblotting as described in Materials and Methods. Cells treated with normal human IgG (10 μg/ml) or EGF (50 ng/ml) were used as controls. The phosphorylation level of each HER2 pY residue was normalized against the expression level of tubulin. (**C**) Time-course experiments. CHO-K6 cells were treated with trastuzumab (10 μg/ml) for 15, 30, 60 and 120 minutes. The phosphorylation of various HER2 pY residues was examined by immunoblotting.

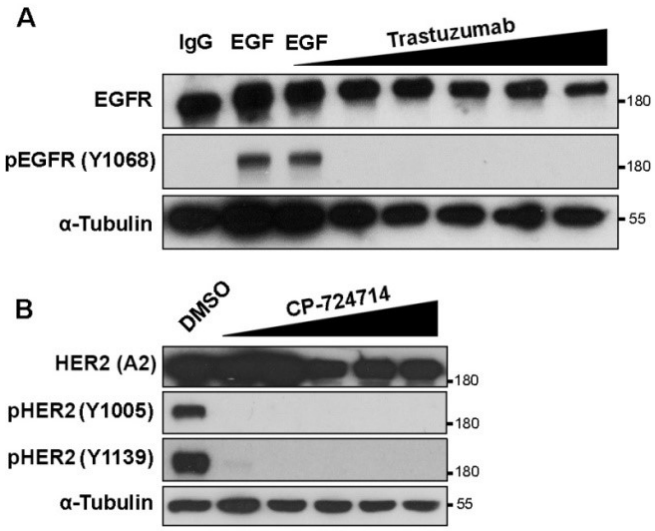

Figure 3.5. Control experiments to show the effects of trastuzumab on EGFR phosphorylation in CHO-EGFR cells and the effects of CP-724714 on HER2 phosphorylation in CHO-K6 cells. (**A**) The effects of trastuzumab on EGFR phosphorylation or EGF-induced EGFR phosphorylation in CHO-EGFR cells. Cells were treated with EGF (50 μg/ml) and/or trastuzumab (0.1, 0.5, 1, 5 and 10 μg/ml) for 1 hour. The phosphorylation of EGFR was determined by immunoblotting with antibody to EGFR pY1068. (**B**) The effects of a chemical inhibitor of HER2, CP-724714 on HER2 phosphorylation (at Y1005 and Y1139 residues) in CHO-K6 cells. Cells were treated with CP-724714 (1, 10, 20, 40 and 100 μM) for 1 hour. The phosphorylation of HER2 was examined by immunoblotting with antibodies against HER2.

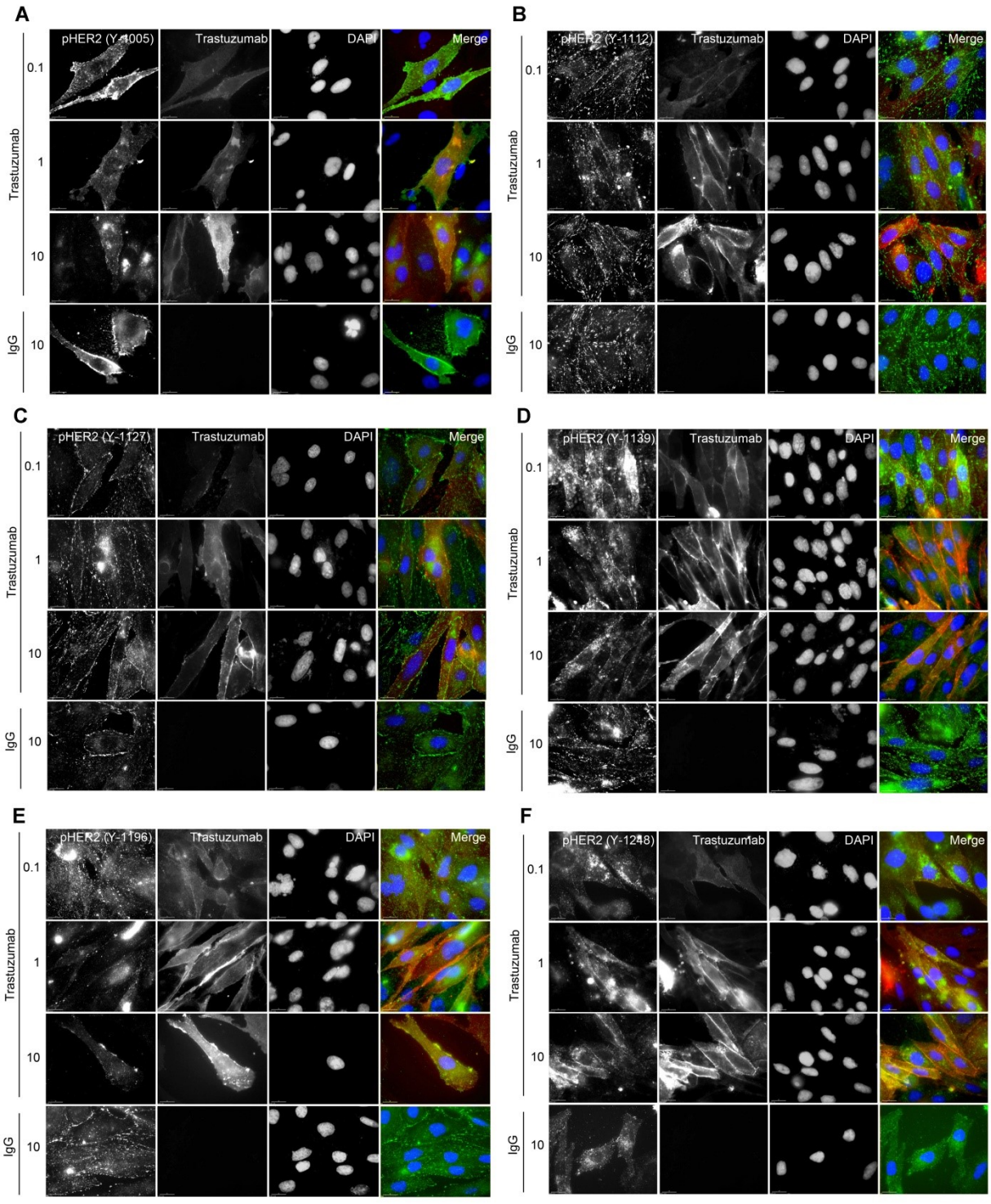

Figure 3.6. The effects of trastuzumab on HER2 phosphorylation in CHO-K6 cells by immunofluorescence. CHO-K6 cells were treated with trastuzumab at concentrations of 0.1, 1 and 10 μg/ml for 1 hour. The phosphorylation of HER2 at Y1005 (**A**), Y1112 (**B**), Y1127 (**C**),

Y1139 (**D**), Y1196 (**E**), and Y1248 (**F**) were then examined by immunofluorescence staining. The localization of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER2 was revealed by the rabbit anti-phosphoHER2 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counterstained with DAPI. Yellow indicated the co-localization of trastuzumab and pHER2. The cells treated with normal human IgG (10 μg/ml) were used as negative controls. Scaled size of each picture width: 100 μm.

3.8.5. The effects of trastuzumab on the activation of PI3K/Akt and MAPK pathways

We finally examined the activation of Erk and Akt. The Erk and Akt activation was measured by their phosphorylation. As shown in Figure 3.7A, the Erk phosphorylation level is higher in CHO-K6 cells than the control CHO cells, which suggests that overexpression of HER2 increased Erk activation. However, we did not observe the increase in Akt phosphorylation, which is not surprising as HER2 homodimer has very limited effects on the activation of PI3K/Akt pathway. We next examined the effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. As shown in Figure 3.7B, treatment with trastuzumab did not block the phosphorylation of Erk and Akt.

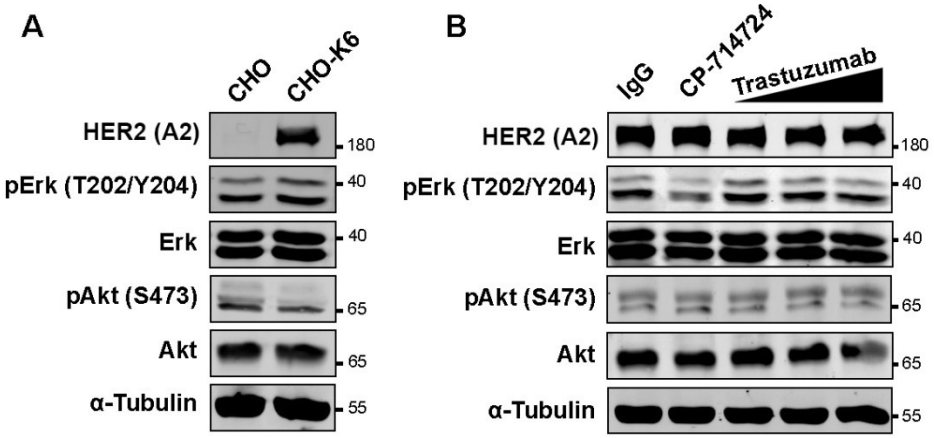

Figure 3.7. The effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. The phosphorylation of Erk and Akt was revealed by immunoblotting. (**A**) The phosphorylation of Erk and Akt in CHO parental cells and in CHO-K6 cells. (**B**) The effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. The cells were

treated with trastuzumab at concentrations of 0.1, 1 and 10 μg/ml for 7 hours. Cells treated with normal human IgG (10 μg/ml) was used as negative control and cells treated with CP-714724 (10 μM) was used as a positive control.

3.8.6. Trastuzumab induces ADCC

The above results suggest that trastuzumab did not inhibit HER2 dimerization and phosphorylation. Thus, it is interesting to find out if trastuzumab can induce ADCC in cells overexpressing HER2. Trastuzumab-induced ADCC in CHO-K6 and CHO-EGFR cells was determined by using Promega ADCC Bioassay kit. As shown in Figure 3.8, trastuzumab induced very strong ADCC in CHO-K6 cells, but not in CHO-EGFR cells.

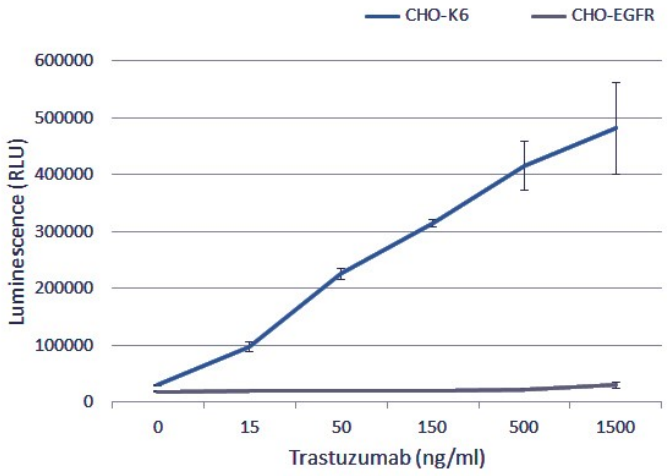

Figure 3.8. Trastuzumab-induced ADCC in CHO-K6 and CHO-EGFR cells. Trastuzumab-induced ADCC was examined in both CHO-K6 and CHO-EGFR cells by using Promega ADCC Bioassay kit.

3.8.7. The effects of trastuzumab on the proliferation of CHO-K6 cells

We next determined if trastuzumab inhibits the proliferation of cells with overexpressed HER2. MTT cell proliferation kit was used to assess the proliferation of various CHO cells including CHO parental cells, CHO-EGFR, CHO-K6, and CHO-HER3 cells. Untreated cells were used as negative controls, and the cells treated with vinorelbine (an anticancer drug) were used as positive controls.

We first determined if overexpression of HER2 in CHO cells stimulates cell proliferation by comparing CHO-K6 cells with the parental CHO cells. As shown in Figure 3.9A, the proliferation rate of CHO-K6 cells is much higher than that of CHO parental cells, which indicates that overexpression of HER2 stimulates cell proliferation. We next examined the effects of trastuzumab on cell proliferation. It is not surprising that treatment with trastuzumab for either 24 or 48 hours had no effects on the proliferation of CHO, CHO-EGFR and CHO-HER3 cells as these cells did not express HER2 (Figures 2.9B-D). Interestingly, even for CHO-K6 cells that overexpressed HER2, trastuzumab at high dosage did not have any effect on their proliferation (Figure 3.9E). However, vinorelbine significantly inhibited the proliferation of all these CHO cells following 24 or 48 hours incubation (Figures 2.9B-E). Moreover, HER2 kinase inhibitors including lapatinib and CP-714724 significantly inhibited the proliferation of CHO-K6 cells when at high dosage (Figure 3.9F). Our data indicated that trastuzumab did not inhibit the proliferation of CHO-K6 cells that overexpressed HER2.

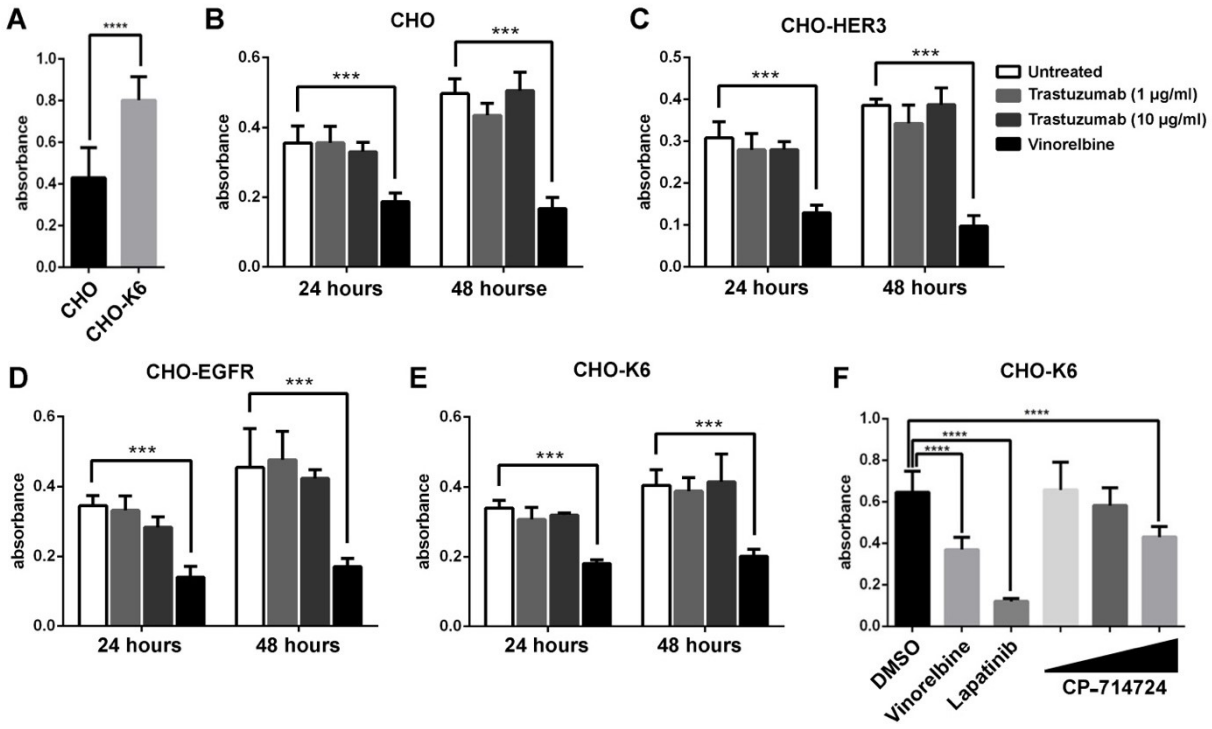

Figure 3.9. The effects of trastuzumab on the proliferation of CHO parental cell line, CHO-EGFR, CHO-K6, and CHO-HER3 cells. The cell proliferation was examined by MTT assay. (**A**) The effects of HER2 overexpression on the proliferation of CHO parental cells. Cell proliferation of both CHO parental cells and CHO-K6 cells was examined. (**B-E**) The effects

of trastuzumab on the proliferation of CHO, CHO-EGFR, CHO-K6 and CHO-HER3 cells. Cells were treated with trastuzumab (1 and 10 μg/ml) as indicated for 24 and 48 hours. Untreated cells were used as negative control and the cells treated with 10 μM of vinorelbine (VR) were used as positive controls. (**B**) CHO cells. (**C**) CHO-HER3 cells. (**D**) CHO-EGFR cells. (**E**) CHO-K6 cells. (**F**) The effects of other HER2 inhibitors on the proliferation of CHO-K6 cells. CHO-K6 cells were treated with HER2 kinase inhibitors lapatinib (20 μM) and CP-714724 (1 μM). Each value is the average of at least three experiments and the error bar is standard error. ***: $P < 0.001$. ****: $P < 0.0001$.

3.9. DISCUSSION

The most controversial mechanism regarding trastuzumab function is its effect on the inhibition of HER2 activation. A major reason behind this controversy is the different cellular backgrounds of various breast cancer cell lines used in those studies. Each breast cancer cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab due to the heterodimerization among HER receptors. In this research, we adopted a CHO cell model. Besides the parental CHO cells that do not express any detectable HER receptors, three stable CHO cell lines that stably express only a single HER receptor including EGFR (CHO-EGFR), HER2 (CHO-K6), and HER3 (CHO-HER3) were employed in this research. Our cell model system avoided the interference of other HER receptors and is very suitable to study the effects of trastuzumab on the homodimerization of HER2 and the phosphorylation of HER2 homodimers. We aim to conclusively determine if trastuzumab specifically binds only to HER2, and blocks HER2 homodimerization and activation.

We showed that trastuzumab only bound to HER2 specifically and with high affinity. Trastuzumab did not bind to EGFR and HER3 even at a high dosage (10 ng/ml) (Figure 3.2). Most HER2-positive breast cancer cells also express EGFR and HER3, our finding suggests that any trastuzumab effects on these cells must be initiated through the interaction between trastuzumab and HER2.

We next examined the effects of trastuzumab on HER2 dimerization. HER2 is an orphan receptor and does not have a ligand. However, HER2 is heterodimerized with EGFR in response

to EGF stimulation and heterodimerized with HER3 in response to HRG [38]. HER2 is also homodimerized when overexpressed in cells. CHO-K6 cells only express a single HER receptor HER2, not EGFR, HER3 or HER4. Thus, our results are regarding the effects of trastuzumab on the homodimerization of HER2.

We showed that in CHO-K6 cells HER2 was mostly dimerized, likely due to the overexpression (Figure 3.3). This is not surprising. As revealed by crystal structures of the HER2 extracellular region, HER2 adopts an extended configuration, which resembles the configuration of EGFR seen in each molecule of an EGFR dimer. Thus, HER2 possesses a constitutive, or ligand-independent, activated conformation, which allows the HER2 homodimerization when overexpressed [4,38,39].

We also showed that trastuzumab did not block the homodimerization of HER2 (Figure 3.3). While it is originally proposed that trastuzumab acts to block HER2 dimerization, so far, no research has been done to determine the effects of trastuzumab on the homodimerization of HER2. Given the fact that trastuzumab binds to the juxtamembrane region of HER2 [39], which is not essential for HER2 dimerization, our results are not surprising. What surprising is that our data suggest that trastuzumab at high dosage actually enhanced the homodimerization of HER2 (Figure 3.3). While we are not certain how trastuzumab stimulates the homodimerization of HER2, it is possible that it functions through the HER2 transmembrane domain. Many data support the role of HER2 transmembrane domain in HER2 dimerization and activation [39]. Parts of the juxtamembrane region has also been implicated in HER2 dimerization and activation [40–42]. As trastuzumab binds to the extracellular juxtamembrane region of HER2, it will likely affect the function of HER2 transmembrane domain and juxtamembrane region in terms of HER2 dimerization. It is possible that somehow the specific effects of trastuzumab enhanced the interaction between two HER2 transmembrane domains and thus increased HER2 homodimerization as we observed here.

It has been believed that trastuzumab functions to inhibit HER2 activation/phosphorylation and HER2-mediated cell signaling [1,15,16]. However, our data indicated that trastuzumab only had very limited effects on HER2 phosphorylation. Among the six pY residues examined in this research, HER2 had no effects on the phosphorylation of pY1005, pY1112, pY1027, pY1196, and pY1248 (Figures 2.5 and 2.6). While HER2 decreased the phosphorylation of pY1139,

which is a much weaker inhibition when compared with CP-724714 (Figures 2.5-2.7). In general, this is consistent with our observation regarding the role of trastuzumab in HER2 dimerization. Trastuzumab did not block HER2 dimerization, thus it did not block HER2 phosphorylation. It is not clear how the effects of HER2 transmembrane domain on HER2 dimerization affect the phosphorylation of HER2. Some research indicated the presence of an alternative dimerization mode of HER2. In this mode, HER2 dimerization is mediated by both the transmembrane domain and the cytoplasmic juxtamembrane region of HER2. Such a dimerization mode exerts inhibiting effects on the HER2 kinase activity [40–42]. Thus, in theory, the enhanced dimerization through the interaction of the transmembrane domain and the juxtamembrane region could result in the inhibition of certain HER2 phosphorylation including pY1139. Recently, some researches with various breast cancer cell lines have shown that trastuzumab did not significantly alter HER2 phosphorylation [16–19,43]. Moreover, there is one research shows the enhanced phosphorylation of pY1248 in response to trastuzumab [15].

Our results suggest that trastuzumab has if any, limited effects on HER2-mediated intracellular signaling. Indeed, when we examined the effects of trastuzumab on the phosphorylation of Erk and Akt, we showed that trastuzumab did not block the phosphorylation of both Erk and Akt in CHO-K6 cells (Figure 3.7). Together, our data indicate that trastuzumab did not significantly alter HER2 activation and HER2 mediated intracellular signaling in the absence of other HER receptors. However, we need to be cautious to apply these findings to breast cancer cells. CHO cell is derived from hamster ovary, thus the expressed human HER2 may not be coupled well with downstream signaling cascades.

We then examined if trastuzumab induces ADCC in CHO-K6 cells. We showed that trastuzumab indeed induces strong ADCC in CHO-K6 cells (Figure 3.8). This is specifically due to the expression of HER2 in CHO-K6 cells as there is no ADCC observed in CHO-EGFR cells (Figure 3.8). The role of trastuzumab in the induction of ADCC in HER2-positive breast cancer cells has been consistently well supported by many pieces of research [8–14]. Our results confirmed the role of trastuzumab in the induction of ADCC in a simple but specific cell setting.

We also showed that trastuzumab did not affect cell proliferation in CHO-K6 cells (Figure 3.9). Some reports indicated that trastuzumab had little effect on proliferation and survival [21,44]. However, other reports indicated that trastuzumab inhibited HER2 activation, and

decreased the activation of Erk and PI3K/Akt pathways, which leads to reduced cell proliferation [20]. Given that trastuzumab has little effects on the phosphorylation of HER2, it is likely that trastuzumab has no effects on HER2-mediated cell signaling leading to cell proliferation. Although trastuzumab induces ADCC in CHO-K6 cells, under the culture conditions used for the MTT assay, no effector cells were present and no ADCC response is expected. It is interesting to note that our above finding is different from the observation by Ghosh [45]. Ghosh et al. reported that trastuzumab inhibited HER2 homodimer-mediated Erk phosphorylation and cell growth. The difference could be due to the different model systems used in these two studies. The HER2 receptor used in the research by Ghosh et al. is fused with FKBP, and the receptor homodimerization is induced by a chemical linker AP1510 that dimerizes the receptor intracellularly through the fused FKBP.

It should be noted that while we observed strong inhibition of HER2 phosphorylation by CP-724714 at 1 μM (Figure 3.5B). We only observed the inhibition of CHO-K6 cell proliferation at much higher CP-724714 concentration (Figure 3.9F). We are not sure what causes this discrepancy, however, there are several possible explanations. Firstly, at 1 μM CP-724714 may not completely inhibit HER2 phosphorylation, we can see weak phosphorylation of Y1139 in Figure 3.5B. There could be weak phosphorylation of HER2 at other pY residues that were not examined. Weak HER2 phosphorylation may still be sufficient to support cell growth. Secondly, there could be the existence of kinase-independent effects of HER2 receptors. There are many reports supporting the existence of kinase-independent cell signaling of various receptor tyrosine kinases including EGFR and insulin receptors [46–49].

3.10. REFERENCES

1. Spector NL, Blackwell KL. Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol. 2009;27: 5838–5847. doi:10.1200/JCO.2009.22.1507

2. Fiszman GL, Jasnis MA. Molecular Mechanisms of Trastuzumab Resistance in HER2 Overexpressing Breast Cancer. Int J Breast Cancer. 2011;2011: 352182. doi:10.4061/2011/352182

3. Rimawi MF, Schiff R, Osborne CK. Targeting HER2 for the treatment of breast cancer.

Annu Rev Med. 2015;66: 111–128. doi:10.1146/annurev-med-042513-015127

4. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DWJ, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421: 756–760. doi:10.1038/nature01392

5. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 1990;50: 1550–1558.

6. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol Immunother. 1993;37: 255–263. doi:10.1007/bf01518520

7. Clynes RA, Towers TL, Presta LG, Ravetch J V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med. 2000;6: 443–446. doi:10.1038/74704

8. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br J Cancer. 2006;94: 259–267. doi:10.1038/sj.bjc.6602930

9. Varchetta S, Gibelli N, Oliviero B, Nardini E, Gennari R, Gatti G, et al. Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res. 2007;67: 11991–11999. doi:10.1158/0008-5472.CAN-07-2068

10. Kute T, Stehle JRJ, Ornelles D, Walker N, Delbono O, Vaughn JP. Understanding key assay parameters that affect measurements of trastuzumab-mediated ADCC against Her2 positive breast cancer cells. Oncoimmunology. 2012;1: 810–821. doi:10.4161/onci.20447

11. Petricevic B, Laengle J, Singer J, Sachet M, Fazekas J, Steger G, et al. Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent in both adjuvant and metastatic HER2/neu breast cancer patients. J Transl Med. 2013;11: 307. doi:10.1186/1479-5876-11-307

12. Duong MN, Cleret A, Matera E-L, Chettab K, Mathe D, Valsesia-Wittmann S, et al. Adipose cells promote resistance of breast cancer cells to trastuzumab-mediated antibody-dependent cellular cytotoxicity. Breast Cancer Res. 2015;17: 57. doi:10.1186/s13058-015-0569-0

13. Shi Y, Fan X, Deng H, Brezski RJ, Rycyzyn M, Jordan RE, et al. Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcgamma receptors on macrophages. J Immunol. 2015;194: 4379–4386.

doi:10.4049/jimmunol.1402891

14. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28: 803–814. doi:10.1038/onc.2008.432

15. Dokmanovic M, Wu Y, Shen Y, Chen J, Hirsch DS, Wu WJ. Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition. Cancer Biol Ther. 2014;15: 1029–1041. doi:10.4161/cbt.29171

16. Gijsen M, King P, Perera T, Parker PJ, Harris AL, Larijani B, et al. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. PLoS Biol. 2010;8: e1000563. doi:10.1371/journal.pbio.1000563

17. Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell. 2002;2: 127–137.

18. Nahta R, Hung M-C, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64: 2343–2346.

19. Longva KE, Pedersen NM, Haslekas C, Stang E, Madshus IH. Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. Int J cancer. 2005;116: 359–367. doi:10.1002/ijc.21015

20. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res. 2002;62: 4132–4141.

21. Xia W, Bisi J, Strum J, Liu L, Carrick K, Graham KM, et al. Regulation of survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. Cancer Res. 2006;66: 1640–1647. doi:10.1158/0008-5472.CAN-05-2000

22. Cuello M, Ettenberg SA, Clark AS, Keane MM, Posner RH, Nau MM, et al. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. Cancer Res. 2001;61: 4892–4900.

23. Austin CD, De Maziere AM, Pisacane PI, van Dijk SM, Eigenbrot C, Sliwkowski MX, et

al. Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol Biol Cell. 2004;15: 5268–5282. doi:10.1091/mbc.e04-07-0591

24. Valabrega G, Montemurro F, Sarotto I, Petrelli A, Rubini P, Tacchetti C, et al. TGFalpha expression impairs Trastuzumab-induced HER2 downregulation. Oncogene. 2005;24: 3002–3010. doi:10.1038/sj.onc.1208478

25. Pietras RJ, Pegram MD, Finn RS, Maneval DA, Slamon DJ. Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. Oncogene. 1998;17: 2235–2249. doi:10.1038/sj.onc.1202132

26. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res. 2001;61: 4744–4749.

27. Izumi Y, Xu L, di Tomaso E, Fukumura D, Jain RK. Tumour biology: herceptin acts as an anti-angiogenic cocktail. Nature. 2002;416: 279–280. doi:10.1038/416279b

28. Wen X-F, Yang G, Mao W, Thornton A, Liu J, Bast RCJ, et al. HER2 signaling modulates the equilibrium between pro- and antiangiogenic factors via distinct pathways: implications for HER2-targeted antibody therapy. Oncogene. 2006;25: 6986–6996. doi:10.1038/sj.onc.1209685

29. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7: 505–516. doi:10.1038/nrm1962

30. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol. 2009;21: 177–184. doi:10.1016/j.ceb.2008.12.010

31. Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J. 1996;15: 2452–2467.

32. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5: 341–354. doi:10.1038/nrc1609

33. Nagy P, Jenei A, Kirsch AK, Szollosi J, Damjanovich S, Jovin TM. Activation-dependent clustering of the erbB2 receptor tyrosine kinase detected by scanning near-field optical microscopy. J Cell Sci. 1999;112 (Pt 1: 1733–1741.

34. Yuste L, Montero JC, Esparis-Ogando A, Pandiella A. Activation of ErbB2 by overexpression or by transmembrane neuregulin results in differential signaling and sensitivity to herceptin. Cancer Res. 2005;65: 6801–6810. doi:10.1158/0008-5472.CAN-

04-4023

35. Wang Q, Zhu F, Wang Z. Identification of EGF receptor C-terminal sequences 1005-1017 and di-leucine motif 1010LL1011 as essential in EGF receptor endocytosis. Exp Cell Res. 2007;313: 3349–3363. doi:10.1016/j.yexcr.2007.06.014

36. Munch RC, Muhlebach MD, Schaser T, Kneissl S, Jost C, Pluckthun A, et al. DARPins: an efficient targeting domain for lentiviral vectors. Mol Ther. 2011;19: 686–693. doi:10.1038/mt.2010.298

37. Mendrola JM, Berger MB, King MC, Lemmon MA. The single transmembrane domains of ErbB receptors self-associate in cell membranes. J Biol Chem. 2002;277: 4704–4712. doi:10.1074/jbc.M108681200

38. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2: 127–137. doi:10.1038/35052073

39. Roskoski RJ. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol Res. 2014;79: 34–74. doi:10.1016/j.phrs.2013.11.002

40. Sajot N, Genest M. Dimer interface of transmembrane domains for neu/erbB-2 receptor dimerization and transforming activation: a model revealed by molecular dynamics simulations. J Biomol Struct Dyn. 2001;19: 15–31. doi:10.1080/07391102.2001.10506717

41. Cymer F, Schneider D. Transmembrane helix-helix interactions involved in ErbB receptor signaling. Cell Adh Migr. 2010;4: 299–312.

42. Bragin PE, Mineev KS, Bocharova O V, Volynsky PE, Bocharov E V, Arseniev AS. HER2 Transmembrane Domain Dimerization Coupled with Self-Association of Membrane-Embedded Cytoplasmic Juxtamembrane Regions. J Mol Biol. 2016;428: 52–61. doi:10.1016/j.jmb.2015.11.007

43. Kostyal D, Welt RS, Danko J, Shay T, Lanning C, Horton K, et al. Trastuzumab and lapatinib modulation of HER2 tyrosine/threonine phosphorylation and cell signaling. Med Oncol. 2012;29: 1486–1494. doi:10.1007/s12032-011-0025-7

44. Xia W, Mullin RJ, Keith BR, Liu L-H, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene. 2002;21: 6255–6263. doi:10.1038/sj.onc.1205794

45. Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, et al. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. 2011;71: 1871–1882. doi:10.1158/0008-5472.CAN-10-1872

46. Weihua Z, Tsan R, Huang W-C, Wu Q, Chiu C-H, Fidler IJ, et al. Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell. 2008;13: 385–393. doi:10.1016/j.ccr.2008.03.015

47. Mielgo A, Seguin L, Huang M, Camargo MF, Anand S, Franovic A, et al. A MEK-independent role for CRAF in mitosis and tumor progression. Nat Med. 2011;17: 1641–1645. doi:10.1038/nm.2464

48. Cossu-Rocca P, Muroni MR, Sanges F, Sotgiu G, Asunis A, Tanca L, et al. EGFR kinase-dependent and kinase-independent roles in clear cell renal cell carcinoma. Am J Cancer Res. 2016;6: 71–83.

49. Tan X, Thapa N, Sun Y, Anderson RA. A kinase-independent role for EGF receptor in autophagy initiation. Cell. 2015;160: 145–160. doi:10.1016/j.cell.2014.12.006

Chapter 4. The effects of pertuzumab on HER2 receptor activation

4.1. SUMMARY

In the previous chapter we demonstrated that trastuzumab alone had not significant effect on HER2 receptor activation and downstream pathways. Adding pertuzumab (Perjeta®) to trastuzumab and docetaxel ameliorated therapy outcomes and improved survival rates of breast cancer patients compared to single agent treatment. Pertuzumab is an anti-HER2 monoclonal antibody that is used for the treatment of HER2-positive breast cancers in combination with trastuzumab and docetaxel and showed promising clinical outcomes. Pertuzumab is suggested to block the heterodimerization of HER2 with EGFR and HER3 that abolishes the canonical function of HER2. However, evidence on the exact mode of action of pertuzumab in the homodimerization of HER2 is limited. In this study, we investigated the effect of pertuzumab and its combination with trastuzumab on HER2 homodimerization, phosphorylation, and whole gene expression profile in CHO cells stably overexpressing human HER2 (CHO-K6). CHO-K6 cells were treated with pertuzumab, trastuzumab, and their combination, and then HER2 homodimerization and phosphorylation at seven pY sites were investigated. The effects of the monoclonal antibodies on the expression of genes involved in cell cycle stages, apoptosis, autophagy, and necrosis were studied by cDNA microarray. Results showed that pertuzumab had no significant effect on HER2 homodimerization, however, trastuzumab increased HER2 homodimerization. Interestingly, pertuzumab increased HER2 phosphorylation at Y1127, Y1139, and Y1196 residues, while trastuzumab increased HER2 phosphorylation at Y1196. More surprisingly, a combination of pertuzumab and trastuzumab blocked the phosphorylation of Y1005 and Y1127 of HER2. Our results also showed that pertuzumab, but not trastuzumab, abrogated the effect of HER2 overexpression on cell cycle in particular G1/S transition, G2/M transition, and M phase, whereas trastuzumab abolished the inhibitory effect of HER2 on apoptosis. Our findings confirm that pertuzumab is unable to inhibit HER2 homodimerization but induces HER2 phosphorylation at some pY sites that abolishes HER2 effects on cell cycle progression. These data suggest that the clinical effects of pertuzumab may mostly through the inhibition of HER2 heterodimers, rather than HER2 homodimers and that pertuzumab binding to

HER2 may inhibit non-canonical HER2 activation and function in the non-HER-mediated and dimerization-independent pathway(s).

4.2. INTRODUCTION

Given the better outcome of pertuzumab treatment in combination with trastuzumab, there seems to be a synergism between the two therapeutics [20]. Adding pertuzumab to trastuzumab and docetaxel showed better outcomes and improved progression-free and overall survival rates of breast cancer patients compared to treatment with trastuzumab and docetaxel alone (Baselga et al., 2012; von Minckwitz et al., 2017; Swain et al., 2013). So far evidences on the exact mode of action of pertuzumab, particularly its role in blocking HER2 homodimerization and HER2-mediated cell cycle progression and cell death still remain controversial. In addition, in the previous chapter, we demonstrated that trastuzumab alone had no significant effect on HER2 receptor activation and downstream pathways. The aim of this part of our study was to study the effects of pertuzumab and its combination with trastuzumab on homodimerization and tyrosine phosphorylation of HER2. We also found that overexpression of HER2 did not increase PI3K/Akt and MAPK signaling pathways activation. We also found that overexpression of HER2 did not increase PI3K/Akt and MAPK signaling pathways activation in CHO cells. In the current study, we also investigated the gene expression profiles of parental CHO and CHO-K6 cells to understand the potential effects of HER2 overexpression in the CHO cells. Moreover, to study the effect of the monoclonal antibody on the biology of CHO-K3 cells, we examined the gene expression profile of CHO-K6 cells under treatment with trastuzumab, pertuzumab as well as a combination of trastuzumab and pertuzumab.

4.3. RESULTS

4.3.1. Specific binding of pertuzumab to HER2

In this study, we used CHO cells stably expressing human HER2 (HER2-K6 [24,25]) as HER2 overexpressing cell model. The expression level of HER2 in CHO-K6 cells was detected significantly higher than that of breast cancer cell lines including SKBR3, BT474, MCF7, and

MDA-MB-231, as well as another clone of HER2-overexpressing CHO cell line HER2-K13 cells [24,25] (Figure 4.1A). To examine the binding of pertuzumab to HER receptors, we treated CHO cells stably overexpressing EGFR (CHO-EGFR), HER2 (CHO-K6) and HER3 (CHO-HER3) with 10 μg/ml pertuzumab for 1 hour. As shown in Figure 4.1B, pertuzumab specifically bound to HER2 in cell membranes, but not to EGFR and HER3. Dose-response experiments (0.1, 0.5, 1, 5 and 10 μg/ml for 60 minutes) indicated that pertuzumab strongly bound to HER2 even at a low concentration of 0.1 μg/ml (Figure 4.1C). Moreover, time-course (10 μg/ml for 5, 15, 30 and 60 minutes) treatment of CHO-K6 cell showed that 5 min incubation is enough to result in strong binding between pertuzumab and HER2. Longer incubation did not increase the binding significantly (Figure 4.1D). Together, these results confirm that pertuzumab specifically binds to the HER2 receptor with high affinity.

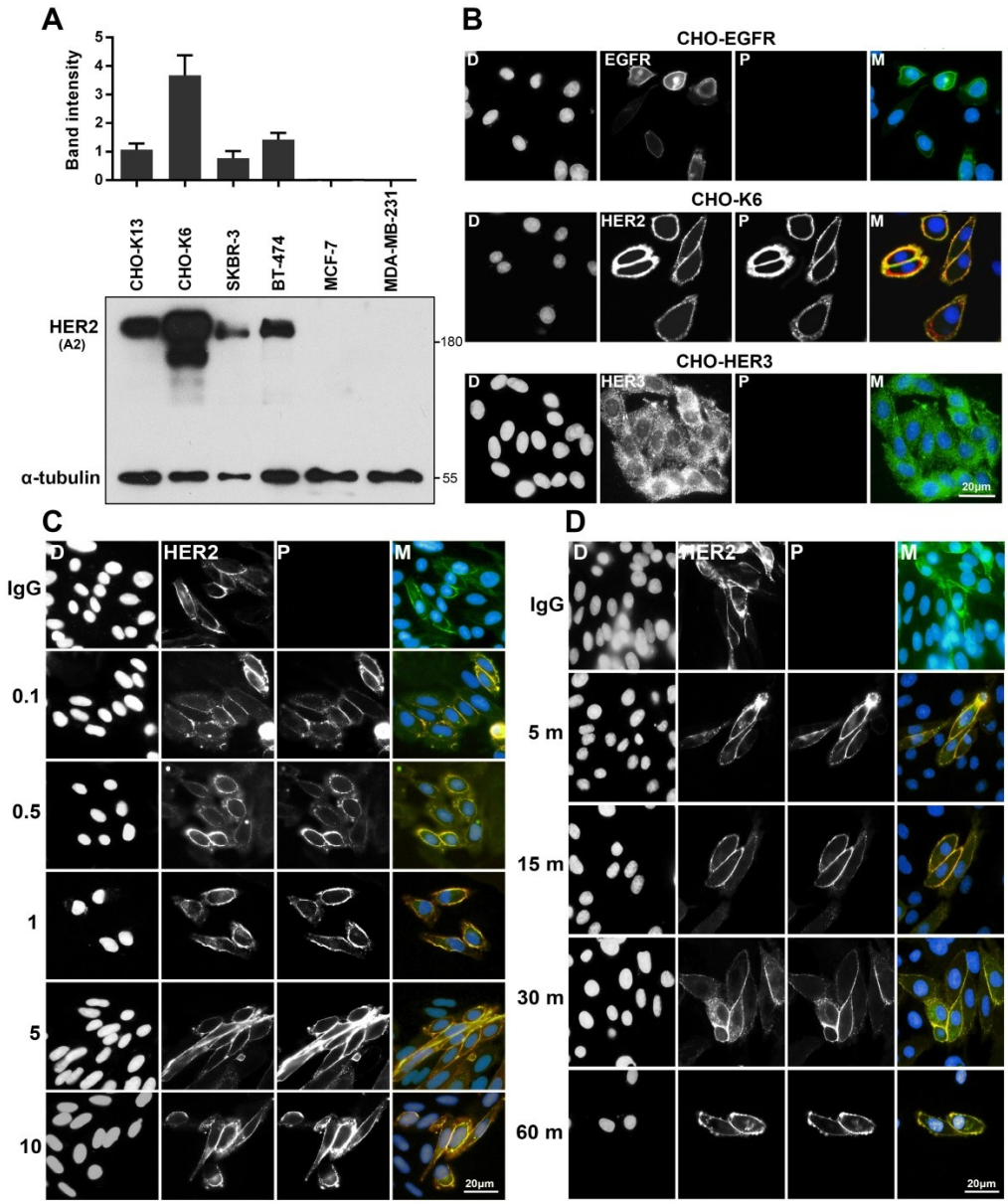

Figure 4.1. Pertuzumab specially binds to HER2. (**A**) The expression levels of HER2 in CHO cell lines stably overexpressing HER2 (CHO-K13 and CHO-K6), two HER2-positive (SKBR3 and BT474) and two HER2-low (MCF7 and MDA-MB-231) breast cancer cell lines. (**B**) Double-immunofluorescence staining of EGFR, HER2, and HER3 (green) and pertuzumab (red) in CHO-EGFR, CHO-K6, and CHO-HER3 cell lines. Pertuzumab binds specifically to HER2 but not to EGFR and HER3. (**C** and **D**) Double-immunofluorescence staining of HER2 (green) and pertuzumab (red) in the cells (**C**) treated with 0.1, 0.5, 1, 5 and 10 μg/ml pertuzumab for 60 minutes (dose-course treatment) and (**D**) treated with 10 μg/ml

pertuzumab for 5, 15, 30 and 60 minutes (time-course treatment). Treatment with 10 μg/ml of human IgG for 60 minutes was used as a mock control.

4.3.2. The effects of the pertuzumab on the HER2 homodimerization

We next examined the effects of pertuzumab, trastuzumab and their combination on the homodimerization of HER2. We previously showed the specific binding of trastuzumab to HER2 [24]. For dose-response experiments, the CHO-K6 cells were treated with 0.1, 1, and 10 μg/ml pertuzumab, trastuzumab, or their combination for 120 minutes. For time-course treatment, the cells were treated with 10 μg/ml pertuzumab for 15, 30, 60 and 120 minutes. Ten μg/ml human IgG and 20 μM HER2 tyrosine kinase inhibitor agent CP-724714 were used as mock and positive controls respectively. HER2 monomer and homodimer were assessed by BS^3-based protein cross-linking assay. As result, the mean ratio of quantified HER2 homodimer to HER2 monomer in the cell treated with CP-724714 was under 1, significantly lower than that of the cells treated with IgG in the dose-response experiments (Figure 4.2A) and the time-course experiments (Figure 4.2B). This indicates a higher level of monomer HER2 than homodimer HER2, confirming that CP-724714 significantly inhibited HER2 homodimerization. In contrast, there was no significant difference between HER2 dimer/monomer ratio of IgG treated cells and that of the cells treated with single-agent pertuzumab in both dose- and time-course treatment experiments. While the cells treated with trastuzumab as well as those treated with a combination of pertuzumab and trastuzumab showed higher HER2 dimer/monomer ratios compared to IgG treated cells (Figure 4.2A). These results implicate that pertuzumab had no considerable effect on HER2 homodimerization. However, trastuzumab increased HER2 homodimerization, which is consistent with our previous observation [24].

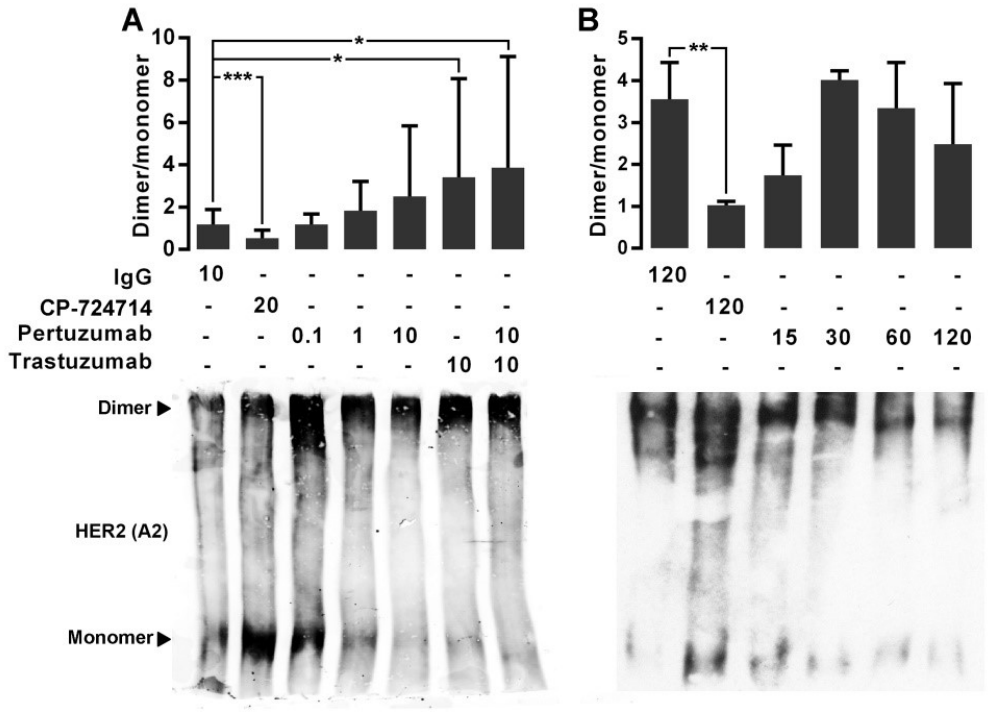

Figure 4.2. The effect of pertuzumab on HER2 homodimerization. (**A**) Immunoblot expression of monomer (185 kDa) and dimer (360-380 kDa) HER2 and quantified dimer/monomer ratios in CHO-K6 cells treated 0.1, 1 and 10 μg/ml pertuzumab, 10 μg/ml trastuzumab or their combination of for 120 minutes (dose-course treatment). (**B**) Time-course treatment of CHO-K6 with 10 μg/ml pertuzumab for 15, 30, 60 and 120 minutes. Ten μg/ml human IgG and 20 μM CP-724714 were used as mock and HER2 inhibitor controls. CP-724714 reduced dimer HER2. Trastuzumab increased dimer HER2 and pertuzumab had no significant effect on HER2 homodimerization. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

4.3.3. The effects of the pertuzumab on the HER2 phosphorylation

We have shown previously that HER2 is strongly phosphorylated in CHO-K6 cells [24]. Here, to examine whether the pertuzumab inhibits HER2 phosphorylation, we investigated the phosphorylation of seven pY sites on HER2 C-terminal including Y1005, Y1112, Y1127, Y1139, Y1196, Y1221/1222, and Y1248. CHO-K6 cells were treated with pertuzumab at final concentrations of 0.1, 1 and 10 μg/ml, 10 μg/ml trastuzumab, and a combination of 10 μg/ml pertuzumab and trastuzumab for 120 minutes, and then the levels of total HER2 and the

phosphorylated HER2 were analyzed by western blotting. A final concentration of 10 μg/ml human IgG and 20 μM CP-724714 were used as mock and positive controls respectively.

As shown in Figure 4A, none of the treatments showed a change in the protein expression levels of total HER2. However, CP-724714 dramatically reduced the phosphorylation of HER2 at all the pY sites (Figure 4.3). Surprisingly, the cells treated with 10 μg/ml pertuzumab showed an increased level of HER2 phosphorylation at Y1127 (Figure 4.3C), Y1139 (Figure 4.3D) and Y1196 (Figure 4.3E), but not at other pY sites. On the other hand, treatment with 10 μg/ml trastuzumab only increased HER2 phosphorylation at pY1196 (Figure 4.3E), but not at any other pY sites. More interestingly, the treatment with combined pertuzumab and trastuzumab reduced the phosphorylation of HER2 at pY1005 (Figure 4.3B) and pY1127 (Figure 4.3C).

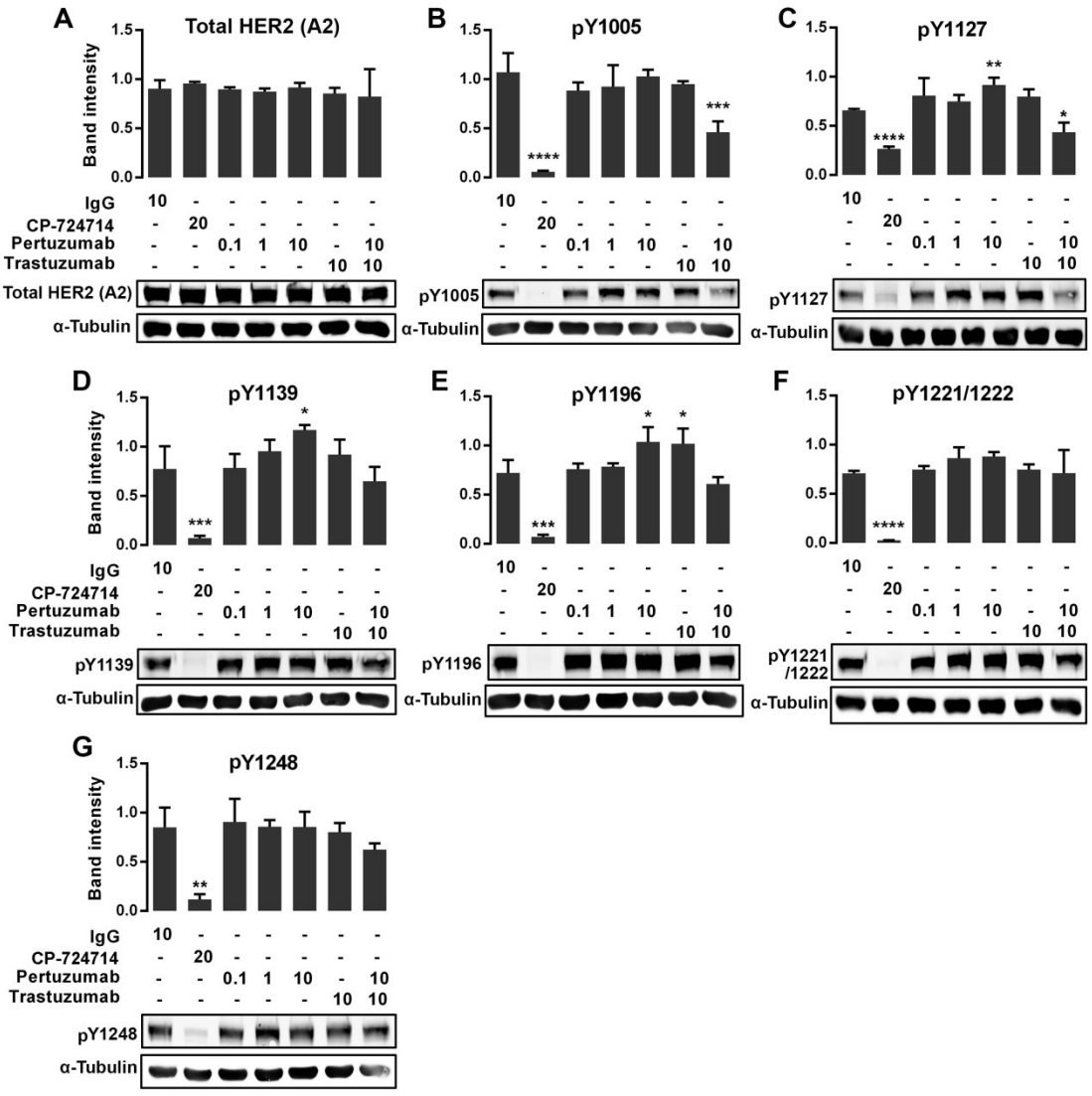

Figure 4.3. Immunoblot expression of pHER2 in pertuzumab treated CHO-K6 cells. CHO-K6 cells were treated with 0.1, 1 and 10 μg/ml pertuzumab, 10 μg/ml trastuzumab and their combination for 120 minutes. Ten μg/ml human IgG and 20 μM CP-724714 were used as mock and positive controls respectively. The expression of (**A**) total HER2, (**B**) pY1005, (**C**) pY1127, (**D**) pY1139, (**E**) pY1196, (**F**) pY1221/1222 and (**G**) pY1248 HER2 was monitored by immunoblotting. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

The effects of pertuzumab on the phosphorylation of HER2 were further examined by immunofluorescence. CHO-K6 cells were treated with 0.1, 1 and 10 μg/ml pertuzumab for 120 minutes. The phosphorylation of HER2 at Y1005, Y1112, Y1122, Y1139, Y1196, and Y1248 was examined by immunofluorescence with specific antibodies as indicated. As shown in Figure 4.4, CP-724714 treatment dramatically decreased HER2 phosphorylation at all the pY sites. Similar to the western blotting results, pertuzumab increased HER2 phosphorylation at pY1127 (Figure 4.4C), pY1139 (Figure 4.4D) and pY1196 (Figure 4.4E) compared to IgG treated cells. Pertuzumab had no significant effects on HER2 phosphorylation at other pY sites (Figure 4.4). Taken together, pertuzumab did not inhibit HER2 phosphorylation, but induced phosphorylation of HER2 at Y1127, Y1139 and Y1196 residues.

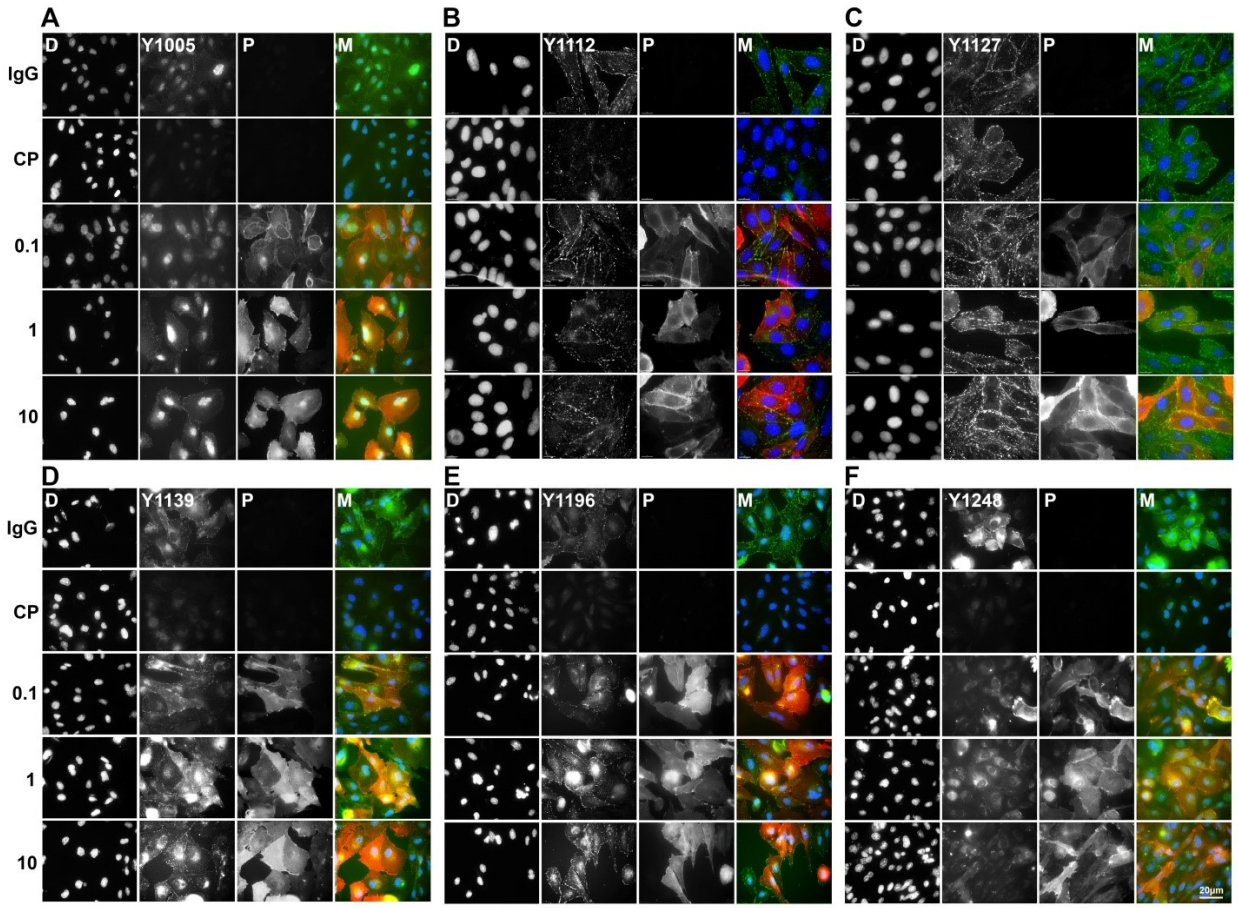

Figure 4.4. Double-immunofluorescence staining of pHER2 in pertuzumab treated CHO-K6 cells. CHO-K6 cells were treated with 0.1, 1 and 10 μg/ml pertuzumab for 2 hours then pHER2 at (**A**) pY1005 (**B**) pY1112, (**C**) pY1127, (**D**) pY1139, (**E**) pY1196, and (**F**) pY1248 HER2 (all green) and pertuzumab (red) were stained. Ten μg/ml human IgG and 20 μM CP-724714 (CP) were used as mock and HER2 positive controls respectively.

4.3.4. The effect of pertuzumab on the gene expression profile of HER2 overexpression cells

To investigate the effect of pertuzumab on the gene expression profile of HER2 overexpressing cells, we treated CHO-K6 and parental CHO (CHO-K1) cells with 10 μg/ml pertuzumab, or 10 μg/ml trastuzumab for 24 hours and then examined the whole transcriptome by microarray analyzing 30,934 unique cDNA. Results showed a minimum of 2-fold changes in the mRNA levels of 606 (1.96%) genes between CHO-K6 and CHO-K1. Of the 606 genes, 427 (1.38%) and 179 (0.58%) genes were respectively upregulated and downregulated in the CHO-K6 cells compared to the CHO-K1 cells (Figure 4.5A). Comparison of the pertuzumab treated

with the untreated CHO-K6 cells revealed a minimum of 2-fold changes in the mRNA levels of 171 (0.55%) genes, of which the expression levels of 19 (0.06%) and 152 (0.49%) genes were respectively upregulated and downregulated in the result of pertuzumab treatment (Figure 4.5B). In the CHO-K6 cells treated with trastuzumab the expression of 27 (0.09%) genes were altered, of which 14 (0.05%) and 13 (0.04%) genes were respectively upregulated and downregulated compared to the untreated cells (Figure 4.5C). Treatment with a combination of pertuzumab and trastuzumab resulted in an altered expression of 35 (11%) genes including 10 (0.03%) upregulated and 25 (0.08%) downregulated genes in the cells compared to the untreated cells (Figure 4.5D). These results suggest that overexpression of HER2 and in CHO cells dramatically changes the gene expression profile of the cells. Also, both pertuzumab and trastuzumab have major effects on the gene expression profile of CHO-K6 cells.

4.3.5. The effect of pertuzumab on cell cycle progression

To examine the effect of pertuzumab on the cell cycle we analyzed the expression of the genes that are highly expressed in each stage of the cell cycle in CHO-K6 cells treated with pertuzumab, trastuzumab, and their combination. The result showed a different expression levels of G1/S transition, S phase and DNA replication, G2/M transition and M phase marker genes between untreated parental CHO (CHO-K1) cells and untreated CHO-K6 cells regard the (Figures 4.5E-H). The different expression profiles most likely due to overexpression of HER2 in CHO-K6 cells. The expression levels of G1/S transition marker genes in the pertuzumab treated CHO-K6 cells were different from those of untreated CHO-K6 cells but similar to those of CHO-K1 cells (Figure 4.5E). However, trastuzumab treated CHO-K6 cells were similar to untreated CHO-K6 cells in terms of the expression of G1/S transition marker genes. These results indicate that pertuzumab but not trastuzumab abrogated the effect of HER2 overexpression on the expression of G1/S transition marker genes. Interestingly, the expression pattern of G1/S transition marker genes in CHO-K6 cells treated with the combination was very similar to those in untreated as well as trastuzumab treated CHO-K6 cells (Figure 4.5E).

CHO-K6 cells showed lower expression levels of S phase marker genes compared with CHO-K1 cells, which suggests that HER2 overexpression inhibits the expression of S phase marker genes. Treatment of CHO-K6 cells with pertuzumab did not change the expression profile of the S phase marker genes. In contrast, the treatment of CHO-K6 cells with trastuzumab

stimulated the expression levels of the S phase marker genes, making similar to those of CHO-K1 cells. Moreover, the expression levels of S phase marker genes in CHO-K6 cells treated with a combination of pertuzumab and trastuzumab were low and similar to those of untreated as well as pertuzumab treated cells (Figure 4.5F). These results indicate that trastuzumab, but not pertuzumab was able to abrogate the effect of HER2 overexpression on the expression of the S phase marker genes.

The expression levels of G2/M transition marker genes in CHO-K1 and CHO-K6 was different, which suggests the role of HER2 overexpression. Pertuzumab changed the expression of some G2/M transition marker genes in CHO-K6 cells. The expression of G2/M transition marker genes in trastuzumab treated cells was very similar to that of untreated CHO-K6 (Figure 4.5G).

Similarly, HER2 overexpression also affects the expression profile of the M phase marker genes as there were significant differences between CHO-K1 and CHO-K6 cells in terms of the expression of M phase marker genes. Hierarchical clustering showed a resemblance between the expression profile of M phase marker genes in the untreated CHO-K6, trastuzumab treated and the cells treated with a combination of trastuzumab and pertuzumab (Figure 4.5H). Whereas, the expression of M phase marker genes in pertuzumab treated CHO-K6 cells was similar to that of CHO-K1 cells, however, this similarity was not significant (Figure 4.5H). Taken together, in CHO-K6 cells, pertuzumab abrogated the effect of HER2 overexpression on the expression of G1/S transition, G2/M transition and M phase genes. Whereas, trastuzumab inhibited the HER2 effect on S phase genes. More interestingly, the effect of combination treatment of pertuzumab and trastuzumab was different compared to the effect of treatment with single-agent pertuzumab or trastuzumab on cell cycle of CHO-K6.

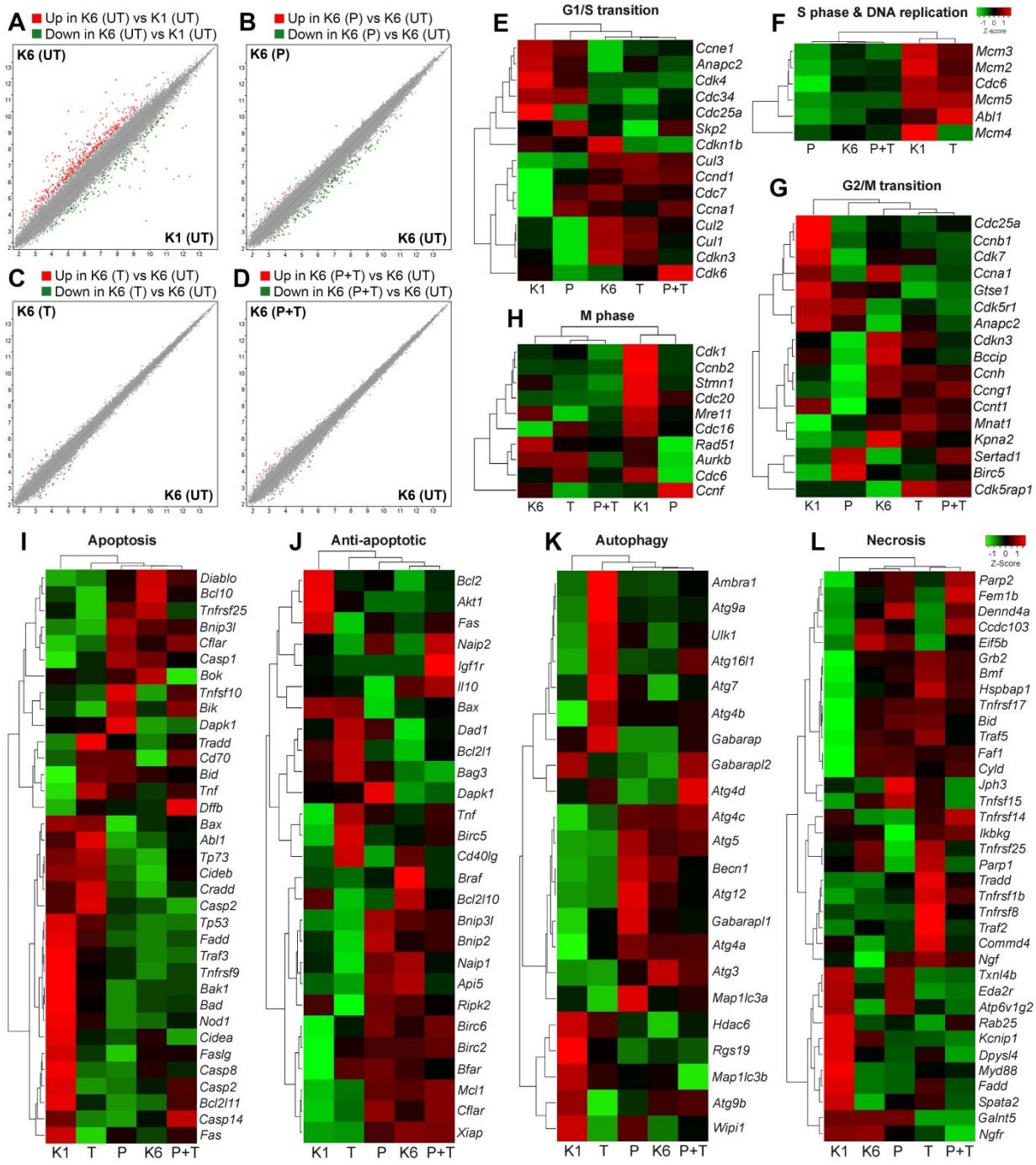

Figure 4.5. The effects of pertuzumab on the gene expression profile of HER2 overexpressing cells. The whole transcript levels were evaluated by microarray for untreated CHO-K1, untreated CHO-K6 and CHO-K6 treated with 10 μg/ml pertuzumab (P), 10 μg/ml trastuzumab (T), and their combination for 24 hours. (**A-D**) Scatter plot showing the whole gene expression profile of (**A**) untreated CHO-K6 (K6) vs untreated

CHO-K1 (K1) cell lines, (**B**) pertuzumab treated vs untreated CHO-K6 cells, (**C**) trastuzumab treated vs untreated CHO-K6 cells and (**D**) CHO-K6 cells treated with the combination of the monoclonal antibodies vs untreated cells. The values with a minimum of 2-fold change are illustrated in red (upregulated) and green (downregulated) dots. (**E-L**) Hierarchal heatmap illustrating Z-score expression of selected marker genes for (**E**) G1/S transition, (**F**) S phase and DNA replication, (**G**) G2/M transition, (**H**) M phase of cell cycle, (**I**) apoptosis, (**J**) anti-apoptosis, (**K**) autophagy and (**L**) necrosis.

4.3.6.The effect of pertuzumab on cell death pathways

To study the effect of the pertuzumab on cell death pathways we investigated the expression of selected marker genes for apoptosis, autophagy, and necrosis in the cells treated with pertuzumab, trastuzumab, or their combination. Similar to the cell cycle marker genes, there were opposite expression profiles of cell death marker genes between CHO-K1 and CHO-K6 cells, implicating the significant effects of HER2 overexpression on the cell death pathways (Figures 5I-L). The expression profiles of apoptotic (Figure 4.5I), anti-apoptotic (Figure 4.5J), autophagy (Figure 4.5K) and necrosis (Figure 4.5L) marker genes in pertuzumab treated CHO-K6 cells were similar to those in untreated CHO-K6, which suggests that pertuzumab does not inhibit HER2 function in regulating cell apoptosis. In contrast, trastuzumab inhibited the function of HER2 in regulating cell apoptosis as on apoptosis and anti-apoptosis gene expression profiles of CHO-K6 cells treated with trastuzumab were very different from untreated CHO-K6 cells, but similar to CHO-K1. Similar results were observed for the expression profile of autophagy and necrosis marker genes (Figures 4.5J-L). Furthermore, the CHO-K6 cells treated with the combination of trastuzumab and pertuzumab were similar to untreated CHO-K6 cells from the point of view of all the cell death expression profiles (Figures 4.5I-J). Taken together, the results suggest that pertuzumab treatment did not have significant effects on the cell death pathways of CHO-K6 cells. However, trastuzumab treatment inhibited the expression of apoptosis, but not autophagy and necrosis marker genes in CHO-K6 cells.

4.3.7. The effect of pertuzumab on cell proliferation

We next investigated the effects of pertuzumab on cell proliferation in CHO-K6 cells. We cultured CHO-K1, CHO-K6, CHO-K13, CHO-EGFR and CHO-HER3 cells in the presence of

10 μg/ml pertuzumab or 10 μg/ml trastuzumab or their combination for 72 hours and then evaluated viable cells by MTT assay. Human IgG (10 μg/ml) was used as mock control for the monoclonal antibodies. CP-724714 (10 μM) was used as a positive control of HER2 inhibition. Paclitaxel (5 μM) was used as a positive control of cell proliferation inhibition.

The results showed that the proliferation rates of all the paclitaxel treated cell lines were significantly lower than that of relevant untreated cells. Among the cell lines treated with CP-724714, CHO-K1, CHO-K6, and CHO-EGFR but not CHO-K13 and CHO-HER3 cells showed lower proliferation rates compared to the relevant untreated cells (Figure 4.6). None of the cells treated with pertuzumab, trastuzumab, and their combination showed a significant change in the proliferation rates in comparison with relevant IgG treated cells (Figure 4.6). These results indicate that treatment with pertuzumab, trastuzumab, and their combination did not have a significant effect on the proliferation of HER2, HER3 and EGFR overexpressing CHO cells.

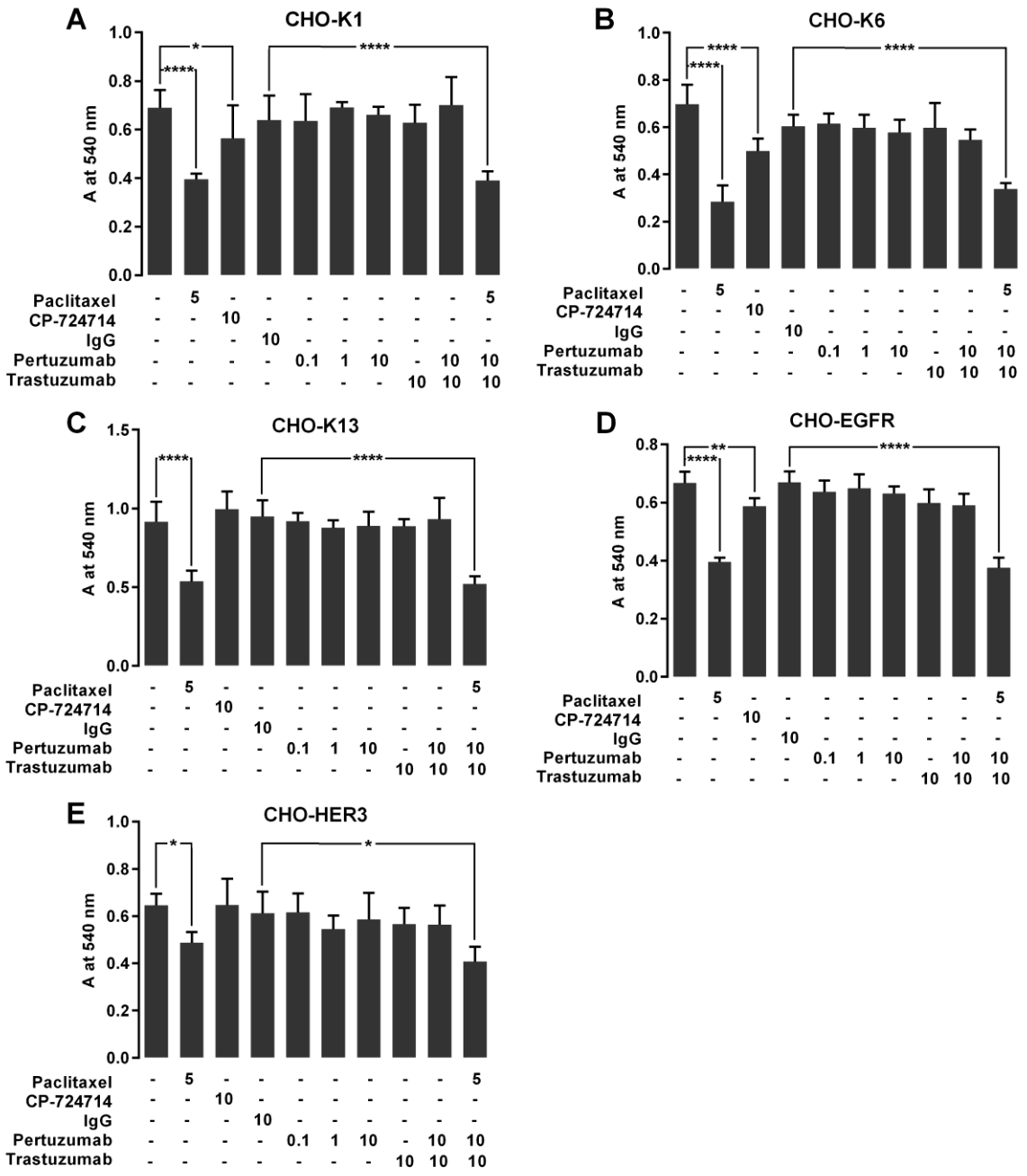

Figure 4.6. The effect of pertuzumab on the proliferation of cells overexpressing EGFR, HER2 and HER3. (**A**) CHO-K1, (**B**) CHO-K6, (**C**) CHO-K13, (**D**) CHO-EGFR and (**E**) CHO-HER3 cell lines were treated with 0.1, 1 and 10 μg/ml pertuzumab, 10 μg/ml trastuzumab and their combination for 72 h, and then the living cell mass was evaluated by MTT assay. Five μM paclitaxel, 10 μg/ml human IgG and 20 μM CP-724714 were used as

respectively antiproliferative, mock and HER2 inhibitor controls. *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$.

4.4. DISCUSSION

In this study, we investigated the effect of pertuzumab on the function of HER2 homodimers. We also compared the effects of pertuzumab with trastuzumab and the combination of pertuzumab and trastuzumab. Three CHO cell lines stably overexpressing human HER2 (CHO-K6), human EGFR (CHO-EGFR) and human HER3 (CHO-HER3), as well as parental CHO (CHO-K1) cells, were used in our study. The parental CHO cell line dose did not express any of HER family receptors per se. CHO-K6 provides an appropriate model cell to study HER2 receptor function that allows monitor HER2 function without interaction by other HER receptors. Although pertuzumab specifically binds only to HER2, studying HER2 homodimerization and its subsequent effects on cellular biology are not quite feasible in breast cancer cell lines. This is because HER2-positive breast cancer cell lines express also other HER receptors particularly EGFR and HER3 in addition to HER2 [26–28]. Endogenous EGFR and HER3 could mediate in HER2 heterodimerization and significantly affect the HER2 phosphorylation. On the other hand, the CHO cell lines allow us to study each HER receptor independently. CHO-K6 cells show high rates of HER2 homodimerization and phosphorylation that can be inhibited by CP-724714. Despite canonical downstream signaling pathways of HER2 (PI3K/Akt and MAPK pathways) do not work in CHO-K6 cells, the cell proliferation rate is higher than that of parental CHO cells (CHO-K1) [24]. This HER2-mediated increased proliferation can be inhibited by lapatinib and CP-724714. Moreover, we showed that the monoclonal antibodies changed the gene expression profile of the cells. These confirm that the CHO-K6 cell line is sensitive to anti-HER2 agents. This sensitivity is revealed by the inhabitation of HER2 dimerization, phosphorylation and CHO-K6 cell proliferation in response to treatment with the agents. These results further confirm that CHO-K6 is a suitable cell model for studying HER2 homodimerization and phosphorylation but is not an appropriate model for studying HER2-mediated PI3K/Akt and MAPK pathways. Furthermore, CHO-K6 cells provide a valuable cell model for studying the oncogenic function of HER2 via non-canonical

mechanism(s) independently of PI3K/Akt and MAPK signaling pathways that deserve paying more attention.

We showed that pertuzumab specifically bound to HER2 with high affinity on CHO-K6 cells. No pertuzumab binding was detected on CHO-EGFR and CHO-HER3 cells. Pertuzumab binds at the dimerization pocket of HER2 located in its extracellular domain II [16], while trastuzumab binds to a site located in extracellular domain IV [29]. Thus, it is suggested that the binding of pertuzumab but not trastuzumab to HER2 disrupts its heterodimerization with EGFR [14] and HER3 [15–18,20]. However, there is no independent research focusing on the effect of pertuzumab on HER2 homodimerization, phosphorylation, and gene expression profile. Structurally, the conformation of monomer HER2 resembled the ligand-bound EGFR receptor. Therefore HER2, when overexpressed, is able to form a homodimer in the absence of ligand, which also resulted in the phosphorylation of HER2 [16,24,29]. In the present study, HER2 was highly dimerized in CHO-K6 cells, most likely due to the overexpression. We showed that pertuzumab does not significantly affect HER2 homodimerization, while trastuzumab and a combination of pertuzumab and trastuzumab increase HER2 homodimerization. Homodimerization of HER2 takes place through the interaction of domain II of one HER2 receptor with the C-shaped pocket formed by domain I, II and III of the adjacent HER2 receptor [30]. A previous study shows that pertuzumab but not trastuzumab inhibits HER2 homodimerization and increases the antiproliferative effect of trastuzumab on HER2-positive breast cancer cells in combination with trastuzumab [31]. In another research Hu et al. [30] report that pertuzumab binding to its epitope on domain II of HER2 prevents interaction of C-shaped pocket from adjacent HER2 with dimerization arm of HER2 by masking the dimerization pocket [30]. According to this study, pertuzumab abolishes HER2 homodimerization in COS-7 cell expressing extracellular domains of HER2, however, trastuzumab had negligible effect of the HER2 homodimerization [30]. However, our results do not support these reports. We found that pertuzumab has no significant effect on HER2 homodimerization. In contrast, trastuzumab and the combination treatment increase homodimerization of HER2. In addition, pertuzumab is not able to abrogate the positive effect of trastuzumab on HER2 homodimerization. There are some reasons that show our results are more reliable compared to previous opposite results. Firstly; in this study we used an originally HER2-negative cell line that stably expresses full-length human HER2, while Hu et al. [30] used a partial extracellular domain of HER2 (residues

1–624). It is quite possible that partial HER2 has a structure distinct from the structure of full-length HER2. Aberrant conformation of partial HER2 most likely effects the binding and function of the monoclonal antibody on HER2. Second; Diermeier-Daucher et al. [31] used BT474 and SKBR3 cell lines to study homodimerization. Both of the cell lines express EGFR, HER3, and HER4 in addition to HER2. The HER receptors have a similar molecular size and can form 10 different dimers with each other. The distinction of HER2 homodimer form heterodimers and homodimers of the other three HER receptors based on molecular weight may be highly erroneous.

We showed that trastuzumab increases HER2 homodimerization which is consistent with our previous results [24]. The epitope of trastuzumab is located near the transmembrane domain of HER2 [29]. Several experimental and molecular dynamics simulation studies support the critical role of transmembrane and juxtamembrane domains in HER2 homo- and heterodimerization [32–40]. HER2 transmembrane has two GXXXG-like motifs, one in the N-terminal close to the extracellular domain and one in the C-terminal close to the intracellular domain [34,39,40]. The N-terminal GXXXG-like motif mediates in heterodimerization, whereas the C-terminal motif had a role in the formation of homodimer [40]. It has been shown that mutation at valine 664 such as Val664Glu which is located in the transmembrane domain between the GXXXG-like motifs leads to constitutive HER2 activation by enhancing the tendency to dimerize [41]. Moreover, the substitution of isoleucine 655 of HER2 transmembrane with valine is found to increase breast cancer risk. This mutation changes the conformation of the receptor that causes constitutive activation of the HER2 tyrosine kinase domain [42]. Further, phosphorylation of threonine 677 in the juxtamembrane domain of HER2 in shown to inhibit HER2/EGFR heterodimerization [43]. These results demonstrate that conformation change in HER2 transmembrane can alter HER2 receptor dynamics resulting in an altered tendency for dimerization. Indeed, binding of trastuzumab [29] and pertuzumab [16] majorly changes HER2 conformation. It is possible that somehow trastuzumab binding confers a new conformation that facilitates homodimerization of HER2, a function that pertuzumab is not able to perform.

We showed that pertuzumab did not inhibit HER2 phosphorylation but induced phosphorylation of HER2 at Y1127, Y1139, and Y1196 residues. Trastuzumab did not have a significant effect on HER2 phosphorylation expect at Y1196. This result is consistent with our

previous reports that show trastuzumab does not affect HER2 phosphorylation at any pY sites [24]. We showed that the combination of pertuzumab and trastuzumab inhibits pY1005 and pY1127. Lack of inhibitory effect of pertuzumab on phosphorylation is expected since it had no inhibitory effect on dimerization. The function of pY1127 is not described yet. pY1005 have been shown to bind Shc [44]. pY1139 is an important pY for HER2 function. pY1139 is a binding site for EGFR and PI3K [45,46]. Phosphorylation of Y1139 activates RAS through GRB2 and increases the transcriptional activity of STAT3 [47,48]. Phosphorylation of Y1196 enhances activation of Erk through a Ras-independent pathway and increases the binding affinity of HER2 to Crk which is a member of an adapter protein family. Crk is required for HER2 to increase Rac-dependent cell motility and HER2-mediated inhibition of apoptosis [49–51].

Phosphorylation of HER2 at canonical pY sites takes place by HER receptor tyrosine kinases following homo- and heterodimerization. HER2 is not phosphorylated only by HER family receptor tyrosine kinases, but it can be also phosphorylated by other kinases in a dimerization-independent manner at tyrosine residues. For example, Src phosphorylates HER2 at tyrosine 877 located in the P-loop of the kinase domain and increases the kinase activity of HER2 [52]. It is possible that pertuzumab has a ligand-like function that confers HER2 a new confirmation providing Y1127, Y1139 and Y1196 residues binding sites for non-HER family tyrosine kinases. However, there is no evidence yet supporting or abrogating this hypothesis. Taken together we suggest that pertuzumab may have roles in non-canonical HER2 activation in a dimerization-independent manner. To test this hypothesis, the effect of pertuzumab and trastuzumab on phosphorylation of non-canonical HER2 phospho-sites, and identity of potential kinases involved in pertuzumab-induced phosphorylation will be investigated in the future research.

Our results showed that HER2 overexpression altered the expression levels of 606 different genes (1.96% of all analyzed transcript) in the CHO cell line. Most of the altered genes (N = 427; 70.46%) were upregulated. These numbers are significant enough to conform the huge impact of HER2 overexpression on cellular biology. A microarray study analyzing 5,184 unique transcripts in HER2 overexpressing breast cancer cells and tumors revealed different expression levels of 136 (2.62%) and 151 (0.03%) genes in respectively HER2 overexpressing cell line and HER2-positive tumor tissues compared to low HER2 levels cell lines and tumors [53]. In another

analysis of 6,000 cDNA array, expression of 61 (1.02%) genes were found altered du to overexpression of HER2 [54]. In our study, pertuzumab treatment changed the expression levels of 171 genes in CHO-K6 that most of them (N = 152; 88.89%) were downregulated. However, trastuzumab only altered the expression levels of 27 genes of which 14 were upregulated and 13 were downregulated. The different effects of pertuzumab and trastuzumab may be due to different mode of action of these two monoclonal antibodies.

We also showed that expression profiles of G1/S transition, G2/M transition and M phase marker genes in pertuzumab treated HER2 overexpressing cells resembled those of HER2-negative cells. This is strong evidence confirming that pertuzumab inhibits HER2-mediated cell cycle progression. However, pertuzumab does not induce cell death pathways including apoptosis, autophagy, and necrosis. In contrast, trastuzumab has no major effect on the cell cycle but induces apoptosis. These results are supported by numerous previous reports that show pertuzumab inhibits cell cycle progression but not apoptosis and trastuzumab effects as vice versa [11,55–58]. HER2 kinase inhibitors lapatinib and CP-724714 significantly inhibit CHO-K6 cell proliferation, however, we did not observe the inhibitory effect of pertuzumab and trastuzumab on cell proliferation. This is probably because pertuzumab was unable to abrogate the HER2 effects on the cell cycle progression completely. Basically, the CHO-K6 cell line grows faster than the CHO-K1 cell line, due to the positive effect of HER2 overexpression on cell cycle progression of CHO-K6 cells [24]. As discussed above, the gene expression profile of almost all the 48 cell cycle regulators between CHO-K6 and CHO-K1 cell lines was considerably opposite. Pertuzumab treatment caused a change in the expression levels of several cell cycle regulators but not all of them and had no major effect on some other important cell cycle regulators. For example, the expression levels of the G1/S transition regulatory genes *Ccna1*, *Ccnd1*, *Cdc7*, and the G2/M transition regulator genes *Sertad1* and *Birc5* in pertuzumab treated CHO-K6 cells were still higher than those of CHO-K1 cells. Also, compared to CHO-K1 cells, the expression levels of the G1/S transition regulator gene *Cdc25a*, the G2/M transition regulator genes *Cdc25a*, *Ccnb1*, *Cdk7*, and the M phase regulator genes *Cdk1*, *Ccnb2*, *Stmn1,* and *Cdc16* were still lower in pertuzumab treated CHO-K6 cells similar to untreated CHO-K6 cells. Probably, regulation of these genes by HER2 is sufficient for induction of cell cycle progression. Furthermore, this result shows that HER2 affects cell cycle progression and gene expression through different pathways which pertuzumab can block. While, we observed that

CP-724714 inhibited the proliferation of CHO-K6 and CHO-EGFR cells. This effect is correlated with the inhibitory effect of CP-724714 on HER2 dimerization and phosphorylation. These results strongly support the notion that kinase activity of HER2 is critical for its stimulatory effects on cell cycle particularly via its canonical pathways. Despite the minor inhibitory effect of pertuzumab on HER2 homodimer-mediated cell cycle progression, lack of inhibitory effects of pertuzumab and trastuzumab on cell proliferation is likely due to their inability to inhibit HER2 phosphorylation. Surprisingly, treatment with combination of pertuzumab and trastuzumab showed distinct effect on the expression of cell cycle and death marker genes compared to single agent treatment. This result suggests that not only binding of single agent pertuzumab and trastuzumab, but dual binding of the monoclonal antibodies to HER2 have unique effects on HER2-mediated cell cycle progression and apoptosis inhibition.

4.5. REFERENCES

1. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 1990;50: 1550–1558.

2. Fleeman N, Bagust A, Beale S, Dwan K, Dickson R, Proudlove C, et al. Pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2-positive metastatic or locally recurrent unresectable breast cancer. Pharmacoeconomics. 2015;33: 13–23. doi:10.1007/s40273-014-0206-2

3. Swain SM, Baselga J, Kim S-B, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N Engl J Med. 2015;372: 724–734. doi:10.1056/NEJMoa1413513

4. Baselga J, Cortes J, Kim S-B, Im S-A, Hegg R, Im Y-H, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med. 2012;366: 109–119. doi:10.1056/NEJMoa1113216

5. von Minckwitz G, Procter M, de Azambuja E, Zardavas D, Benyunes M, Viale G, et al. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer. N Engl J Med. 2017;377: 122–131. doi:10.1056/NEJMoa1703643

6. Swain SM, Kim S-B, Cortes J, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA

study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. Lancet Oncol. 2013;14: 461–471. doi:10.1016/S1470-2045(13)70130-X

7. Toth G, Szoor A, Simon L, Yarden Y, Szollosi J, Vereb G. The combination of trastuzumab and pertuzumab administered at approved doses may delay development of trastuzumab resistance by additively enhancing antibody-dependent cell-mediated cytotoxicity. MAbs. 2016;8: 1361–1370. doi:10.1080/19420862.2016.1204503

8. Deng W, Liu J, Pan H, Li L, Zhou C, Wang X, et al. A Bispecific Antibody Based on Pertuzumab Fab Has Potent Antitumor Activity. J Immunother. 2018;41: 1–8. doi:10.1097/CJI.0000000000000200

9. Collins D, O'Donovan N, Mahgoub T, Gately K, Hughes C, Davies A, et al. Trastuzumab (T) and pertuzumab (P)-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) in tyrosine kinase inhibitor (TKI)-treated breast cancer (BC) cell lines. J Clin Oncol. 2014;32: 643. doi:10.1200/jco.2014.32.15_suppl.643

10. Collins DM, O'Donovan N, McGowan PM, O'Sullivan F, Duffy MJ, Crown J. Trastuzumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) in HER-2-non-amplified breast cancer cell lines. Ann Oncol Off J Eur Soc Med Oncol. 2012;23: 1788–1795. doi:10.1093/annonc/mdr484

11. Brockhoff G, Heckel B, Schmidt-Bruecken E, Plander M, Hofstaedter F, Vollmann A, et al. Differential impact of Cetuximab, Pertuzumab and Trastuzumab on BT474 and SK-BR-3 breast cancer cell proliferation. Cell Prolif. 2007;40: 488–507. doi:10.1111/j.1365-2184.2007.00449.x

12. Nahta R, Hung M-C, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64: 2343–2346.

13. Diermeier-Daucher S, Breindl S, Buchholz S, Ortmann O, Brockhoff G. Modular anti-EGFR and anti-Her2 targeting of SK-BR-3 and BT474 breast cancer cell lines in the presence of ErbB receptor-specific growth factors. Cytometry A. 2011;79: 684–693. doi:10.1002/cyto.a.21107

14. Hughes JB, Berger C, Rodland MS, Hasmann M, Stang E, Madshus IH. Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization. Mol Cancer Ther. 2009;8: 1885–1892. doi:10.1158/1535-7163.MCT-09-0291

15. Baselga J. A new anti-ErbB2 strategy in the treatment of cancer: prevention of ligand-dependent ErbB2 receptor heterodimerization. Cancer Cell. 2002;2: 93–95.

16. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5: 317–328.

17. Sak MM, Szymanska M, Bertelsen V, Hasmann M, Madshus IH, Stang E. Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. Carcinogenesis. 2013;34: 2031–2038. doi:10.1093/carcin/bgt173

18. Sakai K, Yokote H, Murakami-Murofushi K, Tamura T, Saijo N, Nishio K. Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway. Cancer Sci. 2007;98: 1498–1503. doi:10.1111/j.1349-7006.2007.00553.x

19. King CR, Borrello I, Porter L, Comoglio P, Schlessinger J. Ligand-independent tyrosine phosphorylation of EGF receptor and the erbB-2/neu proto-oncogene product is induced by hyperosmotic shock. Oncogene. 1989;4: 13–18.

20. Nami B, Maadi H, Wang Z. Mechanisms Underlying the Action and Synergism of Trastuzumab and Pertuzumab in Targeting HER2-Positive Breast Cancer. Cancers (Basel). 2018;10. doi:10.3390/cancers10100342

21. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A. 1992;89: 4285–4289.

22. Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, et al. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. 2011;71: 1871–1882. doi:10.1158/0008-5472.CAN-10-1872

23. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell. 2009;15: 429–440. doi:10.1016/j.ccr.2009.03.020

24. Maadi H, Nami B, Tong J, Li G, Wang Z. The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2. BMC Cancer. 2018;18: 238. doi:10.1186/s12885-018-4143-x

25. Munch RC, Muhlebach MD, Schaser T, Kneissl S, Jost C, Pluckthun A, et al. DARPins: an efficient targeting domain for lentiviral vectors. Mol Ther. 2011;19: 686–693. doi:10.1038/mt.2010.298

26. Subik K, Lee J-F, Baxter L, Strzepek T, Costello D, Crowley P, et al. The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical

Analysis in Breast Cancer Cell Lines. Breast Cancer (Auckl). 2010;4: 35–41.

27. Kalous O, Conklin D, Desai AJ, O'Brien NA, Ginther C, Anderson L, et al. Dacomitinib (PF-00299804), an irreversible Pan-HER inhibitor, inhibits proliferation of HER2-amplified breast cancer cell lines resistant to trastuzumab and lapatinib. Mol Cancer Ther. 2012;11: 1978–1987. doi:10.1158/1535-7163.MCT-11-0730

28. Jeong H, Kim J, Lee Y, Seo JH, Hong SR, Kim A. Neuregulin-1 induces cancer stem cell characteristics in breast cancer cell lines. Oncol Rep. 2014;32: 1218–1224. doi:10.3892/or.2014.3330

29. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DWJ, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421: 756–760. doi:10.1038/nature01392

30. Hu S, Sun Y, Meng Y, Wang X, Yang W, Fu W, et al. Molecular architecture of the ErbB2 extracellular domain homodimer. Oncotarget. 2015;6: 1695–1706. doi:10.18632/oncotarget.2713

31. Diermeier-Daucher S, Hasmann M, Brockhoff G. Flow cytometric FRET analysis of erbB receptor interaction on a cell-by-cell basis. Ann N Y Acad Sci. 2008;1130: 280–286. doi:10.1196/annals.1430.003

32. Sajot N, Genest M. Dimer interface of transmembrane domains for neu/erbB-2 receptor dimerization and transforming activation: a model revealed by molecular dynamics simulations. J Biomol Struct Dyn. 2001;19: 15–31. doi:10.1080/07391102.2001.10506717

33. Sajot N, Genest M. Structure prediction of the dimeric neu/ErbB-2 transmembrane domain from multi-nanosecond molecular dynamics simulations. Eur Biophys J. 2000;28: 648–662.

34. Cymer F, Schneider D. Transmembrane helix-helix interactions involved in ErbB receptor signaling. Cell Adh Migr. 2010;4: 299–312.

35. Mendrola JM, Berger MB, King MC, Lemmon MA. The single transmembrane domains of ErbB receptors self-associate in cell membranes. J Biol Chem. 2002;277: 4704–4712. doi:10.1074/jbc.M108681200

36. Sharpe S, Barber KR, Grant CWM. Evidence of a tendency to self-association of the transmembrane domain of ErbB-2 in fluid phospholipid bilayers. Biochemistry. 2002;41: 2341–2352.

37. Duneau J-P, Vegh AP, Sturgis JN. A dimerization hierarchy in the transmembrane domains of the HER receptor family. Biochemistry. 2007;46: 2010–2019. doi:10.1021/bi061436f

38. Moriki T, Maruyama H, Maruyama IN. Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. J Mol Biol. 2001;311: 1011–1026. doi:10.1006/jmbi.2001.4923

39. Escher C, Cymer F, Schneider D. Two GxxxG-like motifs facilitate promiscuous interactions of the human ErbB transmembrane domains. J Mol Biol. 2009;389: 10–16. doi:10.1016/j.jmb.2009.04.002

40. Gerber D, Sal-Man N, Shai Y. Two motifs within a transmembrane domain, one for homodimerization and the other for heterodimerization. J Biol Chem. 2004;279: 21177–21182. doi:10.1074/jbc.M400847200

41. Beevers AJ, Kukol A. The transmembrane domain of the oncogenic mutant ErbB-2 receptor: a structure obtained from site-specific infrared dichroism and molecular dynamics. J Mol Biol. 2006;361: 945–953. doi:10.1016/j.jmb.2006.07.004

42. Papewalis J, AYu N, Rajewsky MF. G to A polymorphism at amino acid codon 655 of the human erbB-2/HER2 gene. Nucleic Acids Res. 1991;19: 5452.

43. Kawasaki Y, Sakimura A, Park CM, Tomaru R, Tanaka T, Ozawa T, et al. Feedback control of ErbB2 via ERK-mediated phosphorylation of a conserved threonine in the juxtamembrane domain. Sci Rep. 2016;6: 31502. doi:10.1038/srep31502

44. Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. Mol Syst Biol. 2005;1: 2005.0008. doi:10.1038/msb4100012

45. Hazan R, Margolis B, Dombalagian M, Ullrich A, Zilberstein A, Schlessinger J. Identification of autophosphorylation sites of HER2/neu. Cell Growth Differ. 1990;1: 3–7.

46. Ruiz-Saenz A, Dreyer C, Campbell MR, Steri V, Gulizia N, Moasser MM. HER2 Amplification in Tumors Activates PI3K/Akt Signaling Independent of HER3. Cancer Res. 2018;78: 3645–3658. doi:10.1158/0008-5472.CAN-18-0430

47. Ren Z, Schaefer TS. ErbB-2 activates Stat3 alpha in a Src- and JAK2-dependent manner. J Biol Chem. 2002;277: 38486–38493. doi:10.1074/jbc.M112438200

48. Bourguignon LY, Zhu H, Zhou B, Diedrich F, Singleton PA, Hung MC. Hyaluronan promotes CD44v3-Vav2 interaction with Grb2-p185(HER2) and induces Rac1 and Ras signaling during ovarian tumor cell migration and growth. J Biol Chem. 2001;276: 48679–48692. doi:10.1074/jbc.M106759200

49. Lucs A V, Muller WJ, Muthuswamy SK. Shc is required for ErbB2-induced inhibition of apoptosis but is dispensable for cell proliferation and disruption of cell polarity. Oncogene. 2010;29: 174–187. doi:10.1038/onc.2009.312

50. Marone R, Hess D, Dankort D, Muller WJ, Hynes NE, Badache A. Memo mediates ErbB2-driven cell motility. Nat Cell Biol. 2004;6: 515–522. doi:10.1038/ncb1134

51. Dankort D, Maslikowski B, Warner N, Kanno N, Kim H, Wang Z, et al. Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. Mol Cell Biol. 2001;21: 1540–1551. doi:10.1128/MCB.21.5.1540-1551.2001

52. Xu W, Yuan X, Beebe K, Xiang Z, Neckers L. Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. Mol Cell Biol. 2007;27: 220–228. doi:10.1128/MCB.00899-06

53. Wilson KS, Roberts H, Leek R, Harris AL, Geradts J. Differential gene expression patterns in HER2/neu-positive and -negative breast cancer cell lines and tissues. Am J Pathol. 2002;161: 1171–1185. doi:10.1016/S0002-9440(10)64394-5

54. Mackay A, Jones C, Dexter T, Silva R, Bulmer K, Jones A, et al. DNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. Oncogene. 2003;22: 2680–2688.

55. Sims AH, Zweemer AJM, Nagumo Y, Faratian D, Muir M, Dodds M, et al. Defining the molecular response to trastuzumab, pertuzumab and combination therapy in ovarian cancer. Br J Cancer. 2012;106: 1779–1789. doi:10.1038/bjc.2012.176

56. Mullen P, Cameron DA, Hasmann M, Smyth JF, Langdon SP. Sensitivity to pertuzumab (2C4) in ovarian cancer models: cross-talk with estrogen receptor signaling. Mol Cancer Ther. 2007;6: 93–100. doi:10.1158/1535-7163.MCT-06-0401

57. Yamashita-Kashima Y, Shu S, Yorozu K, Moriya Y, Harada N. Mode of action of pertuzumab in combination with trastuzumab plus docetaxel therapy in a HER2-positive breast cancer xenograft model. Oncol Lett. 2017;14: 4197–4205. doi:10.3892/ol.2017.6679

58. Mohsin SK, Weiss HL, Gutierrez MC, Chamness GC, Schiff R, Digiovanna MP, et al. Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. J Clin Oncol. 2005;23: 2460–2468. doi:10.1200/JCO.2005.00.661

Chapter 5. Trastuzumab inhibits cleavage and nuclear localization of HER2

5.1. SUMMARY

In the previous chapters, we showed that overexpression of HER2 had major effects on the gene expression profile of CHO cells and increased the cell proliferation, however had no major effects on activation of PI3K/Akt and MAPK pathways. We also found that trastuzumab has no major effect on the activation of PI3K/Akt and MAPK pathways, but inhibits cells cycle progression and survival, and induces apoptosis. These results suggest that HER2 exerts important oncogenic function via a non-canonical pathway. Interestingly several previous studies have demonstrated nuclear function of C-terminal truncated HER2 fragments (ctHER2) in breast cancer. ctHER2 can arise by proteolytic cleavage of full-length HER2 (p185HER2), localizes in the nucleus and mediates in the regulation of transcription. Inhibiting ctHER2 resulted in significant inhibition of tumor growth and invasion and overcame trastuzumab resistance. Based on these findings, we hypothesized that trastuzumab may block HER2 cleavage and nuclear function of ctHER2. In the current chapter, we found that trastuzumab induces ADCC of HER2+ breast cancer cells, does not inhibit HER2 receptor activation (homo- and heterodimerization, and phosphorylation), but inhibits activation of EGF-induced PI3K/Akt and MAPK pathways. We found expression and nuclear localization of a ctHER2 with an approximate size of 85 kDa (p85HER2) in the HER2+ breast cancer. However, trastuzumab treatment reduced the level of nuclear p85HER2 in a dose-dependent manner. We also found a synergism between trastuzumab and a proteinase inhibitor (TAPI-2) in the inhibition of HER2 cleavage, p85HER2 production and the cell growth, suggesting that trastuzumab binding to HER2 inhibits cell growth by blocking HER2 cleavage. Further studies showed that trastuzumab inhibits nuclear localization of p85HER2 as well. These results demonstrate that HER2 cleavage and nuclear localization of p85HER2 may be important factors for tumor growth as well as a target for trastuzumab. To investigate the function of p85HER2 we performed mass spectrometry analysis of p85HER2 protein samples pull down from nuclear fraction by immunoprecipitation (IP). Result revealed direct interaction of nuclear p85HER2 with proteins involved in spliceosome and RNA processing. In addition, interaction of p85HER2 with 9 transcription factors was found. These results indicate a novel function of HER2 in the regulation of RNA splicing, processing and gene

expression in the nucleus. Further gene set enrichment analysis showed the expression of the nuclear p85HER client proteins under the regulation of oncogenic/stemness transcription factors. Majority of the upstream transcription factors were found as positive regulators of mesenchymal maintenance involved in induction of EMT of breast cancer. Overall, our findings indicate a novel mechanism of action of trastuzumab through blocking HER2 cleavage and production, nuclear localization and regulatory function of p85HER2 in the nucleus. Our results shed further light on the roles of HER2 oncogene, EMT and cancer stemness in the breast cancer growth, invasion and therapy resistance. We also suggest that blocking HER2 cleavage and nuclear p85HER2 by proteinase inhibitor or chemical inhibitors mimicking trastuzumab function is a promising approach to target HER2-positive breast cancer.

5.2. INTRODUCTION

Growing pieces of evidence have uncovered direct nuclear signaling of HER2. Cohort studies on breast tumors conclusively showed a correlation of ctHER2 expression with breast cancer poor prognosis and trastuzumab resistance warranting ctHER2 expression as a prognostic factor for metastasis, worse outcome and also a predictive marker of trastuzumab resistance [1–4]. A study on breast tumor models overexpressing ctHER2 showed a direct relationship between ctHER2 expression and the emergence of resistant to antitumor effects of trastuzumab, whereas, inhibition of ctHER2 nuclear localization significantly inhibited breast cancer tumor cell growth [5,6]. ctHER2 is well demonstrated to migrates to the nuclear compartment, where it acts as a transcription co-factor. Wang et al. [7] identified icHER2 fragments in the nucleus of both tumor tissue and cultured tumor cells and found that it binds to a specific nucleotide sequence of *PTGS2* (coding for COX2) promoter and regulates gene expression. Chen et al. [8] found a nuclear localization signal (NLS) sequence KRRQQKIRKYTMRR (amino acids 655-668) located in the juxtamembrane region of HER2 which is essential for HER2 nuclear trafficking. Recent studies showed that in HER2-positive breast cancer cells ctHER2 can translocate to the nucleus wherein make a complex with the transcription factor STAT3 and regulate the promoter activity of the *CCND1* gene (coding for Cyclin D1), the regulation which is associated with poor clinical outcome [6,9]. Venturutti et al. [10] revealed a novel function of nuclear HER2 as a

regulator of miRNAs expression in the promotion of breast cancer metastasis. Nuclear HER2 interacts with the promoter of *MIR21* (coding for microRNA-21) an oncogenic microRNA which, by turn downregulated the expression of *PDCD4* gene [10]. Li et al. [11] reported nuclear HER2 functions as a regulator of ribosomal RNA synthesis. Nuclear HER2 also enhances cellular translation by association with β-actin and RNA polymerase I thereby contributes to tumor progression [11]. A more recent study addressed the function of ctHER2 in post-transcriptional regulation of the Na^+-HCO_3^- co-transporter SLC4A7 (NBCn1) which is known associated with breast malignancy. In breast cancer, ctHER2 increases the expression of SLC4A7 protein by binding to 3′UTR, stabilizing and protecting of SLC4A7 mRNA against degradation [12]. These shreds of evidence suggest non-canonical oncogenic pathways of nuclear HER2 warranting an independent role of nuclear HER2 in breast cancer development, metastasis and its resistance to trastuzumab.

Several pieces of evidence show that HSP90 is an important regulator of HER2 trafficking. Inhibition of HSP90 has been shown to reduce HER2 levels in preclinical models of breast cancer [13]. HSP90 inhibitor also blocked icHER2 function and suppressed the growth of trastuzumab-resistant breast tumors [5]. In a recent study, Cordo Russo et al. [6] reported that inhibition of HER2 nuclear localization by deleting NLS sequence of HER2 abrogated breast cancer cell growth and overcame trastuzumab resistance through blockade of Akt signaling and Cyclin D1 expression. This evidence suggests non-canonical oncogenic pathways of nuclear HER2 warranting an independent function of nuclear HER2 in breast cancer development, metastasis and its resistance to trastuzumab. Taken together, targeting nuclear HER2 is suggested to be a useful strategy to arrest breast tumor growth and overcome trastuzumab resistance in breast cancer patients.

In addition, our previous results showed that HER2 overexpression had major effect on the gene expression profile of CHO cells, however, did not change the activation of canonical downstream pathways PI3K/Akt and MAPK. We also found that trastuzumab has no major effect on the activation of PI3K/akt and MAPK pathways, but inhibits cells cycle progression and survival, and induces apoptosis. This evidence suggests that trastuzumab may blocks a non-canonical pathway of HER2 (probably via nuclear ctHER2) in HER2-overexpressing cells. Based on shreds of evidence reviewed above, we hypothesize that (1) Binding trastuzumab to

HER2 prevents cleavage of full-length HER2 and inhibits nuclear expression of C-terminal HER2; (2) nuclear HER2 mediates in the regulation of gene expression by contributing in the transcription complex. The aim of this study was to investigate the effect of trastuzumab in blocking HER2 cleavage and nuclear HER2 localization as well as the function of nuclear HER2 in HER2-positive breast cancer cells.

5.3. RESULTS

5.3.1. Trastuzumab specifically binds to HER2

Using CHO cells stably expressing human EGFR, HER2, and HER3, we previously showed that trastuzumab specifically binds to HER2 but not to EGFR or HER3 (see Figure 3.2) [14]. Here, we further confirmed the binding of trastuzumab to HER2 in breast cancer cell lines SKBR3, BT474, MCF7 and MDA-MB-231 by double immunofluorescence staining of HER2 and trastuzumab (Figure 5.1). We also examined trastuzumab binding in CHO-K6 and CHO-K13 cells as a positive control as well as in MCF10A cells as HER2-negative cell line. To this end, the cells were treated with 10 μg/ml trastuzumab for 1 hour and then HER2 was stained using a monoclonal antibody against the N-terminal end of HER2 (9G6) and a FITC-conjugated secondary antibody. Trastuzumab was stained by a TRITC-conjugated anti-human IgG antibody. Results showed very specific co-localization of trastuzumab and HER2 in all the HER2-expressing cells indicating specific binding of trastuzumab to HER2 (Figure 5.1).

5.3.2. Trastuzumab inhibits proliferation of HER2-positive breast cancer cells

To investigate whether binding of trastuzumab to HER2 inhibits the proliferation of HER2-positive breast cancer cells, we treated SKBR3 and BT474 cell lines with 10 μg/ml trastuzumab for 5 days and then monitored the cell proliferation levels by MTT assay. Cells treated with non-specific human IgG (10 μg/ml), cell cycle inhibitor vinorelbine (10 μM), and HER2 kinase inhibitor CP-714724 (10 μM) were used as respectively mock control, anti-proliferative control, and HER2 inhibitor control. we also tested the effect of pertuzumab (10 μg/ml) alone as well as in combination with trastuzumab. In addition, we examined all the treatments in 293T cells as HER2-negative cell control. As shown in Figure 5.2, trastuzumab ($P < 0.0001$) as well as other HER2-targeting agents CP-714724 ($P < 0.0001$) and pertuzumab ($P < 0.0001$) significantly

inhibited the proliferation of SKBR3 and BT474 cells but not 293T cells. These results show that the binding of trastuzumab to HER2 inhibits cell proliferation of HER2-positive breast cancer cell lines probably via blocking HER2 function.

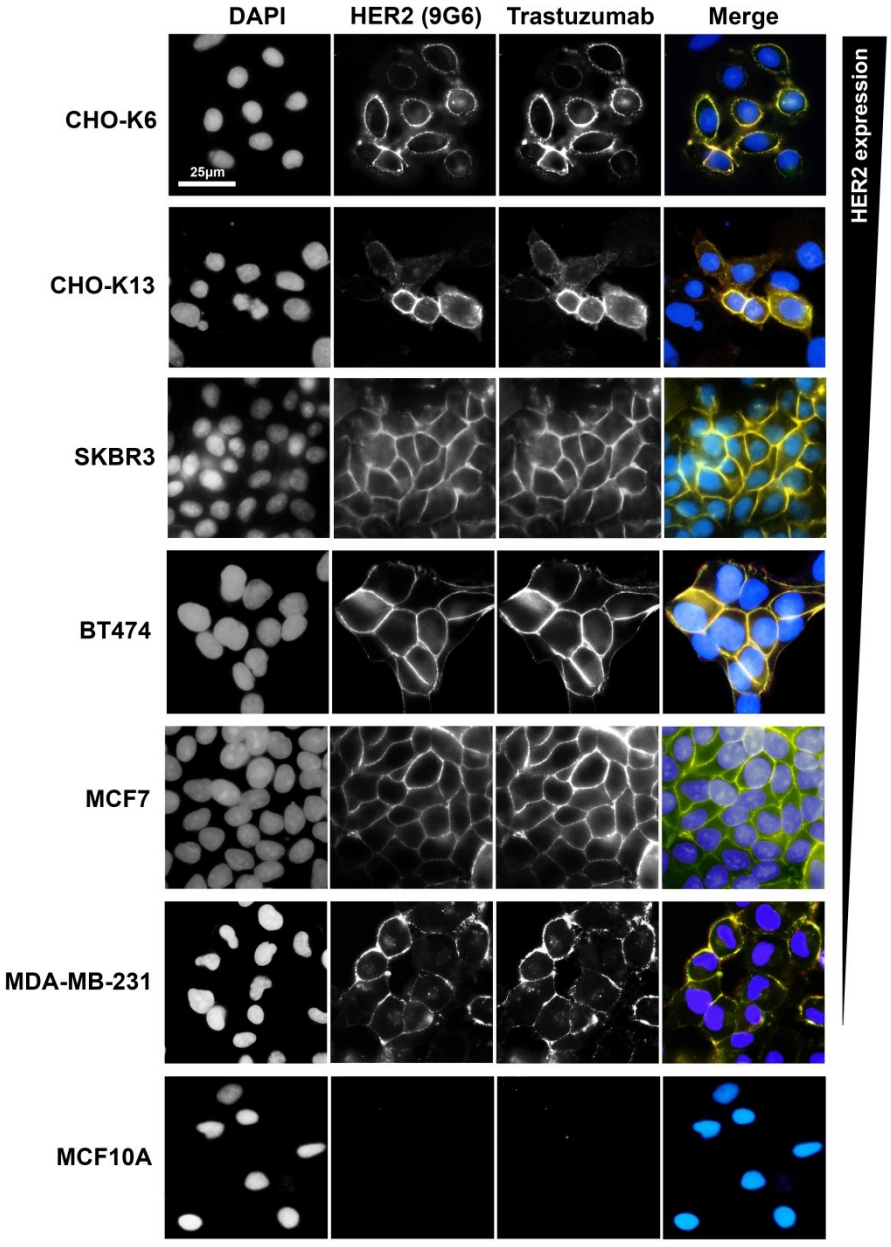

Figure 5.1. Trastuzumab binding to HER2 in breast cancer cell lines. Binding of trastuzumab to HER2 in transgenic high-level HER2 expressing CHO cell lines (CHO-K6 and CHO-K13), breast cancer cell lines with high HER2 expression levels (SKBR3 and BT474), low HER2 expression levels (MCF7 and MDA-MB-231), and HER2-negative breast cell line (MCF10).

The cells were treated with 10 μg/ml trastuzumab for 1 hour prior to immunofluorescence staining. HER2 (9G6, N-terminal) was stained by FITC (green). Trastuzumab was stained by TRITC (red). Scale bar: 25 μm. The gradient bar indicates HER2 expression level.

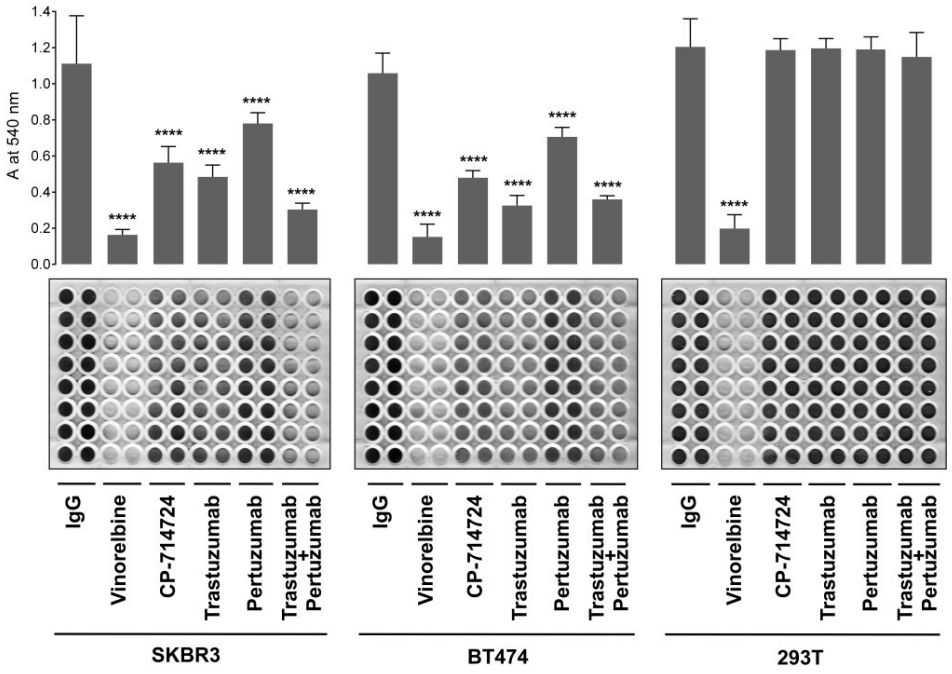

Figure 5.2. Trastuzumab inhibits the proliferation of HER2-positive breast cancer cell lines. SKBR3, BT474, and 293T cells were treated with 10 μg/ml trastuzumab, pertuzumab or their combination for 5 days, and then the living cell mass was evaluated by MTT assay (absorbance at 540-nanometer wave-lengths. Ten μg/ml human IgG, 10 μM vinorelbine, and 10 μM CP-724714 were used as respectively mock, anti-proliferative, and HER2 inhibitor controls. ****: $P < 0.0001$.

5.3.3. Trastuzumab induces ADCC

I previously showed that trastuzumab induces strong *in vitro* ADCC in CHO-K6 cells but not in CHO-GFFR (see Figure 3.8) [14]. We here examined whether trastuzumab induces ADCC in HER2-positive breast cancer cells. To this end, we treated BT474, MCF7 and MDA-MB-231 cells with increasing consecrations of trastuzumab (0, 0.005, 0.015, 0.05, 0.15, 0.5, 1, 1.5 and 2 μg/ml), and then investigated ADCC using ADCC Reported Bioassay kit as the method described in section 6.6. As shown in Figure 5.3, trastuzumab induced ADCC in a concentration-depending manner in all the cells. The ADCC levels in BT474 cells were significantly higher

than those in MCF7 and MDA-MB-231 cells. This may be due to a higher level of trastuzumab binding to the BT474 cell line compared to MCF7 and MDA-MB-231 cell lines.

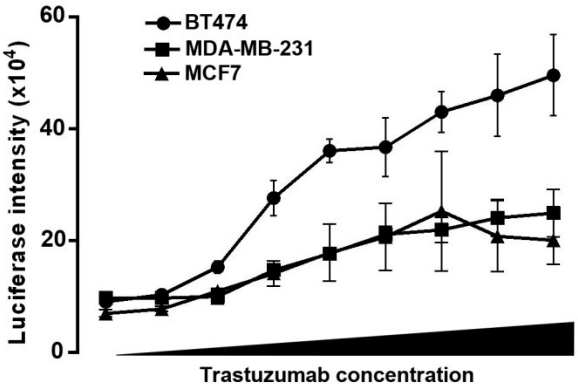

Figure 5.3. Trastuzumab induces ADCC of breast cancer cell lines. BT474, MCF7 and MDA-MB-231 cells were treated with different concentrations of trastuzumab (0, 0.005, 0.015, 0.05, 0.15, 0.5, 1, 1.5, and 2 μg/ml) and then *in vitro* ADCC was investigated.

5.3.4. Trastuzumab induces HER2 homo- and heterodimerization

To investigate the effect of trastuzumab on HER2 homodimerization, we treated CHO-K6 cells which is HER2-positive, EGFR-negative, HER3-negative, and HER4-negative, with 0.1, 0.5, 1, 5, and 10 μg/ml trastuzumab for 2 hours and then HER2 homodimers were cross-linked using BS^3 and were detected by immunoblotting using a monoclonal antibody against C-terminal end of HER2 (A2). The cells treated with IgG (10 μg/ml) were used as mock control for trastuzumab. As shown in Figure 5.4A, trastuzumab treatment increased HER2 dimer and reduced HER2 monomer in a dose-dependent manner. This result shows that binding trastuzumab to HER2 increases HER2 homodimerization.

To examine the effect of trastuzumab on HER2 heterodimerization, we treated MDA-MB-231 cells with 10 μg/ml trastuzumab for 2 hours and then monitored the HER2/EGFR heterodimer levels. Cells treated with human IgG (10 μg/ml) and EGF (50 ng/ml) were used as mock control for trastuzumab and positive HER2 dimerization inducer control respectively. HER2 protein complexes were pulled down by protein IP using A2 antibody, and EGFR levels in IPed and total lysate were detected by immunoblotting. The result showed increased levels of EGFR in EGF

treated as well as in trastuzumab treated cells (Figure 5.4B). This result shows that the binding of trastuzumab increases HER2/EGFR heterodimerization.

5.3.5. Trastuzumab has no effect on HER2 phosphorylation

To investigate the effect of trastuzumab on HER2 phosphorylation, we treated MCF7 and MDA-MB-231 cells with increasing concentrations of trastuzumab (0.1, 1, and 10 μg/ml) for 2 hours and then levels of total HER2 (using A2 antibody) and phosphorylated HER2 at Y1005, Y1127, and Y1139 were studied by immunoblotting. Treatment with human IgG (10 μg/ml) was used as a mock control. The result showed no significant difference in phosphorylated HER2 levels between the cells treated with trastuzumab and those treated with human IgG (Figure 5.4C). Overall, these results indicate that trastuzumab binding to HER2 neither increase nor inhibit HER2 phosphorylation.

5.3.6. Trastuzumab inhibits EGF-induced PI3K/Akt and MAPK pathways

Since trastuzumab has no effect on HER2 receptor activation, we examined the effect of trastuzumab on activation of HER2 downstream pathways PI3K/Akt and MAPK. For this, BT474, MCF7, MDA-MB-231, and COS7 (HER2-negative cell line) cells were treated with 10 μg/ml trastuzumab for 2 hours and then the level of phosphorylated Akt (S473) and Erk (T202/Y204) proteins were investigated by immunoblotting. Phosphorylation of Akt at S473 and Erk at T202/Y204 are the key modification and indicators of activated PI3K/Akt and MAPK pathways respectively. Treatment with human IgG (10 μg/ml) and EGF (50 ng/ml) were used as mock and positive controls.

Results showed that EGF treatment increased the levels of phosphorylated Akt and Erk in all the cell lines. Trastuzumab treatment had no effect on phospho-Akt and phospho-Erk levels in the absence of EGF in all the cell lines (Figure 5.4D). However; MCF7 and BT474 cells treated with a combination of trastuzumab and EGF showed lower levels of phospho-Akt and phospho-Erk compared to the cells treated with EGF alone. This regulation was not observed in COS7 (HER2-negative) cells (Figure 5.4D). These results indicate that trastuzumab inhibited EGF-induced PI3K/Akt and MAPK pathways in HER2+ breast cancer cells but not in HER2-negative cells. In addition, our results suggest that trastuzumab blocks the oncogenic function of HER2

not via inhibiting HER2 receptor activation, but maybe through blocking a non-canonical function of HER2 in HER2-positive breast cancer.

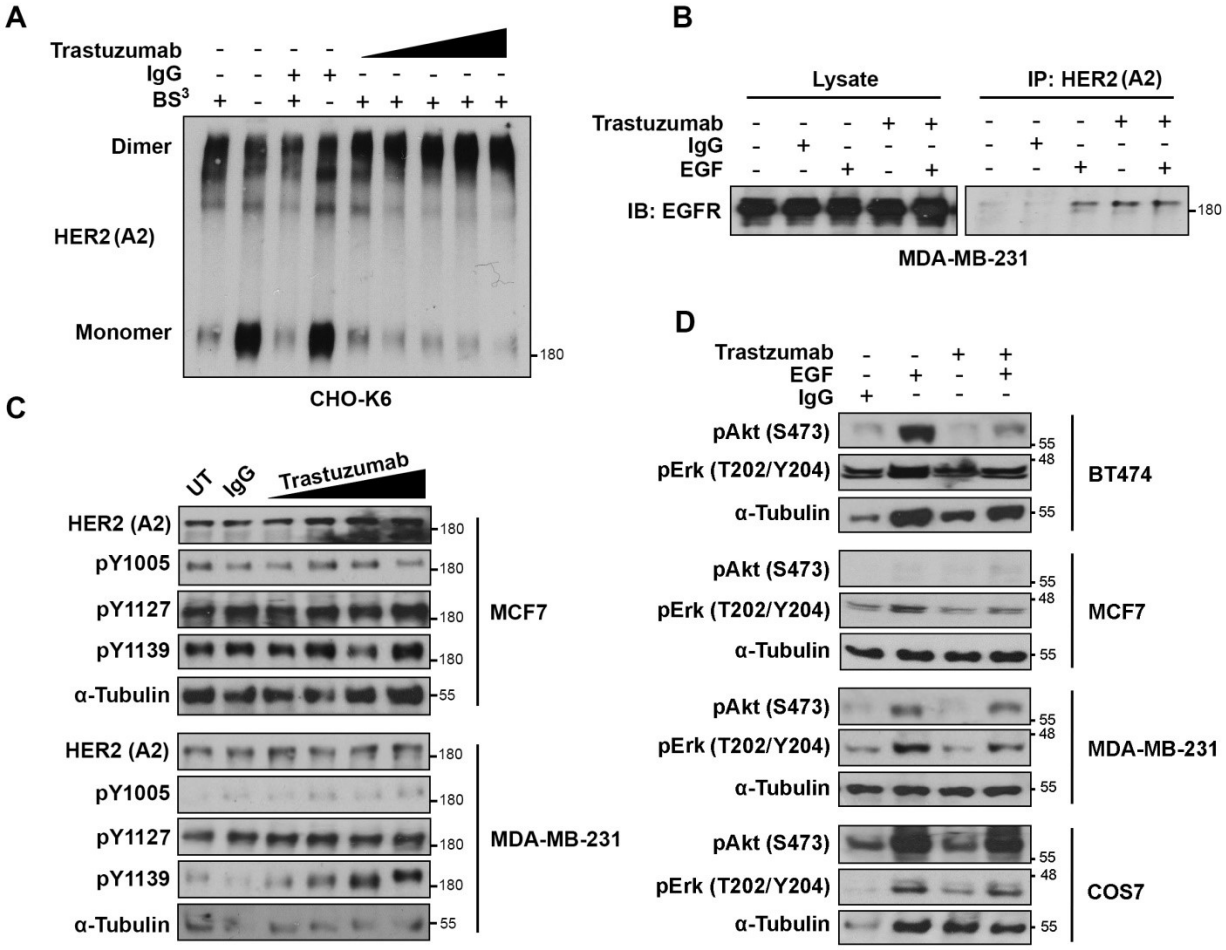

Figure 5.4. The effect of trastuzumab on HER2 dimerization, phosphorylation, and its downstream signaling pathways. (**A**) Immunoblotting result of HER2 monomer and homodimer CHO-K6 cells treated with increasing concentrations of trastuzumab (0.1, 0.5, 1, 5, and 10 μg/ml) for 2 hours and cross-linked by BS^3. (**B**) Immunoblotting (IB) result of EGFR in HER2-immunoprecipitated protein sample (IP) and total lysate from MDA-MB-231 cells treated with trastuzumab (10 μg/ml) for 2 hours. (**C**) Immunoblotting result of the expression of total and phosphorylated HER2 (Y1005, Y1127, and Y1139) in MCF7 and MDA-MB-231 cells treated with increasing concentrations of trastuzumab (0.1, 1 and 10 μg/ml) for 2 hours. (**D**) Immunoblotting result of the expression of phosphorylated Akt (S473) and Erk (T202/Y204) in BT474, MCF7, MDA-MB-231, and COS7 cells treated with

trastuzumab (10 μg/ml) for 2 hours. Treatment with human IgG (10 μg/ml) and EGF (50 ng/ml) were used as mock and positive controls. UT: Untreated.

5.3.7. Trastuzumab inhibits proteolytic cleavage of HER2

In the previous chapters, we showed that overexpression of HER2 had major effects on the gene expression profile of CHO cells (see Figure 4.5A) and increased the cell proliferation (see Figure 3.9A), however had no major effects on activation of PI3K/Akt and MAPK pathways (see Figure 3.7A). Based on these findings, we hypothesized that trastuzumab blocks a non-canonical function of HER2. HER2 (A2) immunoblotting result of CHO-K6 cells treated with trastuzumab for 2 hours revealed decreased levels of two different truncated HER2 with an approximate size of 85 kDa (p85HER2) and 45 kDa (p45HER2) in response to trastuzumab treatment (Figure 5.5A). To confirm this, we treated SKBR3 and BT474 cells with trastuzumab (10 μg/ml), pertuzumab (10 μg/ml) and human IgG (10 μg/ml) each for 6 hours and then immunoblotted HER2 using another antibody against C-terminal end of HER2 (C18). As a result, both the cell lines treated with human IgG showed significant levels of truncated HER2 fragment with a size of smaller than 185 kDa (Figure 5.5B). The levels of p85HER2 fragments were significantly higher than those of other size fragments. Interestingly, trastuzumab treatment decreased HER2 fragmentation in SKBR3 and BT474 cell lines; however, pertuzumab exerted the same effect in BT474 cells but not in SKBR3 cells (Figure 5.5B). To validate this and to investigate whether the HER2 fragments are N-terminal or C-terminal truncated, we stained HER2 in BT474 cells by double-immunofluorescence staining of HER2 using C18 (FITC) and 9G6 (TRITC) antibodies. As a result, the C18 antibody but not 9G6 antibody stained intracellular HER2 localized majorly in the nucleus; however, 9G6 antibody stained only plasma membrane HER2 (Figure 5.5C). Taken together, these results demonstrate that trastuzumab binding to HER2 inhibits HER2 protein fragmentation and production of C-terminal truncated HER2 fragments (ctHER2).

ctHER2 can arise by proteolytic cleavage of full-length HER2 [4,15–23]. To examine whether trastuzumab blocks HER2 cleavage, we treated SKBR3 and BT474 cells with trastuzumab (10 μg/ml), metalloproteinases (ADAMs and MMPs) inhibitor TAPI-2 (10 μM), and combination of trastuzumab and TAPI-2 for 6 hours, and then immunoblotted HER2 using A2 antibody. As shown in Figure 5.5D, the levels of ctHER2 in the cells treated with TAPI-2 as well as in trastuzumab treated cells were lower compared to control cells. More interestingly, the rate of

HER2 fragments in the cells treated with a combination of TAPI-2 and trastuzumab was lower than those in the cells treated with TAPI-2 or trastuzumab alone (Figure 5.5D). This reveals a synergic effect of trastuzumab and TAPI-2 in blocking proteolytic HER2 cleavage. These results indicate that ctHER2 fragments are produced through metalloproteinases-mediated cleavage of full-length HER2 and that trastuzumab binding to HER2 inhibits proteolytic HER2 cleavage and production of C-terminal HER2 fragments.

To investigate the effect of HER2 cleavage on cell growth and proliferation, we treated SKBR3 cells with TAPI-2 (10 μM), trastuzumab (10 μg/ml), and their combination for 72 hours (3 days) and then investigated cell proliferation by MTT assay. A combination of 1 μl DMSO (TAPI-2 vehicle) and human IgG (10 μg/ml) was used as vehicle/mock control, and 5 μM paclitaxel (cell cycle inhibitor agent) was used as anti-proliferative control. The result showed inhibited cell proliferation in the cells treated with TAPI-2 ($P < 0.05$) and those treated with trastuzumab ($P < 0.01$) compared to control cells (Figure 5.5E). Treatment with a combination of TAPI-2 and trastuzumab inhibited the cell proliferation with higher efficiency ($P < 0.0001$) compared to the TAPI-2 or trastuzumab alone (Figure 5.5E). These results indicate that HER2 cleavage is a positive factor for SKBR3 cell growth and proliferation and suggest a new mechanism of anti-cancer action of trastuzumab in HER2-positive breast cancer via blocking proteolytic cleavage of HER2 and probably an oncogenic effect of HER2 cleavage and ctHER2.

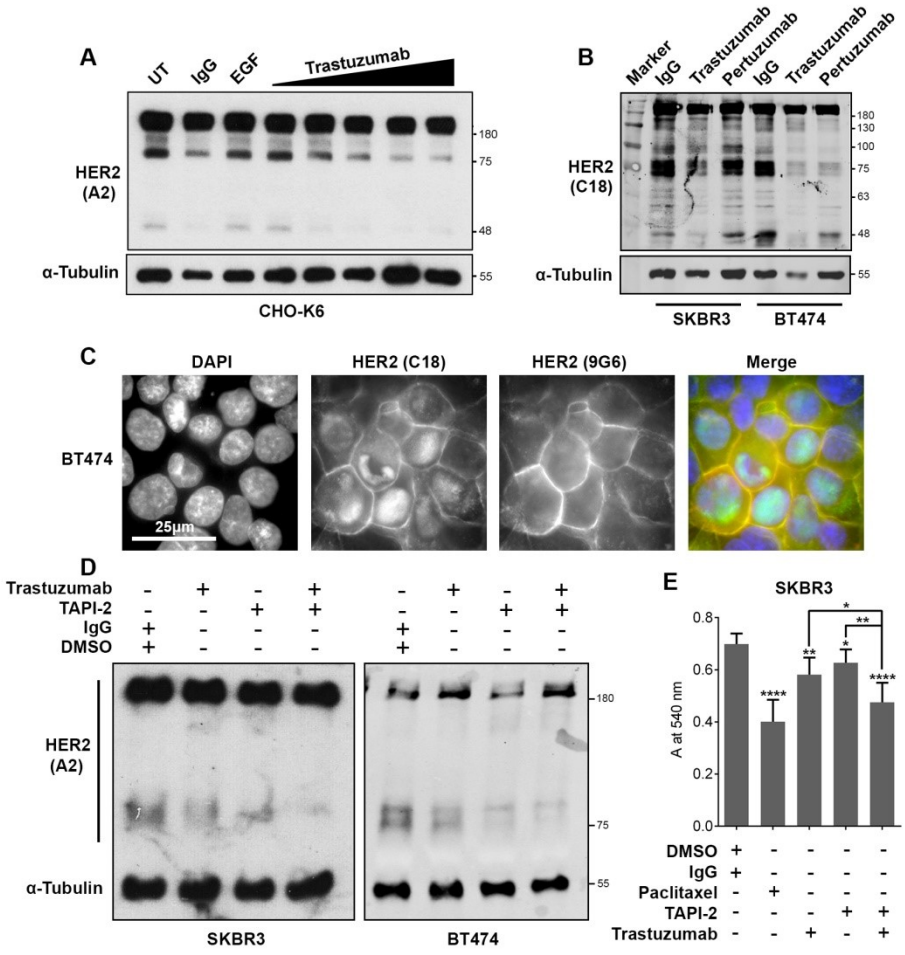

Figure 5.5. Trastuzumab inhibits proteolytic HER2 cleavage. (**A**) HER2 (A2 antibody) immunoblotting result of CHO-K6 cells treated with trastuzumab (0.1, 0.5, 1, 5, and 10 μg/ml) human IgG (10 μg/ml) and EGF (50 ng/ml) for 2 hours. UT: untreated. (**B**) HER2 (C18 antibody) immunoblotting result of SKBR3 and BT474 cells treated with trastuzumab (10 μg/ml), pertuzumab (10 μg/ml) and human IgG (10 μg/ml) for 6 hours. (**C**) Immunofluorescence staining of HER2 by C18 (FITC) and 9G6 (TRITC) antibodies in the BT474 cell line. Scale bar: 25 μm. (**D**) HER2 (A2 antibody) immunoblotting result of SKBR3 and BT474 cells treated with trastuzumab (10 μg/ml), TAPI-2 (10 μM), the combination of trastuzumab and TAPI-2 and combination of human IgG (10 μg/ml) and DMSO (10 μl/ml) for 6 hours. (**E**) MTT cell proliferation assay result of SKBR3 cells under treatment described in (**C**) for 72 hours (3 days). Paclitaxel (5 μM) was used as an anti-proliferative agent control. *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$.

HER2 can be cleaved from its juxtamembrane domain by zinc-containing metalloproteinases particularly ADAMs [4,15–23]. For further confirmation, we investigated the effect of trastuzumab binding to HER2 on proteolytic cleavage of HER2 by computational protein-protein binding simulation. For this, we used crystal structures the extracellular part of rat HER2 (ecHER2; PDB ID: 1N8Y [24]), catalytic domain of ADAM17 (PDB ID: 1BKC [25]) and the extracellular domain of human HER2 complexed with trastuzumab Fab (PDB ID: 1N8Z [24]) available from RCSB PDB. ADAM17 was virtually blind docked to the HER2 as well as to HER2-trastuzumab fab complex using ZDOCK server [26] without a pre-defining binding site.

As shown in Figure 5.6A, ADAM17 was appropriately bound to the juxtamembrane region of the HER2, and the cleavage site of the HER2 was perfectly located in the zinc endopeptidase active site of ADAM17. In contrast, the docking of ADAM17 to HER2 complexed with trastuzumab fab was aberrant. ADAM17 could not bind to the juxtamembrane region of the HER2, and the zinc endopeptidase active site of ADAM17 was too far to the HER2 cleavage site (Figure 5.6A). This is due to the binding of the trastuzumab fab to domain IV of the HER2 where is very close to the ADAM17 target site. There are two possible mechanisms explaining how binding trastuzumab to HER2 prevents enzymatic interaction of ADAM17 with HER2; (1) directly by burying HER2 juxtamembrane, and (2) indirectly by disrupting 3D protein conformation of HER2. Both of the mechanisms can inhibit HER2 cleavage by preventing accessibility of HER2 cleavage site to ADAM17 enzymatic active site. However, the exact mechanism is still to be studied by further analysis examination. Taken together computational simulation supports the hypothesis that trastuzumab inhibits proteolytic cleavage of HER2.

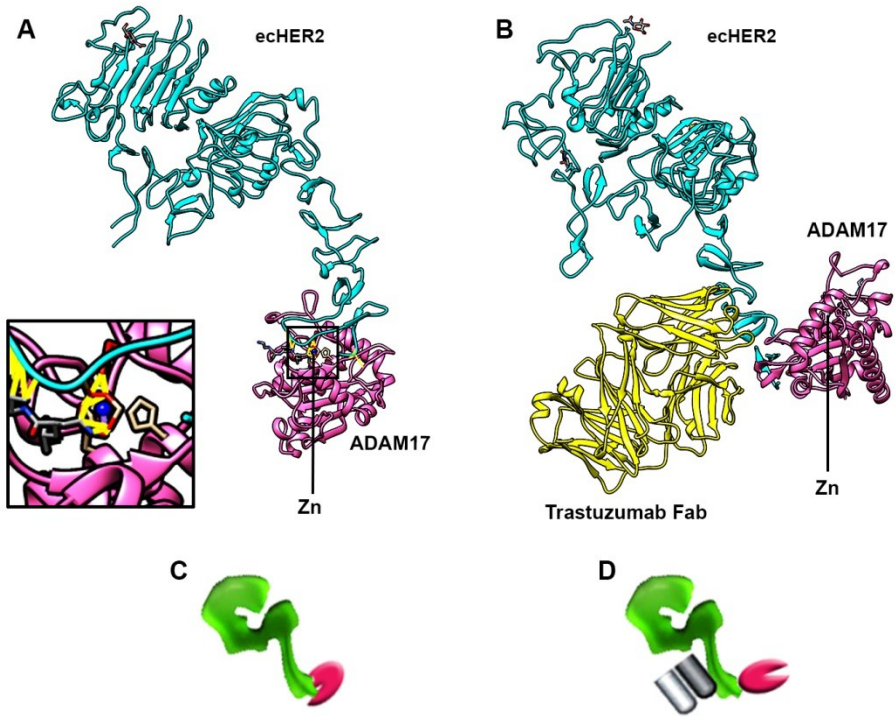

Figure 5.6. Crystal structure of extracellular HER2 in complex with ADAM17 and trastuzumab Fab. (**A**) Crystal structure of extracellular HER2 (ecHER2; PDB ID: 1N8Y; cyan) in the complex with the catalytic domain of ADAM17 (PDB ID: 1BKC; pink). The structures were docked using ZDOCK server. Zn indicates the endopeptidase enzymatic active site of ADAM17. Inset shows a region of ADAM17 enzymatic active site (pink) interacted with a juxtamembrane region of HER2 (cyan). Yellow connections represent chemical bonds (**B**) Crystal structure of ecHER2 complexed with trastuzumab fab (PDB ID: 1N8Z; ecHER2 in cyan; trastuzumab fab in yellow), and the catalytic domain of ADAM17 (PDB ID: 1BKC; pink). (**C** and **D**) Schematized of ecHER2/ADAM17 (**C**), and ecHER2/trastuzumab Fab/ADAM17 (**D**) structures shown in (A) and (B) respectively.

5.3.8. Trastuzumab inhibits nuclear localization of HER2

To confirm whether trastuzumab blocks HER2 cleavage and nuclear localization of C-terminal truncated HER2, we treated MCF7, SKBR3, and BT474 cell lines with trastuzumab (10 μg/ml) for 6 hours, and then stained HER2 using C18 antibody. As shown in Figures 4.8A-C, the cells treated with human IgG (10 μg/ml) showed strong HER2 localization in the nucleus. However, nuclear p85HER2 were not seen in the trastuzumab treated cells.

To further confirm nuclear p85HER2 and the effect of trastuzumab on nuclear localization of p85HER2, we monitored HER2 separately in the plasma membrane, cytosolic and nuclear fractions of SKBR3 and BT474 cells treated with trastuzumab. To this end, the cells were treated with trastuzumab (10 μg/ml) or human IgG (10 μg/ml) for 6 hours and then plasma membrane, cytosolic and nuclear proteins were isolated by subcellular fractionation. The HER2 content of each fraction was investigated by immunoblotting using the C18 antibody. As result, trastuzumab treated SKBR3 and BT474 cells showed lower levels of p85HER2 in the plasma membrane fraction compared to the relevant IgG treated cells (Figure 5.7D). No major difference was detected between the cytosolic fraction of trastuzumab treated cells and that of the cells treated with human IgG in terms of p85HER2 content (Figure 5.7E). Nuclear fractions from both IgG and trastuzumab treated cells revealed full-length as well as several different sized HER2 fragments. However, the levels of p85HER2 in trastuzumab treated cells were significantly lower than that in the cells treated with human IgG (Figure 5.7F).

We validated these results by investigating the effect of trastuzumab on production and nuclear localization of transgene HER2. For this, we transfected pEGFP-ERBB2 plasmid (coding full-length HER2 with green fluorescence protein (GFP) flag fused to its C-terminal end) into COS7 cells and then treated the cells with trastuzumab (10 μg/ml) or human IgG (10 μg/ml) for 6 hours. C-terminal HER2 localization was studied by immunofluorescence staining of HER2 using C18 antibody. In addition, the cells were subjected to subcellular fractionation, and truncated HER2 fragments were then investigated in the plasma membrane, cytosolic and nuclear fractions by immunoblotting using C18 antibody. As result, GFP was detected in the nucleus of the cells treated with human IgG. The cells treated with trastuzumab showed GFP localization only at the plasma membrane fraction and no nuclear GFP was detected in the nucleus (Figure 5.8A). Trastuzumab treated cells also showed lower rates of p85HER2 fragments in the nuclear fraction that was confirmed by immunoblotting using antibodies against C18 and GFP flag (Figure 5.8B). Results showed localization of full-length HER2 in the cytosol as well as in the nucleus. The cytosolic full-length HER2 might be the nascent HER2 proteins not yet localized in the plasma membrane. There is no documented evidence regarding the localization of full-length HER2 in the nucleus. The most possible explanation for presence of full-length HER2 in the nucleus is the contamination of nuclear fraction by plasma membrane.

Our previous results showed that HER2 phosphorylated at Y1005 and Y1139 but no other pY sites majorly localized in the nucleus (see Figure 4.4). This result suggests that phosphorylation of HER2 at Y1005 and Y1139 may be required and specific modifications for HER2 cleavage and nuclear localization. To examine this and as further evidence supporting our hypothesis, we immunofluorescence stained phosphorylated HER2 at Y1005 and Y1139 in BT474 cells treated with trastuzumab. Results revealed strong nuclear localization of pY1005 and pY1139 HER2 in human IgG treated cells. Whereas, nuclear pY1005 and pY1139 HER2 disappeared in the cells treated with trastuzumab (Figure 5.9). Since trastuzumab does not inhibit HER2 phosphorylation (see Figure 5.4C), absence of pY1005 and pY1139 HER2 in the nucleus of trastuzumab treated cells, should be due to inhibition of HER2 nuclear translocation by trastuzumab. This evidence strongly supports our hypothesis on this function of trastuzumab. Taken together, these results demonstrate that trastuzumab binding to HER2 inhibits proteolytic cleavage of full-length HER2 and nuclear localization of p85HER2 fragments.

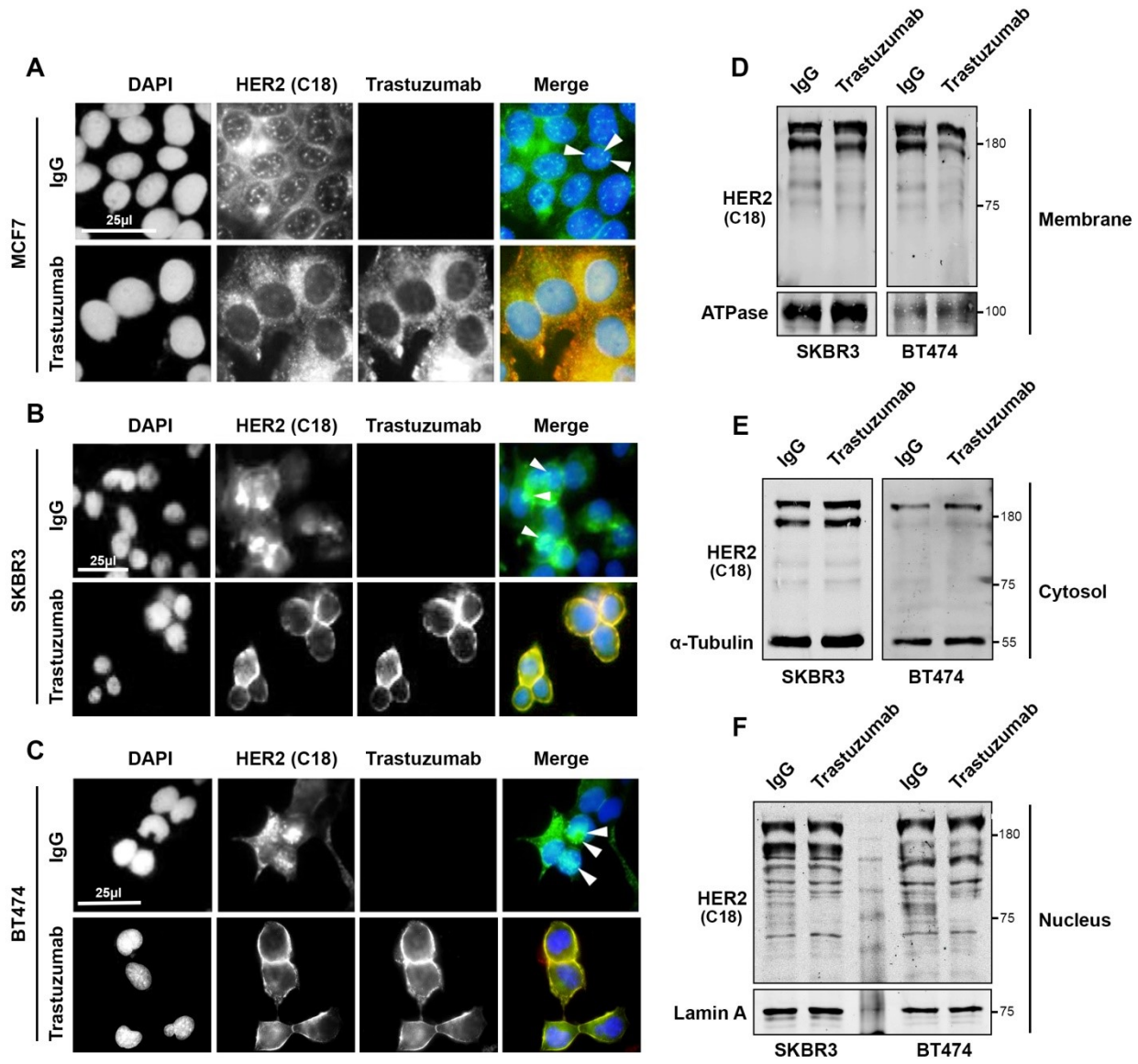

Figure 5.7. Trastuzumab inhibits nuclear localization of endogenous HER2 in breast cancer cell lines. (**A**-**C**) Immunofluorescence staining of HER2 (C18; FITC) and trastuzumab (TRITC) in MCF7 (**A**), SKBR3 (**B**), and BT474 (**C**) cells treated with trastuzumab (10 μg/ml) or human IgG (10 μg/ml) for 6 hours. Arrowheads indicate nuclear HER2. Scale bar: 25 μm. (**D**-**F**) Immunoblotting of HER2 (C18) in the plasma membrane (**D**), cytosolic (**E**) and nuclear (**F**) fractions of SKBR3 and BT474 cells treated with trastuzumab (10 μg/ml) or human IgG (10 μg/ml) for 6 hours. Na^+/K^+-ATPase, α-tubulin, and lamin A proteins were used as specific makers and loading controls for the plasma membrane, cytosolic and nuclear fractions respectively.

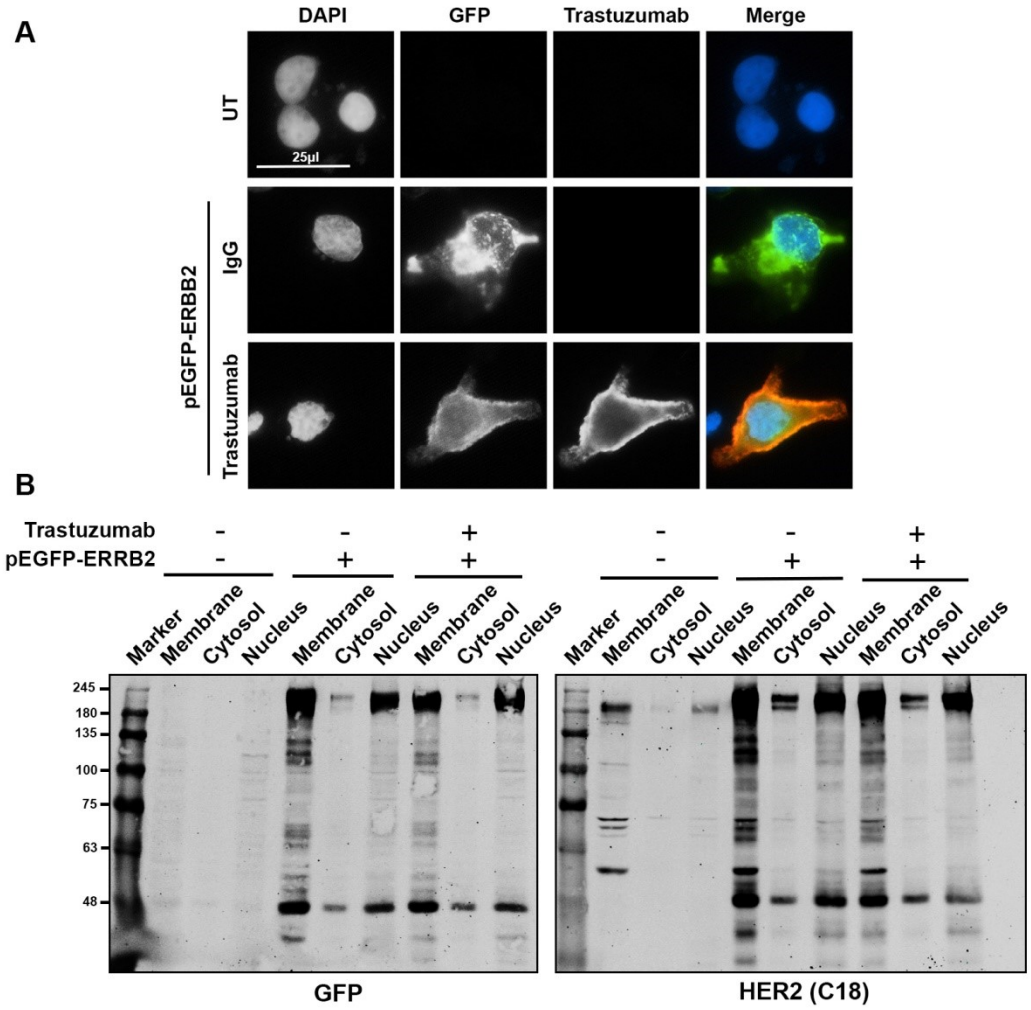

Figure 5.8. Trastuzumab inhibits nuclear localization of transgene HER2. (**A**) Fluorescence image of GFP flag and immunofluorescence staining of trastuzumab (TRITC) in COS7 cells transfected by pEGFP-ERBB2 plasmid and treated with trastuzumab (10 µg/ml) or human IgG (10 µg/ml) for 6 hours. UT: Untransfected. Scale bar: 25 *µm*. (**B**) Immunoblotting of GFP flag and HER2 (C18) in untransfected and transfected COS7 by pEGFP-ERBB2 plasmid treated with trastuzumab (10 µg/ml) for 6 hours.

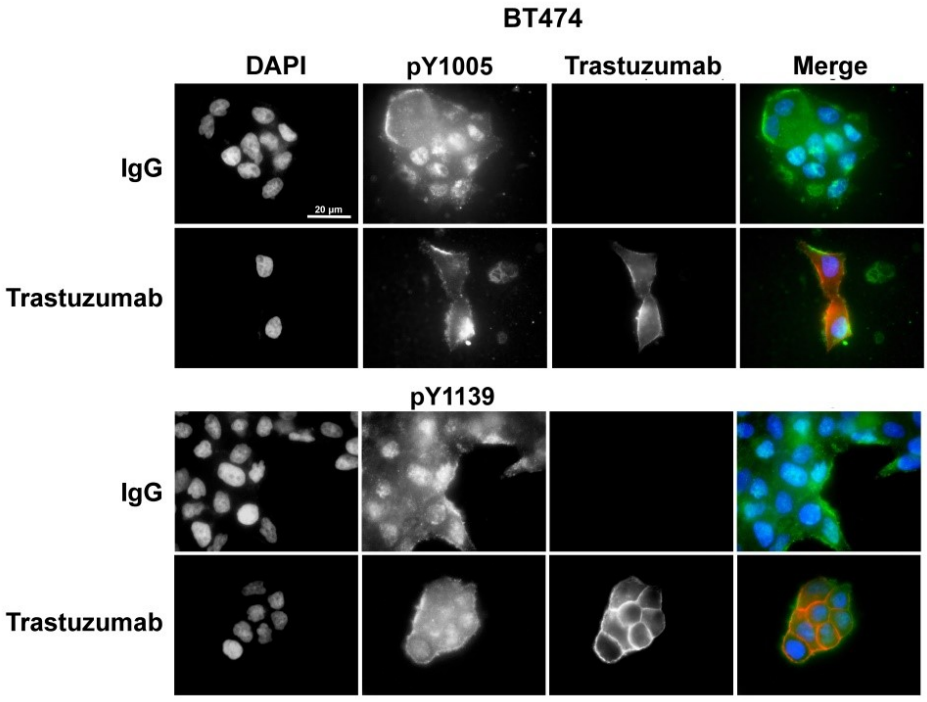

Figure 5.9. Trastuzumab inhibits nuclear localization of pY1005 and pY1139 HER2. Immunofluorescence staining of phosphorylated HER2 (Y1005 and Y1139) BT474 cells treated with human IgG (10 μg/ml) or trastuzumab (10 μg/ml) for 6 hours. Scale bar: 20 μm.

5.3.9. HER2 cleavage at the IJM and nuclear localization signal (NLS) are essential for nuclear localization of p85HER2

We investigated the cleavage site of HER2 required for HER2 nuclear localization. To this end, we constructed expression vectors of full-length HER2 (FL; amino acids 1-1255), HER2 with deleted extracellular part (TM+IC; amino acids 648-1255), icHER2 (IC; amino acids 672-1255), and icHER2 with deleted NLS sequence (IC_ΔNLS; amino acids. 693-1255). The schematic structure and initial amino acid sequence of the truncated HER2 proteins are shown in Figure 5.10A. The open reading frames (ORFs) of truncated *ERBB2* genes were amplified from a previously constructed expression vector possessing full-length human *ERBB2* ORF by polymerase chain reaction (PCR) using specific primer pairs. The sequence of PCR primers is shown in Table 6.1. We cloned the ORFs into pcDNA3.1/*Myc*-HisA(-) plasmid vector (Figure 5.10B). This vector confers a Myc epitope and 6x histidine (6xHis) protein flags both fused to the C-terminal end of the HER2 proteins and allows expressing HER2-Myc-H_6 fusion proteins.

We also cloned the ORFs into pEGFP-N3 plasmid vectors (Figure 5.10B) in order to tag a GFP flag at the C-terminal end of HER2 proteins that express HER2-GFP fusion proteins.

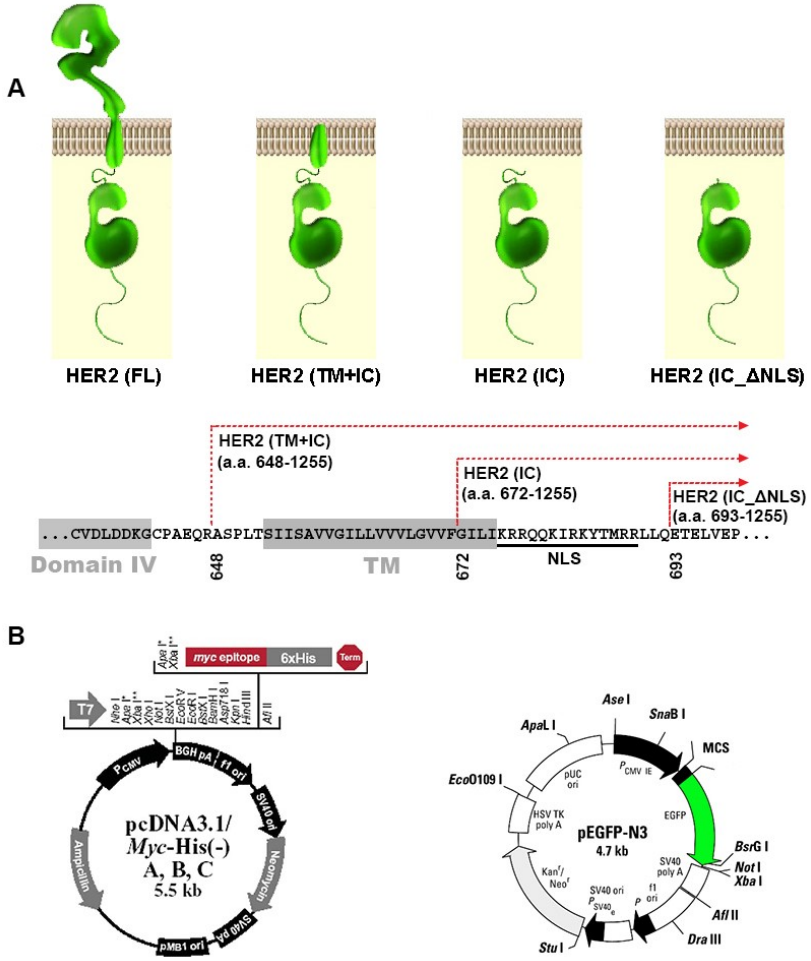

Figure 5.10. Structures and initial amino acid sequences of transgene HER2 proteins. (**A**) Schematic structures and truncation start sites of full-length HER2 (FL; amino acids 1-1255), HER2 with deleted extracellular part (TM+IC; amino acids 648-1255), icHER2 (IC; amino acids 672-1255), and icHER2 with deleted NLS sequence (IC_ΔNLS; amino acids. 693-1255). Gray highlights indicate helix regions (TM and a pert of Domain IV). (**B**) Structural map of pcDNA3.1/*Myc*-HisA(-) and pEGFP-N3 vectors.

We transfected the HER2-GFP and HER2-His expression plasmid constructs as well as empty vectors into 293T cells using calcium phosphate gene transfer method as described in section 6.13 and then monitored GFP and 6x histidine by immunofluorescence staining assay. The result showed cytosolic localization of the flags (GFP and 6xHis) in the cells transfected with empty

vectors. The cells transfected with FL and TM+IC showed the flags at the plasma membrane and in the cytosol but not in the nucleus. Importantly, the cells transfected with IC revealed the localization of the flags in the cytosol as well as in the nucleus but not at the plasma membrane. However, only cytosolic localization of flags was detected in the cells transfected with IC_ΔNLS (Figure 5.11A). Due to the higher transfection rate of ERBB2-His constructs than that of ERBB2-EGFP constructs, we used ERBB2-His constructs in the next experiments.

We confirmed the immunofluorescence staining results by immunoblotting detection of HER2 (A2) and 6xHis flag in the plasma membrane, cytosolic and nuclear fractions from 293T cells transfected by the ERBB2-His expression constructs. As result shown in Figure 5.11B, high levels of full-length HER2, as well as the same size 6xHis flag, were detected only in the plasma membrane fraction of the cells transfected with FL. Low levels of 185 kDa endogenous HER2 were detected in the membrane fraction from untransfected cells, the cells transfected with empty vector as well as the cells transfected with truncated HER2 constructs. The cells transfected with TM+IC showed 95 kDa truncated HER2 and the same size 6xHis flag majorly in the plasma membrane fraction and at a lower level in the cytosolic fraction. The cells transfected with IC showed approximately 85 kDa truncated HER2 and the same size 6xHis flag in the plasma membrane fraction, the cytosolic fraction as well as high in the nuclear fraction. However, the cells transfected with IC_ΔNLS showed truncated HER2 and the same size 6xHis flag in the plasma membrane fraction and in the cytosolic fraction but not in the nuclear fraction. Despite the lack of TM domain, IC and IC_ΔNLS HER2 proteins were detected in the plasma membrane fraction. This may be due to the binding of the truncated HER2s to membranous protein(s) that lets the HER2s localize in at the plasma membrane; however, the exact reason for this observation is still unclear.

Taken together, these results indicate that only HER2 without TM domain but possessing NLS sequence can get into the nucleus confirming that cleavage at the IJM region is required for HER2 nuclear localization.

5.3.10. Nuclear p85HER2 contributes to RNA processing and mRNA spicing

To study the function of nuclear p85HER2 in the nucleus, we pulled down HER2 from nuclear fractions of 293T cells transfected with IC, as well as SKBR3 and BT474 cell lines by IP

using A2 antibody. We then, analyzed whole IP protein samples by mass spectrometry to identify the protein content. The schematic work follow is shown in Figure 5.12A.

Immunoblotting of HER2 (C18) showed nuclear p85HER2 in the IP protein samples from SKBR3, BT474 and the 293T cells transfected with IC. We also detected full-length HER2 in the nuclear fraction of BT474 cells. Heavy (50 kDa) and light (25 kDa) chains of A2 antibody were also detected (Figure 5.12B). These results show successful HER2 pull-down from the nuclear fractions and further validate p85HER2 localization in the nucleus.

To identify client proteins of p85HER2 in the nucleus, their functions and involved pathways, we profiled protein content of the HER2 pull-down protein samples by mass spectrometry analysis, and then performed a gene set enrichment analysis (GSEA) of the identified proteins in terms of Gene Ontology molecular and biological functions, ENCODE and ChIP Enrichment Analysis (ChEA) Consensus transcription factors from ChIP-X as well as KEGG pathways by using Enrichr tool [27,28].

Mass spectrometry profiling of HER2 pull-down protein samples from nuclear fraction revealed 265 proteins in 293T-IC (Figure 5-13A), 157 proteins in SKBR3 (Figure 5-13B), and 120 proteins in BT474 cells (Figure 5-13C). Of all detected proteins, 76 proteins were common in the three cell lines (Figure 5-13D).

As shown in Figure 5.14, gene ontology analysis of the gene sets in terms of the molecular function showed that a big majority (208 proteins) of identified proteins have RNA binding activity. Next top 9 found enriched functional terms of the proteins ranked by combined score of *P*-value and Z-score were as follow: mRNA binding (34 proteins), cadherin binding (35 proteins), double-stranded RNA binding (13 proteins), mRNA 5'-UTR binding (9 proteins), ATP-dependent helicase activity (14 proteins), mRNA 3'UTR binding (12 proteins), poly-pyrimidine tract binding (8 proteins), RNA helicase activity (12 proteins), and ATP-dependent RNA helicase activity (14 proteins).

As shown in Figure 5.15, gene ontology analysis of the gene sets in terms of biological process revealed top 10 enriched biological functions terms as follow: gene expression (81 proteins), RNA splicing via transesterification reactions with bulged adenosine as nucleophile (53 proteins), mRNA splicing via spliceosome (68 proteins), mRNA processing (68 genes), SRP-

dependent cotranslational protein targeting to membrane (45 proteins), nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (50 proteins), cotranslational protein targeting to membrane (45 proteins), protein targeting to ER (45 proteins), viral transcription (46 proteins), and viral gene expression (45 proteins).

As shown in Figure 5.16. enrichment analysis of KEGG pathways showed that the majority of identified proteins were involved in spliceosome pathways (46 proteins). The next top 9 enriched KEGG pathway terms were found as follow: ribosome pathway (45 proteins) Huntington disease (17 proteins), pathogenic Escherichia coli infection (10 proteins), Parkinson disease (14 proteins), RNA transport (11 proteins), mRNA surveillance pathway (10 proteins), protein processing in endoplasmic reticulum (10 proteins), Alzheimer disease (9 protein), and Salmonella infection (8 proteins).

In all the cell lines, 9 transcription factors inclusing BCLAF1, FUS, KHDRBS1, LIMA1, MYBBP1A, SND1, TAF15, THOC2, and THRAP3 were detected interacted with nuclear p85HER2, of which FUS and KHDRBS1 were common in the three cell lines (Figure 5-13E). Gene ontology analysis of the identified transcription factors in terms of the biological function revealed top 5 functions of the transcription factors as follow:

BCLAF1: positive/negative regulation of transcription initiation, positive regulation of apoptotic signaling pathway, positive regulation of response to DNA damage stimulus, positive regulation of protein complex assembly.

FUS: RNA splicing via transesterification reactions with bulged adenosine as nucleophile, mRNA splicing, via spliceosome, mRNA processing.

KHDRBS1: positive regulation of nucleobase-containing compound transport, positive regulation of RNA export from nucleus, positive regulation of nucleocytoplasmic transport, cell cycle G2/M phase transition, negative regulation of nucleic acid-templated transcription.

LIMA1: regulation of actin filament depolymerization, ruffle organization, negative regulation of protein depolymerization, negative regulation of cytoskeleton organization, negative regulation of supramolecular fiber organization.

MYBBP1A: regulation of anoikis, nuclear transport, cellular response to starvation, intrinsic apoptotic signaling pathway by p53 class mediator, positive regulation of cell cycle arrest.

SND1: RNA catabolic process, nucleobase-containing compound catabolic process, cellular macromolecule catabolic process, RNA metabolic process.

TAF15: transcription elongation from RNA polymerase II promoter, regulation of signal transduction by p53 class mediator, regulation of intracellular signal transduction, positive regulation of nucleic acid-templated transcription, positive regulation of gene expression.

THOC2: termination of RNA polymerase II transcription, termination of DNA-templated transcription, RNA transport, mRNA export from nucleus, neuron development.

THRAP3: positive regulation of mRNA splicing via spliceosome, regulation of alternative mRNA splicing via spliceosome, mRNA stabilization, steroid hormone-mediated signaling pathway, initiation of DNA-templated transcription.

For further understanding of the pathways of nuclear p85HER2, we investigated the enrichment of upsteam transcription factors regulating the transcription of HER2 client protein identified by mass spectrometry. To this end, we analyzed the enrichment of consensus transcription factors whose target genes are profiled by ChIP-X experiments and are present in ENCODE and ChEA. The analysis result showed enrichment of 97 transcription factors in the gene set identified in SKBR3 cell line (Figure 5.17A), 96 transcription factors in the gene set identified in BT474 cell line (Figure 5.17B), and 100 transcription factors in the gene set identified in 293-IC cells (Figure 5.17C). Of the transcription factors, 88 were common in the three cell lines (Figure 5.17D). Analysis of KEGG pathways enrichment in the transcription factors gene sets revealed that most of them are associated with cancer development and are positive regulators of stemness and EMT including but not limited to transcriptional misregulation in cancer (17 transcription factors), acute myeloid leukemia (9 transcription factors), chronic myeloid leukemia (8 transcription factors), prostate cancer (8 transcription factors), Thyroid cancer (4 transcription factors), breast cancer (8 transcription factors), panceratic cancer (5 transcription factors) and other cancer types, signaling pathways regulating pluripotency of stem cells (9 transcription factors), cell cycle (8 transcription factors), as well as in many oncogenic/stemness signaling pathways including TGF-β signaling pathway (4

transcription factors) TNF signaling pathways (5 transcription factors), Wnt signaling pathways (5 transcription factors), PI3K/Akt signaling pathways (6 transcription factors) MAPK signaling pathways (7), Notch signaling pathways (2 transcription factors), Estrogen signaling pathways (5 transcription factors), Hippo signaling pathways (4 transcription factors), and JAK/STAT signaling pathways (3 transcription factors). These results show that nuclear HER2 interacts with downstream elements of oncogenic/stemness transcription factors.

Taken together, these results indicate that HER2 regulates RNA processing including RNA export from the nucleus, mRNA splicing by interaction with RNA helicases, RNA transporters, and spliceosome complex proteins. The Client proteins of nuclear p85HER2 are downstream targets of master oncogenic/stemness regulators, suggesting the oncogenic function of nuclear p85HER2 in HER2-positive breast cancer cells.

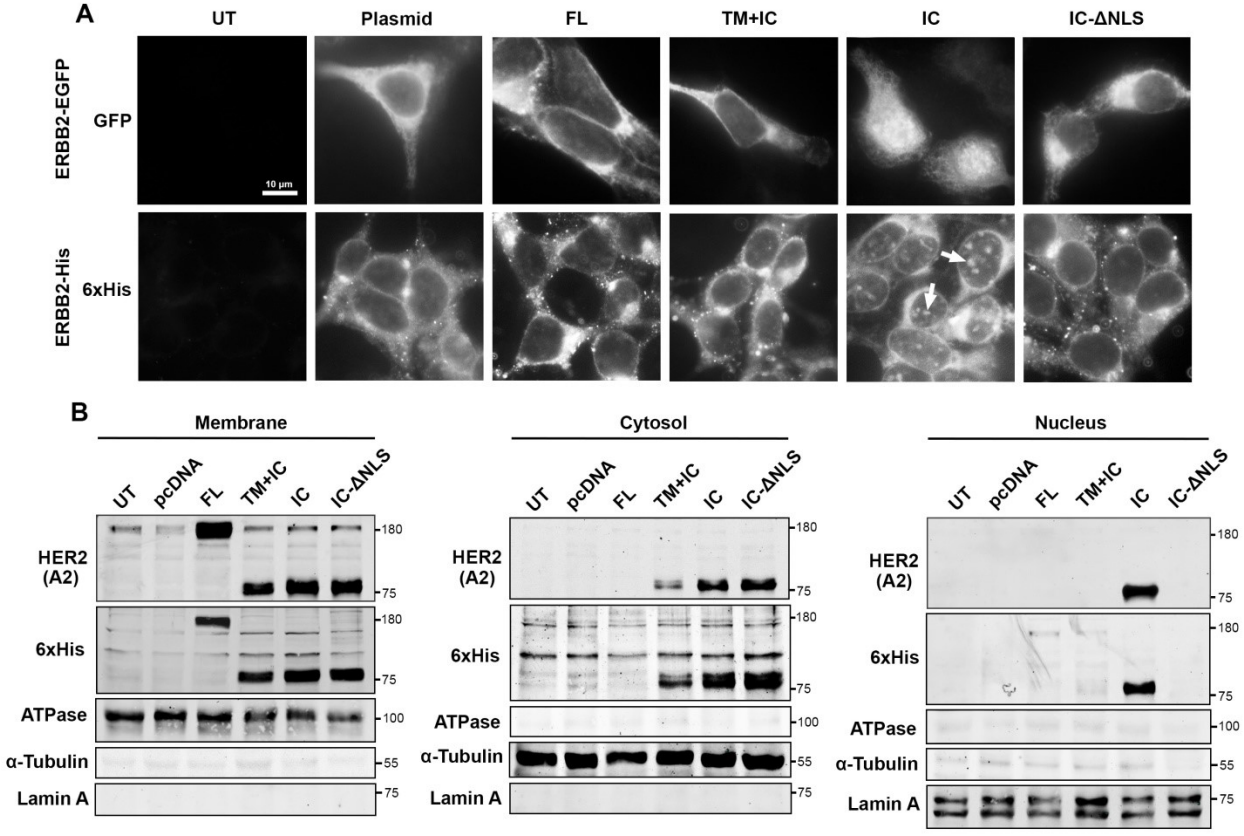

Figure 5.11. Subcellular localization of full-length and C-terminal truncated HER2 proteins. (**A**) immunofluorescence staining of GFP and 6x histidine (6xHis) flags in 293T cells transfected with the ERBB2-EGFP and the ERBB2-His constructs. UT: Untreansfected.

Plasmid: empty pcDNA3.1/*Myc*-HisA(-) or pEGFP-N3 vectors. FL: constructs coding full-length HER2 ORF, TM+IC: constructs coding HER2 ORF with deleted extracellular. IC: constructs coding icHER2 ORF, IC-ΔNLS: constructs coding icHER2 ORF with deleted NLS sequence. Arrows show nuclear HER2. Scale bar: 10 μm. (**B**) Immunoblotting of HER2 (A2) and 6x histidine flag (6xHis) in the plasma membrane, cytosolic and nuclear fractions from 293T cells transfected with the HER2 constructs. Na^+/K^+-ATPase, α-tubulin, and lamin A proteins were used as specific makers and loading controls for the plasma membrane, cytosolic and nuclear fractions respectively.

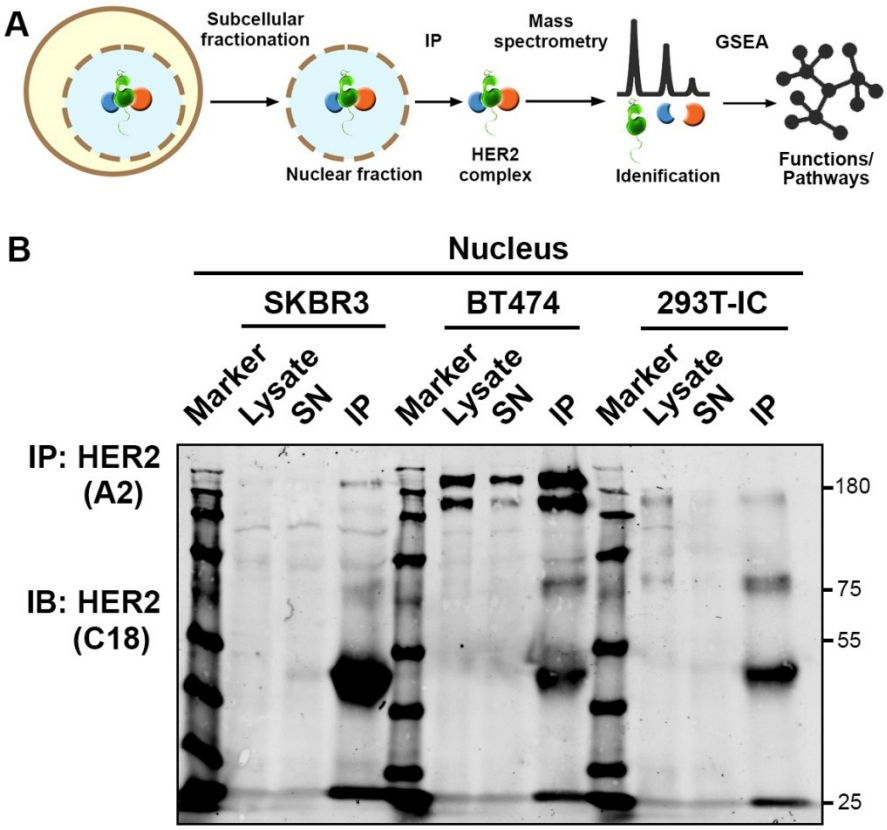

Figure 5.12. Functional analysis of nuclear p85HER2. (**A**) The schematic illustrating the workflow of the identification of nuclear p85HER2 client proteins in the nucleus. SKBR3, BT474 and the 293T transfected with IC (293T-IC) were subjected to subcellular fractionation. HER2 was pulled down from the nuclear fractions by IP using A2 antibody. The IP protein samples were then subjected to proteomic profiling by mass spectrometry to identify the p85HER2 client proteins in the nucleus. The mass spectrometry identified

proteins was subjected to gene set enrichment analysis (GSEA) in terms of functional gene ontology, KEGG pathway analysis and upstream ENCDE/ChEA transcription factors. (**B**) Immunoblotting results of HER2 (C18) in total lysate, supernatant (SN) and IP samples from the nuclear fractions of the cells.

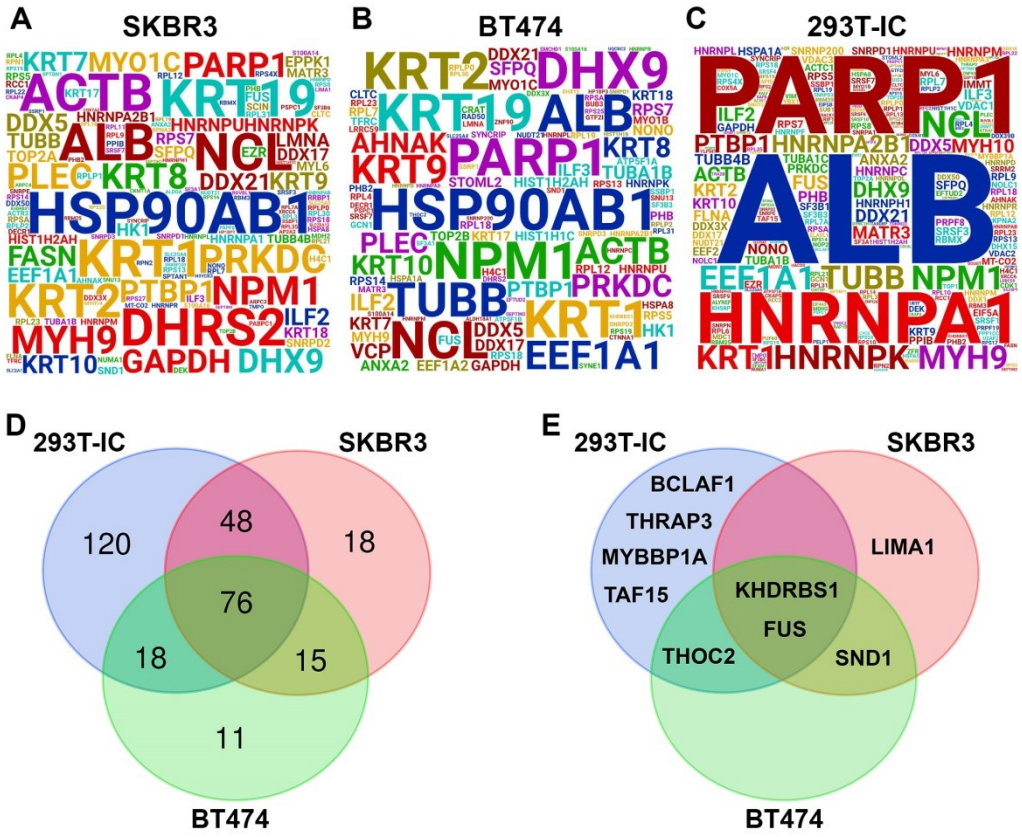

Figure 5.13. The list of proteins detected in p85HER2 pull-down IP samples from the nuclear fractions by mass spectrometry. (**A-C**) Word could diagram illustrating the gene names of all detected proteins in SKBR3 (**A**), BT474 (B) and 293T-IC (**C**) cells. The size of words indicates a mass spectrometry score. Colors peaked randomly and are not informative. (**D and E**) Venn diagram illustrating the number (**D**) and gene name (**E**) of proteins detected in each cell samples and detection overlaps.

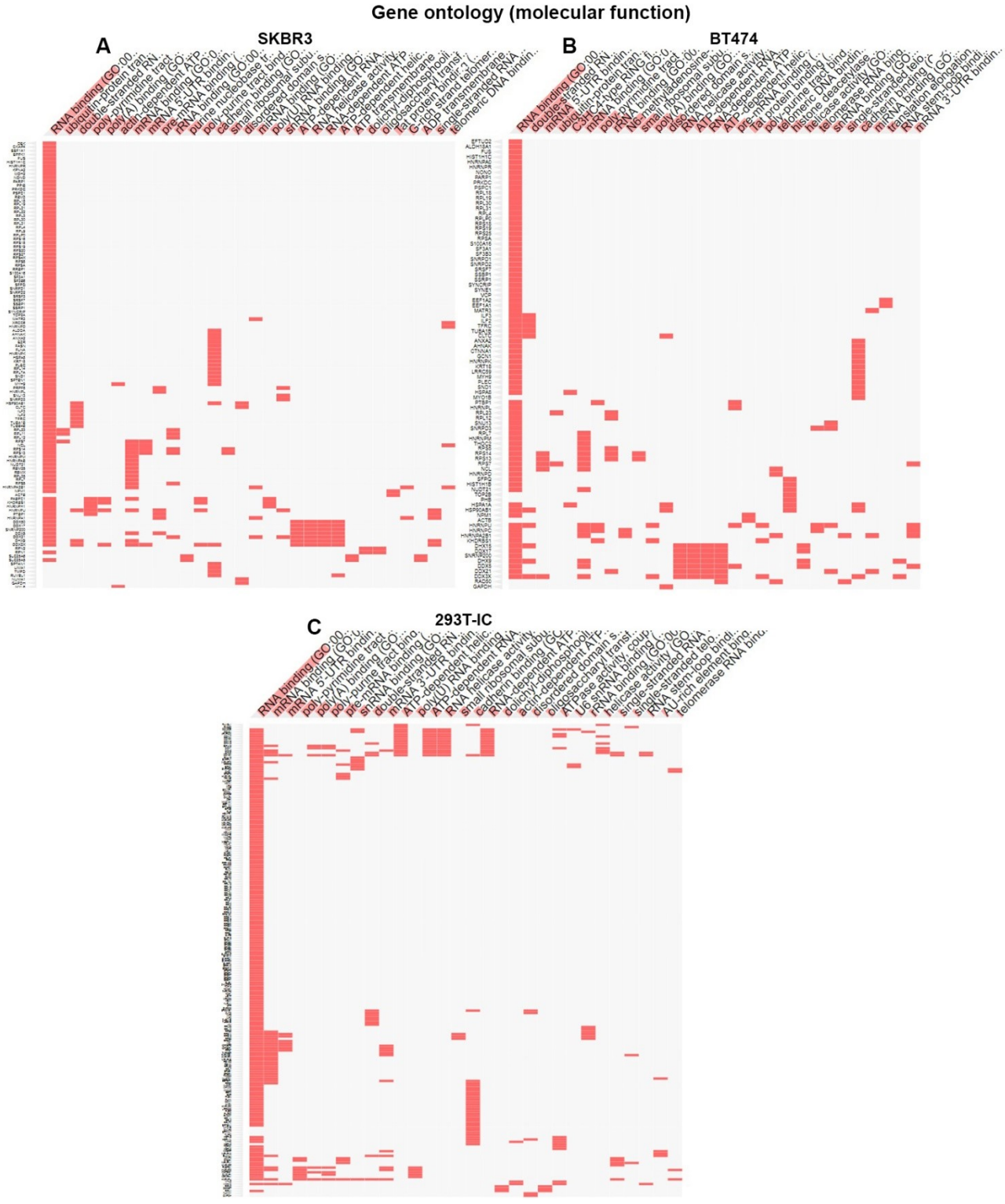

Figure 5.14. Molecular functions of nuclear p85HER2 client proteins. Gene Ontology molecular functions of proteins detected in HER2 pull-down IP samples from nuclear

fractions. Clustergram of top 30 enriched molecular functions of proteins detected in p85HER2 pull-down IP samples from nuclear fractions of (**A**) SKBR3, (**B**) BT474, and (**C**) 293T-IC cells. Enriched functions are the columns, input genes are the rows, and cells in the matrix indicate if a gene is associated with a function. The functions are ranked based on the combined enrichment score of *P*-value and

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Figure 5.15. Biological functions of nuclear p85HER2 client proteins. Gene Ontology biological function of proteins detected in HER2 pull-down IP samples from nuclear fractions. Clustergram of top 30 enriched Gene Ontology biological functions of proteins

detected in p85HER2 pull-down IP samples from nuclear fractions of (**A**) SKBR3, (**B**) BT474, and (**C**) 293T-IC cells. Enriched functions are the columns, input genes are the rows, and cells in the matrix indicate if a gene is associated with a function. The functions are ranked based on the combined enrichment score of *P*-value and Z-score.

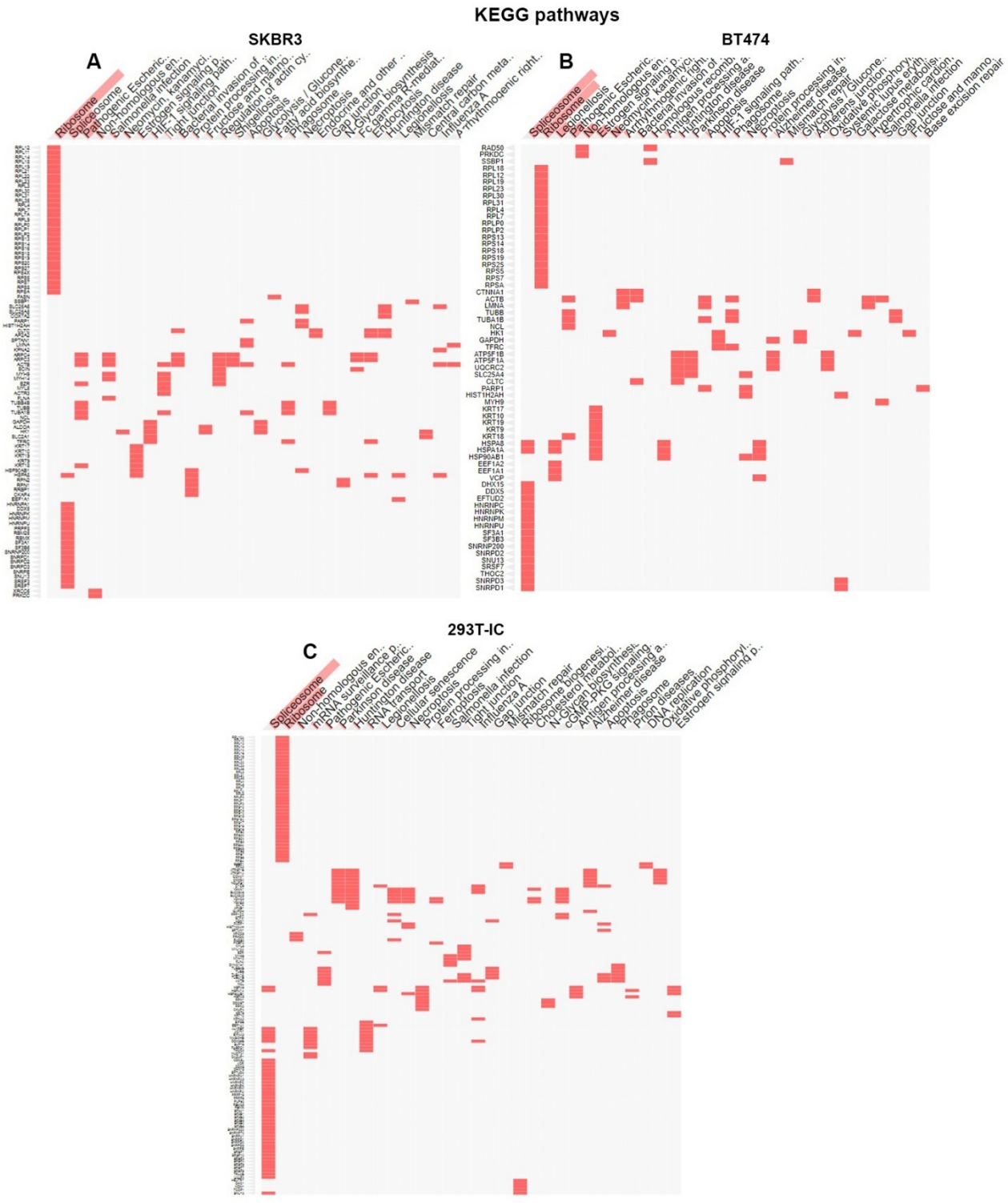

Figure 5.16. Pathways that nuclear p85HER2 client proteins are involved in. KEGG pathways of proteins detected in p85HER2 pull-down IP samples from nuclear fractions. Clustergram of top 30 enriched KEGG pathways of proteins detected in p85HER2 pull-down

IP samples from nuclear fractions of (**A**) SKBR3, (**B**) BT474, and (**C**) 293T-IC cells. Enriched pathways are the columns, input genes are the rows, and cells in the matrix indicate if a gene is associated with a pathway. The pathways are ranked based on the combined enrichment score of *P*-value and Z-score.

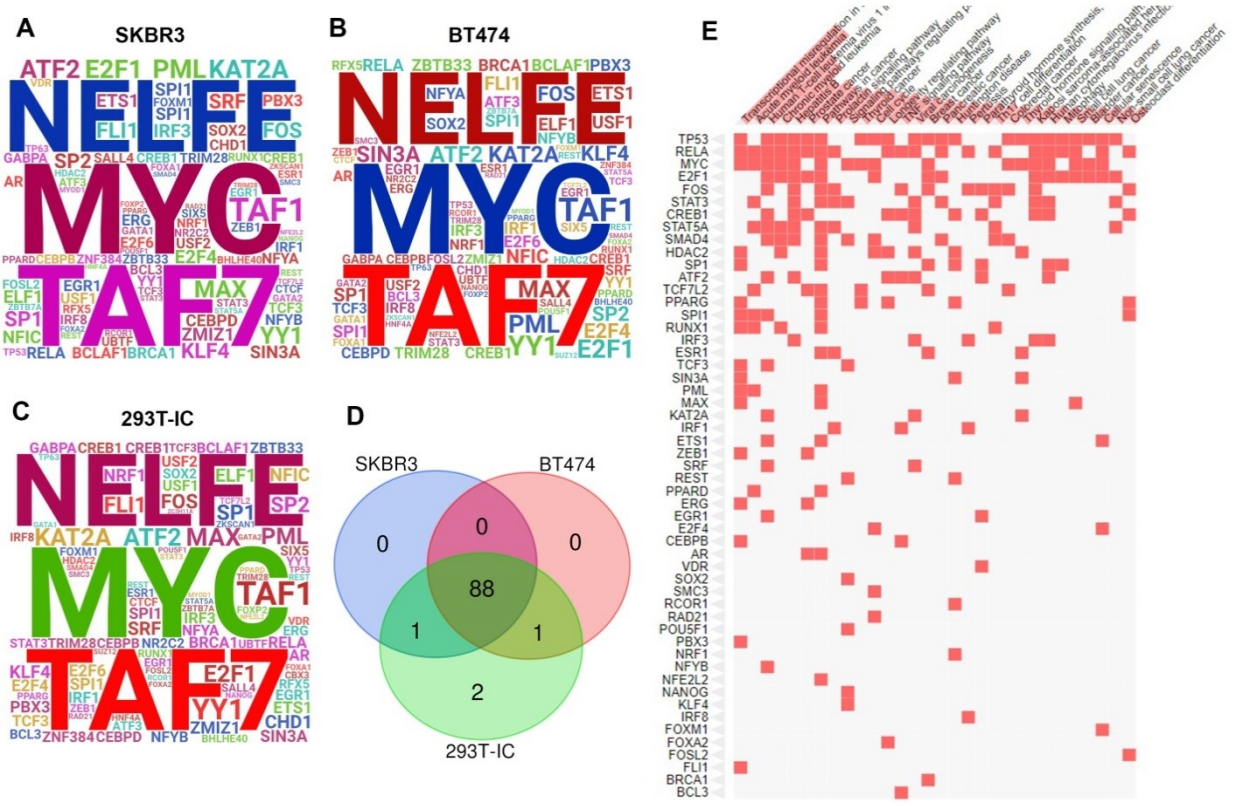

Figure 5.17. Oncogenic/stemness transcription factors regulate nuclear p85HER2 client proteins. (**A-C**) Word cloud diagram showing upstream transcription factor regulators of nuclear p85HER2 client proteins identified in p85HER2 pull-down IP samples from nuclear fractions of SKBR3 (**A**), BT474 (**B**) and 293T-IC (**C**) cells. The size of words indicates a mass spectrometry score. Colors peaked randomly and are not informative. (**D**) Venn diagram illustrating the number of upstream transcription factors of nuclear p85HER2 client proteins detected in each cell sample and overlaps between them. Clustergram showing top 30 enriched KEGG pathways of 100 upstream transcription factor regulators of nuclear p85HER2 client proteins identified in p85HER2 pull-down IP samples from nuclear fractions of 293T-IC. Enriched pathways are the columns, input genes are the rows, and cells in the

matrix indicate if a gene is associated with a pathway. The pathways are ranked based on the combined enrichment score of *P*-value and Z-score.

5.4. DISCUSSION

Clinical studies showed that trastuzumab successfully enhanced the survival rate of metastatic and non-metastatic HER2-positive breast cancer patients. Unfortunately, only 30-40% of patients respond to trastuzumab. Translational medicine researchers and clinicians intensively sought the exact mode of action of trastuzumab and its resistance factor(s) to improve patients' response to trastuzumab as well as HER2 targeting approach. This information is important for some reasons. It will help us to understand the molecular mechanism of trastuzumab therapy which by turn, will shed light on this question why some patients show resistance to trastuzumab and who should or not receive this drug (personalized medicine). Trastuzumab is an effective but also expensive drug because of its production procedure. This information will also lead us to design cheaper chemical drugs acting like trastuzumab and will also shed light on more detail of HER2 proto-oncogene function to understand molecular pathology, strengths and Achilles' heel of breast cancer.

Our previous studies using CHO cell line stably overexpressing human HER2 showed that trastuzumab does not inhibit HER2 receptor activation and downstream pathways, however, it inhibits cell cycle progression and survival, and induces apoptosis of the cells. We also found that overexpression of HER2 in the CHO cells does not induces RTK downstream pathways PI3K/Akt and MAPK pathways but increases cell growth of the cells. By taking these results together, we hypothesize that trastuzumab inhibits a non-canonical pathway(s) of HER2 rather than its RTK pathways. Some previous studies show proteolytic cleavage of HER2 and production of ctHER2 with the ability and nuclear localization and contribution in the regulation of gene expression as a transcription co-factor. HER2 cleavage and nuclear ctHER2 in HER2-positive breast cancer tumors are reported to be associated with fast tumor growth, high invasion, poor prognosis and worse response to trastuzumab treatment. production of ctHER2 via cleavage of full-length HER2 by metalloproteinases (ADAMs and MMPs) is well-documented. The upregulation of the metalloproteinases is a poor prognosis factor and is commonly seen in

mesenchymal-like breast cancer cells. Interestingly, several metalloproteinases including ADAMs 9, 10, 12, 15, 17, 28 [29–34] as well as MMPs 1, 2, 7, 9 11, 12, 13, 14 and -16 are reported overexpressed in breast cancers and potential target for treatment of breast cancer [35–38]. ADAMs 10 and 17 and are the major sheddase enzymes involved in HER2 cleavage shedding [19]. Recent studies showed that ADAMs 10 and 17 is associated with poorer relapse-free survival in HER2-positive breast cancer patients and inhibition of them by chemicals overcame trastuzumab resistance in both naïve and trastuzumab-resistant HER2-positive cell lines [18,39,40]. These data suggest ADAMs 10 and 17 as key drivers of trastuzumab resistance and potential targets to overcome trastuzumab resistance in HER2-positive breast cancer [41]. Moreover, a preclinical study revealed that inhibitors of MMPs 1, 2, 3 and 9 may suppress HER2 shedding [42]. Despite poor outcomes of chemical inhibitors of MMPs in clinical trials, this approach may still be a significant way to prevent HER2 shedding and production of C-terminal truncated HER2 fragments in breast cancer [43]. HER2 cleavage can explain why secondary metastatic tumors with a high percentage of mesenchymal-like cancer stem cells are mostly resistant to trastuzumab but still sensitive to lapatinib which targets HER2 kinase domain. These pieces of evidence demonstrate the oncogenic function of icHER2 through its canonical pathway as well as maybe a non-canonical pathway.

Nuclear HER2 is shown to interact with several transcription factors that resulted in transcriptional upregulation of target genes leading to more cell growth and proliferation. These reports suggest the transcription co-factor function of HER2. In this chapter, we examined the hypothesis that trastuzumab binding to HER2 may inhibit proteolytic cleavage and therefore nuclear localization of ctHER2 as a novel mechanism of trastuzumab action. First of all, we confirmed that trastuzumab binding to HER2 induces ADCC, and inhibits in vitro proliferation of HER2-positive breast cancer cells in the absence of immune cells. However, it did not inhibit HER2 receptor homo- and heterodimerization and phosphorylation but partially inhibited downstream PI3K/Akt and MAPK pathways. This result suggests that the molecular mechanism of anti-cancer function of trastuzumab still remain unknown. Further investigations revealed that trastuzumab reduced the level of an 85 kDa C-terminal HER2 protein (called p85HER2). Results also showed a synergic effect between trastuzumab and metalloproteinase inhibitor TAPI-2 on blocking HER2 cleavage, suggesting that trastuzumab inhibits metalloproteinase-mediate cleavage of HER2. Protein docking simulation analysis confirmed that binding trastuzumab to

domain IV of HER2 blocks enzymatic binding of ADAM17, a HER2-targeting metalloproteinase, to HER2 cleavage site at its extracellular juxtamembrane region. It is not clear whether metalloproteinase-mediated HER2 cleavage results in releasing p85HER2 from the membrane, however cytoplasmic and nuclear localization of p85HER2 had been demonstrated by independent studies. The role of intracellular proteases/sheddases such as calpain4, the common subunit of cysteine protease isoforms calpain1 and calpain 2 in HER2 pathway has been shown. Kulkarni et al. [44] showed that calpain4 is activated in HER2-positive breast cancer and is required for the activation of HER2. Calpain4 was also associated with the worse response of HER2-positive breast cancer to trastuzumab treatment. In addition, cleavage and nuclear localization of HER4 by calpains and transmembrane proteinase γ-secretase have been demonstrated [45–48], but there is no clear evidence showing HER2 cleavage by intracellular or transmembrane proteinases.

Since trastuzumab blocks nuclear localization of HER2, we were interested in understanding the function of p85HER2 in the nucleus. In order to validate our results, and to investigate whether cleavage of HER2 is essential for p85HER2 nuclear localization, we generated two different expression plasmid constructs (one series tagged by GFP flag, and other series tagged by 6x histidine flag) of 4 different length HER2 including full-length HER2 (FL), HER2 deleted extracellular part but possessing transmembrane domain and intracellular part (TM+IC), intracellular HER2 (IC), and intracellular HER2 with deleted nuclear localizing signal (NLS) sequence (IC_ΔNLS). We transfected the construct to 293T cell line (HER2-negative) and then isolated a nuclear fraction of the cells to investigate which variant of HER2 can be localized in the nucleus. Results showed that only IC (p85HER2) localized in the nucleus that was detected by immunofluorescence staining and blotting. To investigate the function of nuclear p85HER2, we pull-down C-terminal HER2 from nuclear fractions of IC transfected 293T cells (called 293T-IC) as well as SKBR3 and BT474 by protein IP using a monoclonal antibody specific for C-terminal end of HER2. Immunoblotting of HER2 in the pull-down samples showed the presence of p85HER2 in the nucleus of all the three cell lines. We then studied the protein profile of the p85HER2 pull-down IP samples by mass spectrometry to identify client proteins of nuclear p85HER2. Mass spectrometry identified 157, 120 and 265 proteins in SKBR3, BT474, and 293T-IC cells respectively. Of the identified proteins, 76 proteins were common in the 3 cell lines.

To understand the function of the proteins as well as nuclear p85HER2, the identified proteins were subjected to GSEA applying Gene Ontology-molecular and biological functions (to determine functions of the proteins), KEGG pathways (to determine pathways that the proteins are involved in) and ENCODE and ChEA Consensus transcription factors from ChIP-X (to determine upstream transcription factor regulators of the proteins). Gene Ontology and pathway analysis showed that a big majority of the proteins possess RNA binding activity and contribute to spliceosome complex, ribosome assembly, RNA processing, RNA trafficking, mRNA splicing, and non-coding RNA processing. In total, 9 transcription factors were detected interacted with nuclear p85HER2. Transcription factor enrichment analysis revealed that expression of the proteins are directly under regulation of oncogenic/stemness transcription factors such as MYC, MAX, ATF2/3, E2F1/4/6, YY1, ETS1, FOXM1, FOXA1, OCT4, SOX2, FOS, STAT3, SMAD4, BCL3, ZEB1, GATA1/2, and other oncogenic/stemness transcription factors. These transcription factors are negative regulators of epithelial maintenance, positive regulators of mesenchymal maintenance and have pivotal roles in the induction of EMT of breast cancer. On the other hand, many pieces of evidence support a negative feedback regulation between activation of stemness pathways (including TGF-β, Wnt/β-catenin, JAK/STAT, and Notch pathways) and HER2 expression. HER2 is also a positive regulator of cell junction and adhesion [49,50]. HER2 cross-talks with the stemness pathways that results in the upregulation and activation of EMT mediators including metalloproteinases leading to increased cleavage of HER2. A well-documented example of negative feedback between HER2 and stemness is crosstalk between HER2 and Wnt/β-catenin pathway. HER2 can upregulate the Wnt/β-catenin pathway, whereas β-catenin activation induces EMT that decrease HER2 expression leading to development of trastuzumab resistance [51]. The decreased response to trastuzumab can be due to proteolytic cleavage of HER2 cleavage as well as *ERBB2* gene silencing by epigenetic reprogramming during EMT that will be studied in the next chapter. Thus, we suggest that HER2 cleavage is a consequence of activation of EMT demonstrating a negative feedback between HER2 expression and EMT. Though, further study is needed to address the mechanism of the regulation.

5.5. REFERENCES

1. Duchnowska R, Sperinde J, Chenna A, Haddad M, Paquet A, Lie Y, et al. Quantitative measurements of tumoral p95HER2 protein expression in metastatic breast cancer patients treated with trastuzumab: independent validation of the p95HER2 clinical cutoff. Clin Cancer Res. 2014;20: 2805–2813. doi:10.1158/1078-0432.CCR-13-2782

2. Lipton A, Goodman L, Leitzel K, Cook J, Sperinde J, Haddad M, et al. HER3, p95HER2, and HER2 protein expression levels define multiple subtypes of HER2-positive metastatic breast cancer. Breast Cancer Res Treat. 2013;141: 43–53. doi:10.1007/s10549-013-2665-0

3. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, et al. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res. 2006;12: 424–431. doi:10.1158/1078-0432.CCR-05-1807

4. Sperinde J, Jin X, Banerjee J, Penuel E, Saha A, Diedrich G, et al. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin Cancer Res. 2010;16: 4226–4235. doi:10.1158/1078-0432.CCR-10-0410

5. Chandarlapaty S, Scaltriti M, Angelini P, Ye Q, Guzman M, Hudis CA, et al. Inhibitors of HSP90 block p95-HER2 signaling in Trastuzumab-resistant tumors and suppress their growth. Oncogene. 2010;29: 325–334. doi:10.1038/onc.2009.337

6. Cordo Russo RI, Beguelin W, Diaz Flaque MC, Proietti CJ, Venturutti L, Galigniana N, et al. Targeting ErbB-2 nuclear localization and function inhibits breast cancer growth and overcomes trastuzumab resistance. Oncogene. 2015;34: 3413–3428. doi:10.1038/onc.2014.272

7. Wang S-C, Lien H-C, Xia W, Chen I-F, Lo H-W, Wang Z, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell. 2004;6: 251–261. doi:10.1016/j.ccr.2004.07.012

8. Chen QQ, Chen XY, Jiang YY, Liu J. Identification of novel nuclear localization signal within the ErbB-2 protein. Cell Res. 2005;15: 504–510. doi:10.1038/sj.cr.7290320

9. Beguelin W, Diaz Flaque MC, Proietti CJ, Cayrol F, Rivas MA, Tkach M, et al. Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. Mol Cell Biol. 2010;30: 5456–5472. doi:10.1128/MCB.00012-10

10. Venturutti L, Romero L V, Urtreger AJ, Chervo MF, Cordo Russo RI, Mercogliano MF, et al. Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis. Oncogene. 2016;35: 2208–2222. doi:10.1038/onc.2015.281

11. Li L-Y, Chen H, Hsieh Y-H, Wang Y-N, Chu H-J, Chen Y-H, et al. Nuclear ErbB2 enhances translation and cell growth by activating transcription of ribosomal RNA genes. Cancer Res. 2011;71: 4269–4279. doi:10.1158/0008-5472.CAN-10-3504

12. Gorbatenko A, Olesen CW, Loebl N, Sigurdsson HH, Bianchi C, Pedraz-Cuesta E, et al. Oncogenic p95HER2 regulates Na+-HCO3- cotransporter NBCn1 mRNA stability in breast cancer cells via 3'UTR-dependent processes. Biochem J. 2016;473: 4027–4044. doi:10.1042/BCJ20160054

13. Modi S, D'Andrea G, Norton L, Yao TJ, Caravelli J, Rosen PP, et al. A phase I study of cetuximab/paclitaxel in patients with advanced-stage breast cancer. Clin Breast Cancer. 2006;7: 270–277. doi:10.3816/CBC.2006.n.040

14. Maadi H, Nami B, Tong J, Li G, Wang Z. The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2. BMC Cancer. 2018;18: 238. doi:10.1186/s12885-018-4143-x

15. Sanderson MP, Dempsey PJ, Dunbar AJ. Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. Growth Factors. 2006;24: 121–136. doi:10.1080/08977190600634373

16. Garrett TPJ, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell. 2003;11: 495–505.

17. Pham D-H, Kim J-S, Kim S-K, Shin D-J, Uong N-T-T, Hyun H, et al. Effects of ADAM10 and ADAM17 Inhibitors on Natural Killer Cell Expansion and Antibody-dependent Cellular Cytotoxicity Against Breast Cancer Cells In Vitro. Anticancer Res. 2017;37: 5507–5513. doi:10.21873/anticanres.11981

18. Feldinger K, Generali D, Kramer-Marek G, Gijsen M, Ng TB, Wong JH, et al. ADAM10 mediates trastuzumab resistance and is correlated with survival in HER2 positive breast cancer. Oncotarget. 2014;5: 6633–6646. doi:10.18632/oncotarget.1955

19. Liu PCC, Liu X, Li Y, Covington M, Wynn R, Huber R, et al. Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells. Cancer Biol Ther. 2006;5: 657–664. doi:10.4161/cbt.5.6.2708

20. Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst. 2007;99: 628–638. doi:10.1093/jnci/djk134

21. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. Cancer Res. 1999;59: 1196–1201.

22. Tural D, Akar E, Mutlu H, Kilickap S. P95 HER2 fragments and breast cancer outcome. Expert Rev Anticancer Ther. 2014;14: 1089–1096. doi:10.1586/14737140.2014.929946

23. Yuan C-X, Lasut AL, Wynn R, Neff NT, Hollis GF, Ramaker ML, et al. Purification of Her-2 extracellular domain and identification of its cleavage site. Protein Expr Purif. 2003;29: 217–222.

24. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DWJ, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421: 756–760. doi:10.1038/nature01392

25. Maskos K, Fernandez-Catalan C, Huber R, Bourenkov GP, Bartunik H, Ellestad GA, et al. Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. Proc Natl Acad Sci U S A. 1998;95: 3408–3412. doi:10.1073/pnas.95.7.3408

26. Pierce BG, Wiehe K, Hwang H, Kim B-H, Vreven T, Weng Z. ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. Bioinformatics. 2014;30: 1771–1773. doi:10.1093/bioinformatics/btu097

27. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics. 2013;14: 128. doi:10.1186/1471-2105-14-128

28. Kuleshov M V, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44: W90-7. doi:10.1093/nar/gkw377

29. O'Shea C, McKie N, Buggy Y, Duggan C, Hill ADK, McDermott E, et al. Expression of ADAM-9 mRNA and protein in human breast cancer. Int J cancer. 2003;105: 754–761. doi:10.1002/ijc.11161

30. Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA. ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem. 2004;279: 51323–51330. doi:10.1074/jbc.M409565200

31. Lendeckel U, Kohl J, Arndt M, Carl-McGrath S, Donat H, Rocken C. Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. J Cancer Res Clin Oncol. 2005;131: 41–48. doi:10.1007/s00432-004-0619-y

32. Mitsui Y, Mochizuki S, Kodama T, Shimoda M, Ohtsuka T, Shiomi T, et al. ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res. 2006;66: 9913–9920. doi:10.1158/0008-5472.CAN-06-0377

33. McGowan PM, Ryan BM, Hill ADK, McDermott E, O'Higgins N, Duffy MJ. ADAM-17 expression in breast cancer correlates with variables of tumor progression. Clin Cancer Res. 2007;13: 2335–2343. doi:10.1158/1078-0432.CCR-06-2092

34. Mullooly M, McGowan PM, Kennedy SA, Madden SF, Crown J, O' Donovan N, et al. ADAM10: a new player in breast cancer progression? Br J Cancer. 2015;113: 945–951.

doi:10.1038/bjc.2015.288

35. Köhrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer. 2009. p. 188. doi:10.1186/1471-2407-9-188

36. Benson CS, Babu SD, Radhakrishna S, Selvamurugan N, Ravi Sankar B. Expression of matrix metalloproteinases in human breast cancer tissues. Dis Markers. 2013;34: 395–405. doi:10.3233/DMA-130986

37. Bartsch JE, Staren ED, Appert HE. Matrix metalloproteinase expression in breast cancer. J Surg Res. 2003;110: 383–392.

38. Merdad A, Karim S, Schulten H-J, Dallol A, Buhmeida A, Al-Thubaity F, et al. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res. 2014;34: 1355–1366.

39. Gijsen M, King P, Perera T, Parker PJ, Harris AL, Larijani B, et al. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. PLoS Biol. 2010;8: e1000563. doi:10.1371/journal.pbio.1000563

40. Caiazza F, McGowan PM, Mullooly M, Murray A, Synnott N, O'Donovan N, et al. Targeting ADAM-17 with an inhibitory monoclonal antibody has antitumour effects in triple-negative breast cancer cells. Br J Cancer. 2015;112: 1895–1903. doi:10.1038/bjc.2015.163

41. Duffy MJ, Mullooly M, O'Donovan N, Sukor S, Crown J, Pierce A, et al. The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? Clin Proteomics. 2011;8: 9. doi:10.1186/1559-0275-8-9

42. Burns DM, He C, Li Y, Scherle P, Liu X, Marando CA, et al. Conversion of an MMP-potent scaffold to an MMP-selective HER-2 sheddase inhibitor via scaffold hybridization

and subtle P1' permutations. Bioorg Med Chem Lett. 2008;18: 560–564. doi:10.1016/j.bmcl.2007.11.086

43. Radisky ES, Radisky DC. Matrix metalloproteinases as breast cancer drivers and therapeutic targets. Frontiers in bioscience (Landmark edition). 2015. pp. 1144–1163.

44. Kulkarni S, Saju L, Farver C, Tubbs R. Calpain4 is required for activation of HER2 in breast cancer cells exposed to trastuzumab and its suppression decreases survival and enhances response. Int J cancer. 2012;131: 2420–2432. doi:10.1002/ijc.27510

45. Merilahti JAM, Elenius K. Gamma-secretase-dependent signaling of receptor tyrosine kinases. Oncogene. 2019;38: 151–163. doi:10.1038/s41388-018-0465-z

46. Ni CY, Murphy MP, Golde TE, Carpenter G. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science. 2001;294: 2179–2181. doi:10.1126/science.1065412

47. Merilahti JAM, Ojala VK, Knittle AM, Pulliainen AT, Elenius K. Genome-wide screen of gamma-secretase-mediated intramembrane cleavage of receptor tyrosine kinases. Mol Biol Cell. 2017;28: 3123–3131. doi:10.1091/mbc.E17-04-0261

48. Lu Y, Gao Y, Tao R, Liao M, Huang J, Wu G, et al. Calpain-Dependent ErbB4 Cleavage Is Involved in Brain Ischemia-Induced Neuronal Death. Mol Neurobiol. 2016;53: 2600–2609. doi:10.1007/s12035-015-9275-2

49. Jeon M, Lee J, Nam SJ, Shin I, Lee JE, Kim S. Induction of fibronectin by HER2 overexpression triggers adhesion and invasion of breast cancer cells. Exp Cell Res. 2015;333: 116–126. doi:10.1016/j.yexcr.2015.02.019

50. Brennan K, McSherry EA, Hudson L, Kay EW, Hill ADK, Young LS, et al. Junctional adhesion molecule-A is co-expressed with HER2 in breast tumors and acts as a novel regulator of HER2 protein degradation and signaling. Oncogene. 2013;32: 2799–2804. doi:10.1038/onc.2012.276

51. Wu Y, Ginther C, Kim J, Mosher N, Chung S, Slamon D, et al. Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like phenotype in trastuzumab-

resistant HER2-overexpressing breast cancer cells. Mol Cancer Res. 2012;10: 1597–1606. doi:10.1158/1541-7786.MCR-12-0155-T

Chapter 6. EMT-mediated epigenetic mechanism of trastuzumab resistance

6.1. SUMMARY

There are several studies indicating that mesenchymal-like breast cancer cells with high stemness properties are HER2-low and trastuzumab-refractory, while epithelial-like differentiated breast cancer cells are HER2-high and trastuzumab-sensitive cells. Some other reports demonstrate a negative feedback regulation loop between HER2 expression and activation of stemness pathways. This regulation reflects also as negative feedback between trastuzumab response and EMT of breast cancer. In chapter 4, we showed that trastuzumab prevents nuclear localization of HER2 by blocking proteolytic cleavage of HER2. Cleavage and nuclear function of HER2 seem to be a part of EMT-mediated cell reprogramming. HER2 can upregulate EMT of HER2-positive breast cancer cells by crosslinking with the stemness pathways that result in the increased subpopulation of HER2-low/negative and proteinase-high mesenchymal cells (such as CD44+/CD24- BSCSs) which are trastuzumab-resistant. Downregulation of extracellular HER2 by EMT-mediated proteinases in trastuzumab-resistant/lapatinib-sensitive cells has been shown by limited studies, however, the mechanism of *ERBB2* gene silencing during EMT and in the mesenchymal-like cells derived from trastuzumab-resistant/lapatinib-resistant HER2-positive breast tumors was entirely unknown. In this chapter, we found HER2 expression is positively and negatively correlated with the expression of epithelial and mesenchymal phenotype marker genes respectively. We found that chromatin of *ERBB2* gene in HER2-high epithelial-like breast cancer cells is active while the chromatin is inactive in HER2-low mesenchymal-like cells. HER2-low breast cancer cell line also revealed less promoter-enhancer interaction small chromatin loops, super-enhancers and less topologically associating domains (TADs) compared to the HER2-high cell lines. The lower HER2 expression, the higher EMT phenotype, and inactivated chromatin all were found correlated with a lower response to lapatinib. The higher EMT phenotype was found correlated with a lower response to lapatinib. We also found that induction of EMT of HER2-positive cells results in downregulated HER2 expression and lower binding rate of trastuzumab. These results show that the downregulation of HER2 in mesenchymal-like cells in the culture of HER2-positive breast cancer cell lines was due to *ERBB2* gene silencing by epigenetic reprogramming of the cells

during EMT. These results indicate that *ERBB2* gene silencing by epigenetic regulation during EMT is the main mechanism of resistance of HER2-positive breast cancer cells to trastuzumab and lapatinib.

6.2. INTRODUCTION

Development of de novo resistance to HER2-targeting agents lapatinib and trastuzumab still remain a big challenge in the treatment of HER2-positive breast cancer. As discussed before, one suggested mechanism of trastuzumab resistance is cleavage and shedding of HER2. Cleavage from EJM region will result in shedding of the extracellular part of HER2 and production of p95HER2 still encored at the plasma membrane. While, in the case of cleavage from IJM region, the intracellular part of HER2 will shed and the extracellular part will remain at the plasma membrane. In both the cleavage cases that can take place by EMT-mediated proteinases, the cell would be resistant to trastuzumab but still sensitive to lapatinib. Notably, the exact mechanism of lapatinib resistance in HER2-positive breast cancer is still to be identified. One suggested mechanism is downregulation of HER2 expression by epigenetic-mediated *ERBB2* gene silencing. We believe that wide-scale epigenetic reprogramming during EMT could be the mechanism of *ERBB2* gene silencing.

HER2 cleavage Generally, epithelial-like cells highly express HER2, whereas mesenchymal cells are majorly HER2-negative or HER2-low. This shows that mesenchymal-like cells show resistance to trastuzumab, suggesting that trastuzumab- resistance may link to EMT. JIMT-1 cell line is HER2+ breast cancer cells that quickly develop resistance to HER2. Studies showed that JIMT-1 was composed of approximately 10% CD44+/CD24- BCSC in initial cultures. This level rose to 85% at the late-passages [1]. Concurrently, the level of HER2 expression significantly reduced in late-passage cultures when compared to the early cultures. This regulation was associated with the development of trastuzumab-resistance. High passage JIMT-1 cells that were enriched mesenchymal CD44+/CD24- BCSCs expressing a lower level of HER2 also exhibited a highly-migratogenic phenotype and produced pro-invasive/metastatic proteins more than low-passage JIMT-1 cells culture [104]. This phenomenon may explain the resistance of HER2-high breast tumors to trastuzumab due to an increased population of HER2-low CD44+/CD24

mesenchymal cells at the late-passages. Further, the CD44+/CD24- cells escape from trastuzumab-mediated ADCC. The cells could survive the immunoselection process in breast cancer cells co-cultured with NK cells and trastuzumab. This resistance may be attributed to the reduced HER2 expression levels on their surface [2]. In addition, our previous results showed that HER2 can be cleaved by metalloproteinases which are hallmark regulators of breast cancer EMT. We also showed that in the nucleus, cleaved HER2 interacts with proteins whose expression is under control of stemness transcription factors. These results suggest the downregulation of membranous HER2 during EMT. These pieces of evidence show that downregulated HER2 and therefore decreased response to trastuzumab are parts of the intrinsic regulation of mesenchymal breast cancer cells. However, the mechanism of this regulation is not yet studied.

In the previous chapter, we discussed that the upregulation of metalloproteinases during EMT results in proteolytic cleavage of HER2 warranting the role of EMT in HER2 protein downregulation and development of trastuzumab resistance. In the current chapter, we test the hypothesis that EMT of HER2-positive breast cancer cells causes chromatin-based epigenetic silencing of *ERBB2* gene that abrogates HER2 expression and leads to resistance against trastuzumab and lapatinib. In this chapter, we study the mechanism of *ERBB2* gene silencing during EMT.

6.3. RESULTS

6.3.1. Mesenchymal breast cancer cells show lower *ERBB2* gene expression

To investigate whether expression level of *ERBB2* gene is correlated with the expression of EMT marker genes, we analyzed the RNA-seq expression of *ERBB2* gene, 12 epithelial marker genes (*ELCAM*, *CD24*, *CDH1*, *F11R*, *FOXA1*, *KRT7*, *KRT8*, *KRT18*, *KRT19*, *MUC*, *NECTIN2*, *NECTIN4*) as well as 12 mesenchymal marker genes (*CD44*, *CTNNB1*, *FOXC1*, *MYC*, *NOTCH1*, *NOTCH2*, *SNAI2*, *SOX10*, *TWIST2*, *VIM*, *ZEB1*, *ZEB2*) in 1,904 breast cancer tumor samples studied by METABRIC study [3]. We used cBioPortal portal [4] to investigate correlations between the mRNA levels of *ERBB2* and the EMT markers in each tumor sample. The result showed a significant positive correlation between the expression of *ERBB2* and all the epithelial

marker genes (Figure 6.1) and a negative correlation between ERBB2 and all the mesenchymal marker genes (Figure 6.2).

We also analyzed the expression of *ERBB2*, epithelial marker gene *MUC1*, mesenchymal marker gene *VIM*, and GAPDH in 38 breast cancer cell lines (AU565, BT-20, BT474, BT-549, CAL-51, CAMA-1, DU4475, HCC-1143, HCC-1187, HCC-1395, HCC-1419, HCC-1428, HCC-1500, HCC-1569, HCC-1599, HCC-1806, HCC-1937, HCC-1954, HCC-202, HCC-2218, HCC-3, HCC-38, HCC-70, Hs578T, MCF7, MDA-MB-175-VII, MDA-MB-231, MDA-MB-361, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, T47D, UACC-812, UACC-893, ZR-75-1, ZR-75-30, ZRT) to study the correlation between mRNA expression levels of ERBB2 and EMT marker genes. For this, two normalized microarray expression datasets (GEO accession numbers: GSE50811 [5] and GSE66071 [6]) available from NCBI GEO (Gene Expression Omnibus) database were analyzed. As shown in Figure 6.3, comparatively, the expression of *ERBB2* was positively and negatively correlated with the expression of respectively *MUC1* and *VIM* in most of the cell lines.

These results show positive coloration between *ERBB2* gene expression and epithelial phenotype, and a negative correlation between *ERBB2* gene expression and mesenchymal phenotype in breast cancer. This suggests that the expression of *ERBB2* gene in epithelial-like breast cancer cells is higher than that in mesenchymal-like breast cancer cells, suggesting that mesenchymal cells mostly show low *ERBB2* gene expression compared to epithelial-like breast cancer cells.

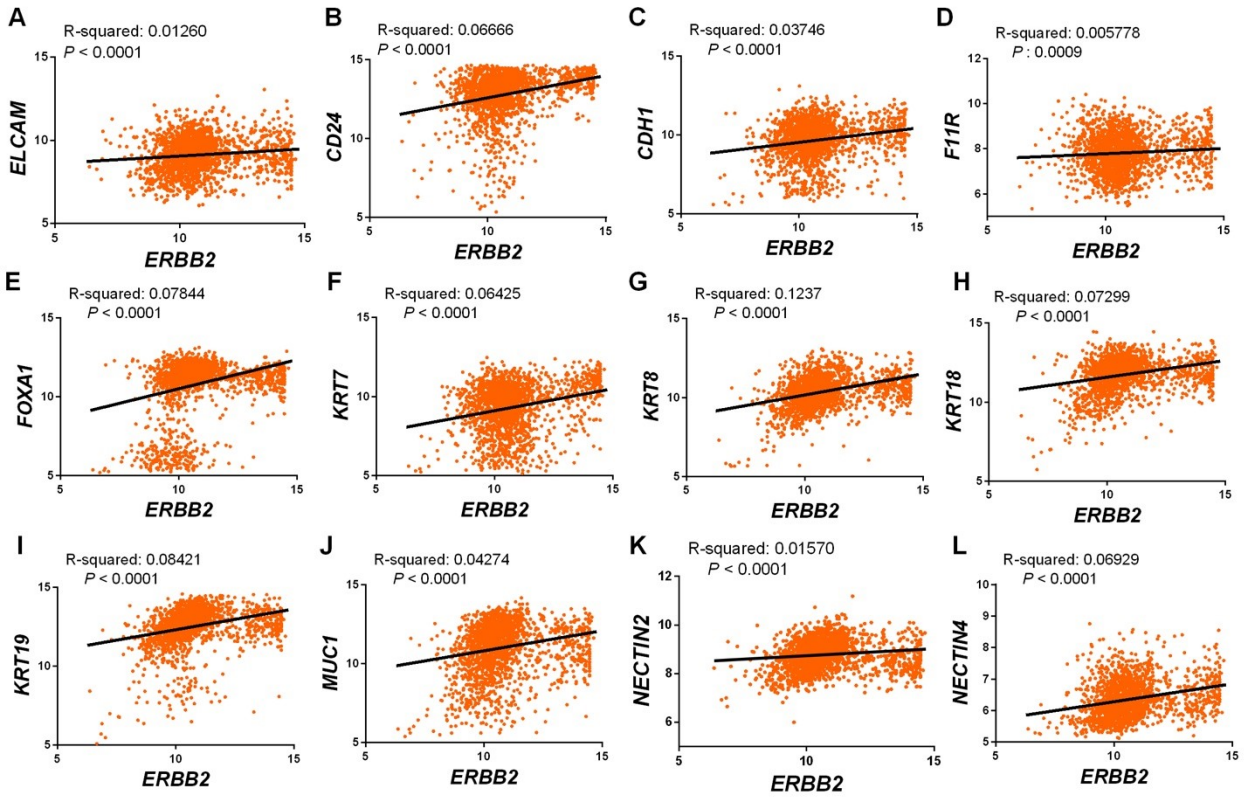

Figure 6.1. Correlation between mRNA expression of *ERBB2* and epithelial-like cell markers in breast cancer tumors. The expression value is presented by Z-score fold changes RNA-seq expression (v2 RSEM). Data source: normalized RNA-seq data from 1,904 breast tumors studied by METABRIC study [3] and available from cBioPortal portal [4] available at "https://cbioportal.org".

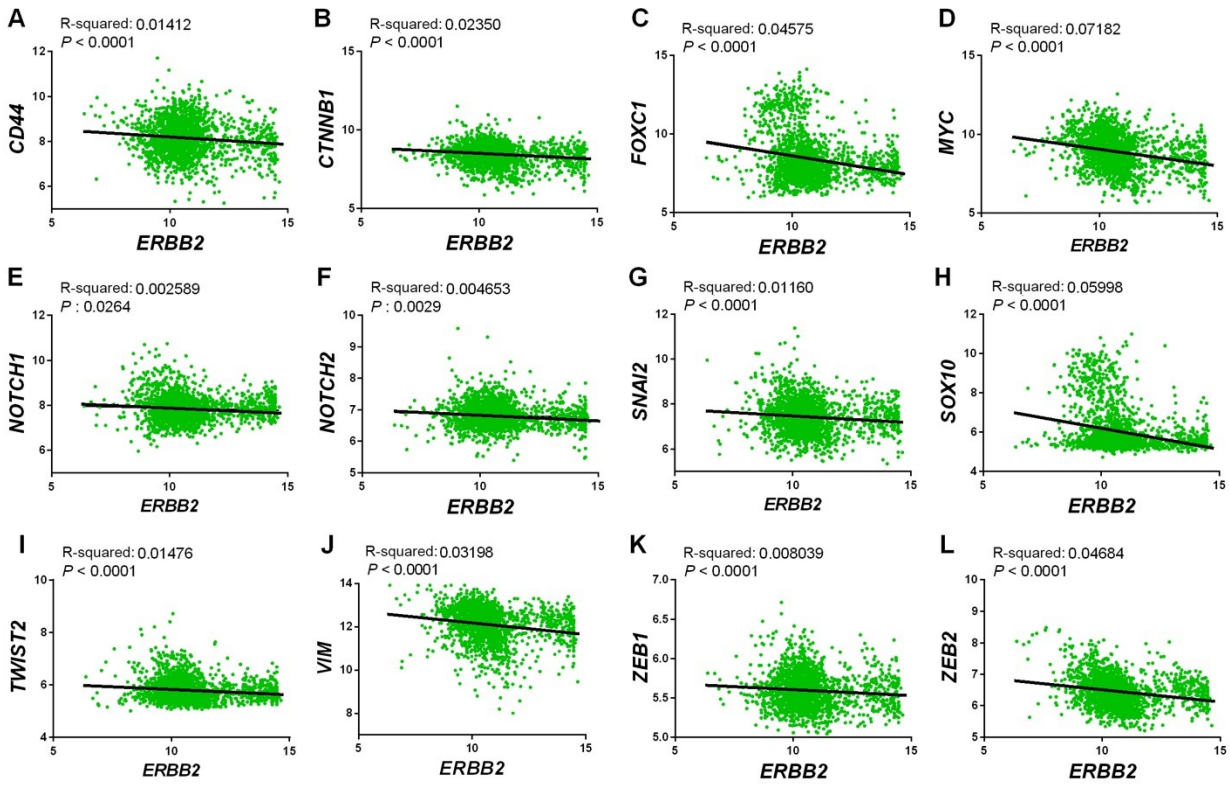

Figure 6.2. Correlation between mRNA expression of *ERBB2* and mesenchymal-like cell markers in breast cancer tumors. The expression value is presented by Z-score fold changes RNA-seq expression (v2 RSEM). Data source: normalized RNA-seq data from 1,904 breast tumors studied by METABRIC study [3] and available from cBioPortal portal [4] available at "https://cbioportal.org".

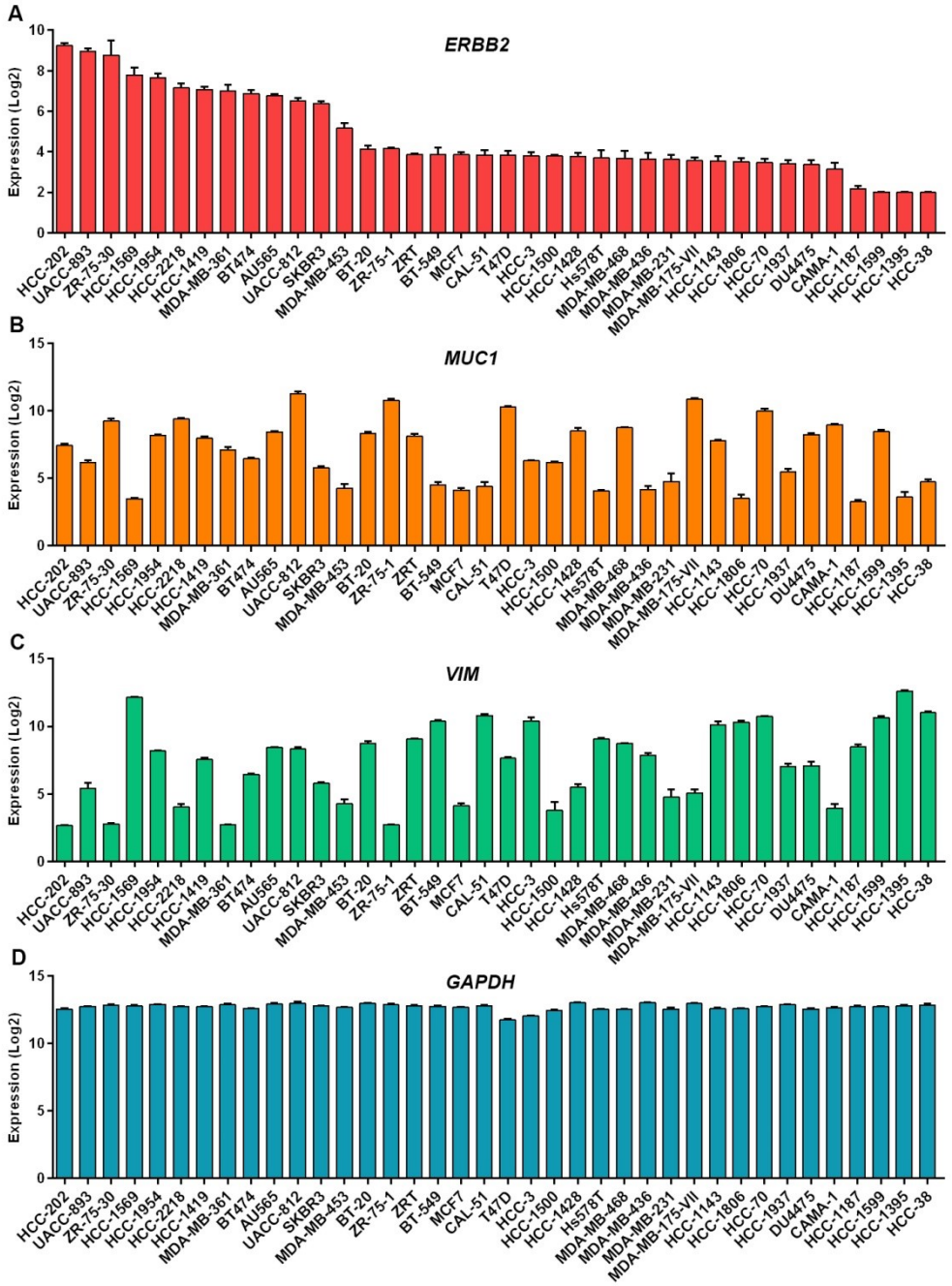

Figure 6.3. mRNA expression levels of *ERBB2*, *MUC1*, *VIM* and *GAPDH* in breast cancer cell lines. Data source: Microarray expression profiling (GEO accession numbers: GSE50811 [5] and GSE66071 [6] available from GEO database.

6.3.2. Promoter CpG islands methylation signature of *ERBB2* in epithelial-like and mesenchymal-like breast cancer cells

Transcription of genes is highly under control of promoter epigenetic regulation in DNA (CpG island methylation) and chromatin (histone protein modification) levels. To study the mechanism of low *ERBB2* gene expression we investigated promoter CpG island methylation signature of *ERBB2* gene in breast cancer cell lines with high *ERBB2* expression (BT474, HCC-1954, MDA-MB-453, SKBR3), and those with low *ERBB2* expression (BT20, MCF7, MDA-MB-231, MDA-MB-468, SUM-159PT, T47D). Array expression and genome tiling array methylation data of the cells were obtained from GEO database (Accession number: GSE44838 [7])

The mRNA expression levels of *ERBB2* in the cells are shown in Figure 6.4A. The result showed a positive correlation between the expression of *ERBB2* and *FOXA1* (epithelial-like cell marker), and the negative correlation between *ERBB2* expression and the expression of *FOXC1* (mesenchymal-like cell marker) in all cell lines except HCC-1954 (Figure 6.4A-C). Despite the different *ERBB2* expression levels of the cell lines, no significant difference was found between the cell lines in terms of the CpG island methylation (Figure 6.4D). These results show that low *ERBB2* expression levels in the mesenchymal-like cells are not due to promoter CpG island methylation.

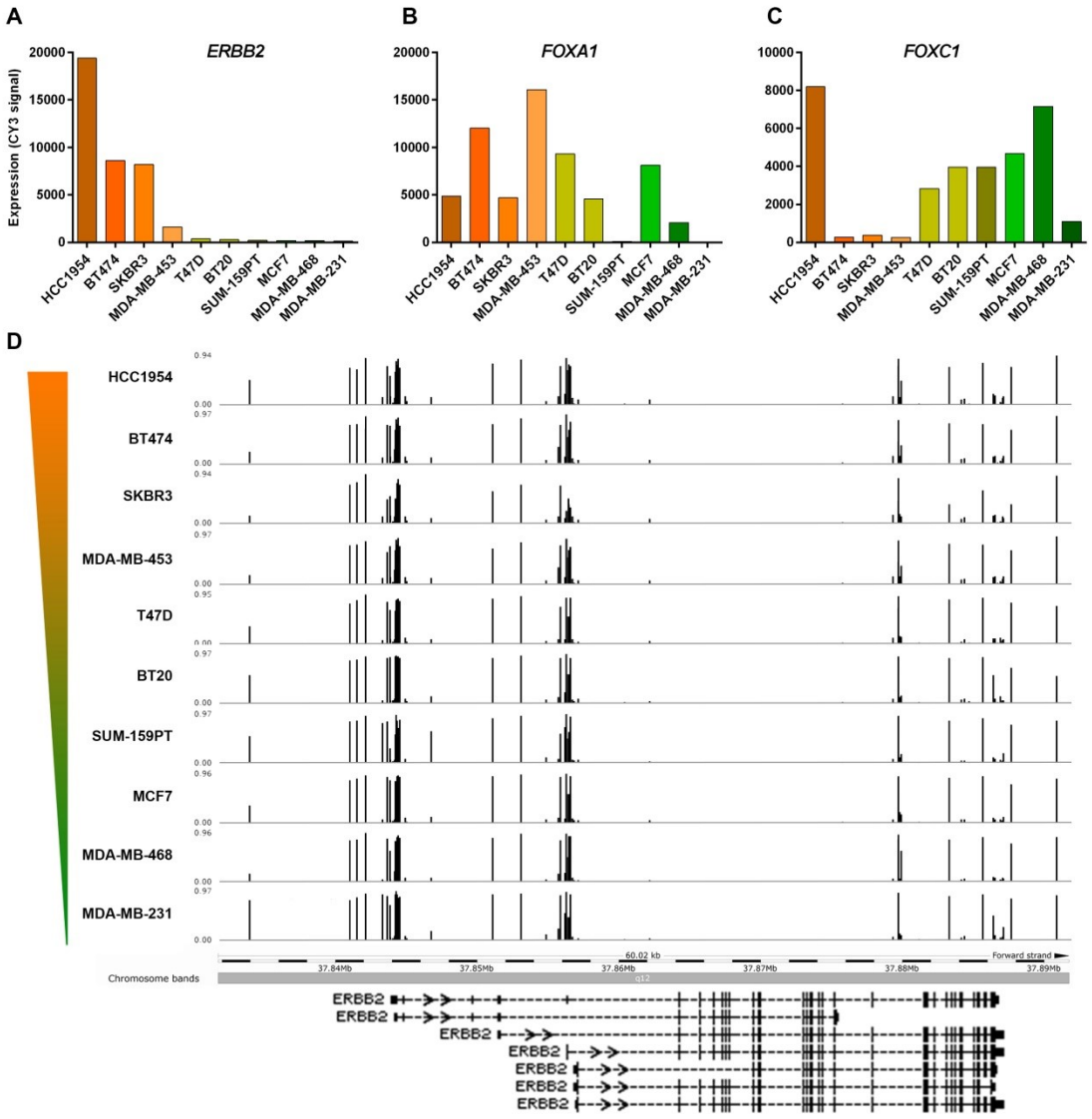

Figure 6.4. CpG island methylation profiles of *ERBB2* promoter in 10 breast cancer cell lines. (**A**) mRNA expression levels of *ERBB2*, *FOXA1*, and *FOXC1* genes in breast cancers. (**B**) Methylation levels of promoter CpG islands in the cell lines. Data source: Array expression profiling and genome tiling array methylation profiling (GEO Accession number: GSE44838 [7]) available from GEO database. The color gradient bar indicates HER2 expression level. Genomic coordinate: chr17:37,834,978-37,897,500 (GRCh37/hg19 assembly).

6.3.3. EMT regulator transcription factors bind to ERBB2 regulatory elements

Since DNA level epigenetic regulation (promoter CpG island methylation) has no role in the expression of *ERBB2*, we hypothesized that chromatin level epigenetic regulation (promoter and enhancer histone protein modification) may control different levels of *ERBB2* expression in the breast cancer cells. To examine this, we first studied transcription factors that directly bind to *ERBB2* chromatin identified by the ChIP-seq experiment. Result identified enrichment of a totally 82 transcription factors at the region of 10 kbp upstream and 10 kbp downstream of *ERBB2* gene motif Y in 3,740 human biological samples (Figure 6.5A). Of 82 transcription factors 8 (CDX2, FOXA1, FOXA2, KLF9, MBD3, MXI1, RUNX3, SP1) were epithelial status maintenance regulators, and 31 (ATF2, E2F1, E2F6, E2F7, EGR1, ELF2, ETS1, ETV1, FOS, FOXM1, FOXP1, FOXP2, GATA1, GATA2, GATA3, GATA6, HOXC9, JUNB, JUND, KDM5A, MAX, MAZ, MYC, MZF1, NANOG, RELA, SMAD4, STAT4, STAT5A, TEAD6, ZBTB7A) were EMT inducers which are master regulators of mesenchymal status maintenance (Figure 6.5B).

We then investigated chromatin accessibility/activity of *ERBB2* regulatory elements (promoter and enhancer) by analyzing ATAC-seq and DNase hypersensitivity data of MCF7 and MDA-MB-231 cell lines to explore the mode of correlation of *ERBB2* expression with accessibility/activity of *ERBB2* regulatory element chromatin. The result showed higher ATAC-seq (Figure 6.5A) and DNase I hypersensitivity (Figure 6.5B) signals at *ERBB2* promoters and enhancer chromatin of MCF7 cell lines compared to MDA-MB-231 cell line. These results show that *ERBB2* promoter and enhancer chromatin of cells with higher *ERBB2* expression level is more accessible/active than that of cells with a lower level of *ERBB2* expression.

To examine whether higher *ERBB2* expression is also correlated with higher enrichment of epithelial (FOXA1) and mesenchymal (E2F1) phenotype inducer transcription factors at regulatory regions of *ERBB2*. The normalized ChIP-seq values were obtained from Cistrome Data Browser [8] and the enrichment peaks were visualized by using WashU Epigenome Browser [9]. Results revealed higher enrichment of FOXA1 at promoter and enhancer chromatin of the MDA-MB-453 cell line compared to the MDA-MB-231 cell line (Figure 6.5C). In contrast, the enrichment of E2F1 at promoter chromatin of *ERBB2* gene in the MA-MB-231 cell line was higher than that in MCF7 cell line (Figure 6.5D). These results demonstrate that higher *ERBB2* expression is correlated with higher enrichment of epithelial phenotype inducer

transcription factors as well as with lower enrichment of mesenchymal phenotype inducer transcription factors at regulatory elements of *ERBB2* gene.

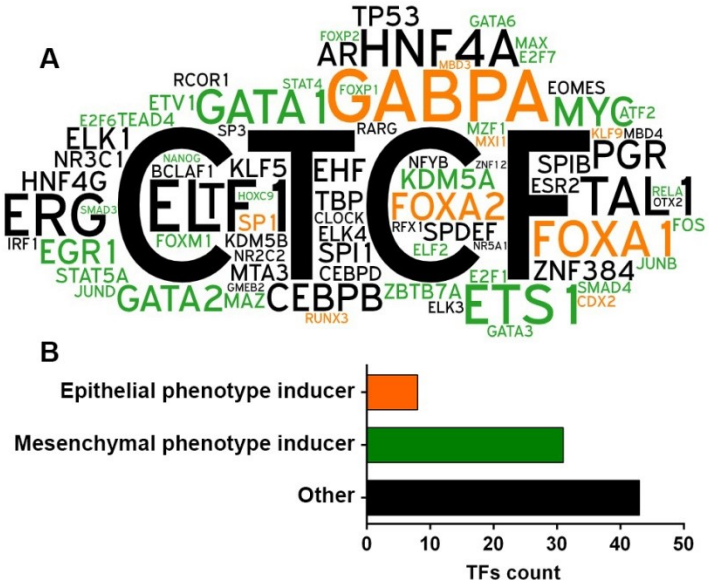

Figure 6.5. *ERBB2* gene binding transcription factors identified by ChIP-seq. (**A**) Word cloud diagram of transcription factors (TFs) detected bound to *ERBB2* gene at 10 kb upstream and downstream of motif Y. The different number of binding sites for each TF at the query region is illustrated as a different word size. TFs promoting epithelial and mesenchymal phenotypes are illustrated in orange and green colors respectively. (**B**) The number of the identified TFs based on their function in EMT procedure. Data obtained from ChIPbase v2.0 database available at "http://rna.sysu.edu.cn/chipbase/index.php".

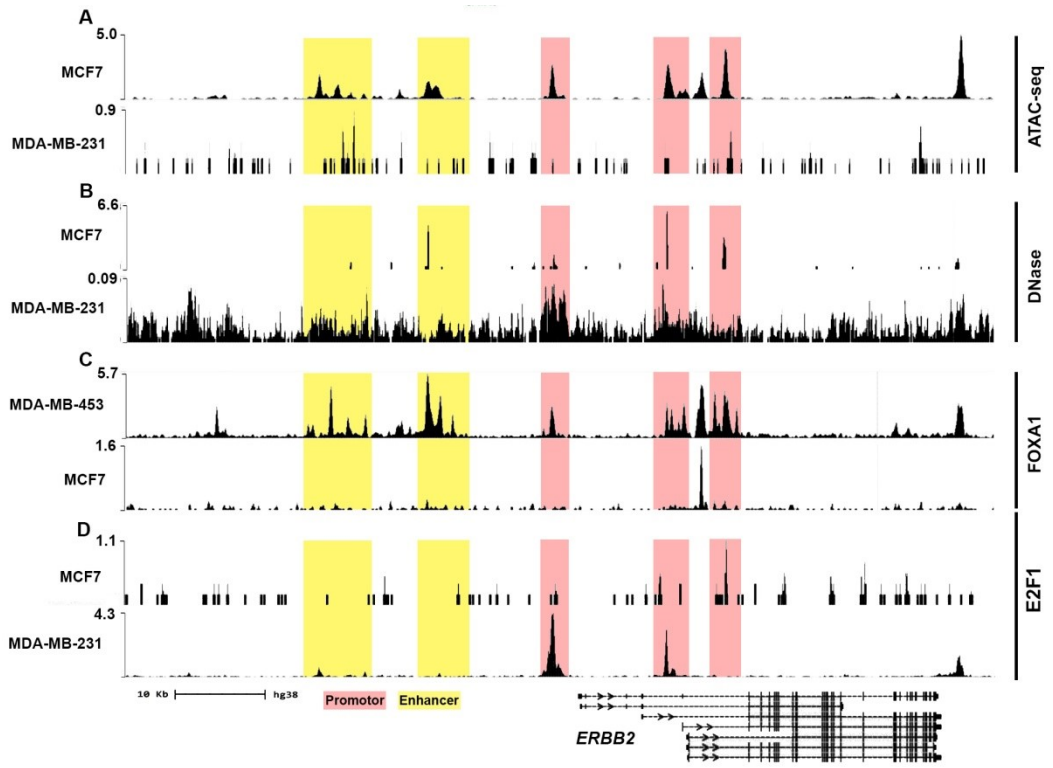

Figure 6.6. *ERBB2* chromatin accessibility/activity and enrichment of FOXA1 and E2F1. (A) ATAC-seq and (B) DNase I hypersensitivity value peaks of *ERBB2* gene and upstream regions in MCF7 and MDA-MB-231 cell lines. (C) ChIP-seq enrichment value peaks of FOXA1 at *ERBB2* gene and upstream regions in MDA-MB-452 and MCF7 cell lines. (D) ChIP-seq enrichment value peaks of E2F1 at *ERBB2* gene and upstream regions in MCF7 and MDA-MB-231 cell lines. Genomic coordinate: chr17:39,643,771-39,735,523 (GRCh38/hg38 assembly).

6.3.4. Histone modification of *ERBB2* chromatin in epithelial-like and mesenchymal-like breast cancer cells

To investigate whether different *ERBB2* expression in epithelial-like and mesenchymal-like cells is due to different *ERBB2* chromatin architecture, we studied the *ERBB2* chromatin dynamics in HER2-high (AU565, BT474, HCC-1954, MDA-MB-361, SKBR3) and HER2-low (MCF7, MDA-MB-231, MDA-MB-468) breast cancer cell lines. To this, we analyzed enrichment of open/active gene body chromatin histone marks (H2BK120ub, H3K39me3, H3K79me2), open/active promoter chromatin histone marks (H3K4me1, H3K4me3),

open/active enhancer chromatin histone marks (H3K9ac, H3K27ac, H4K8ac), as well as closed/inactive promoter and enhancer chromatin histone marks (H3K9me, H3K27me3) at *ERBB2* gene chromatin in the cells. The normalized ChIP-seq values were obtained from Cistrome Data Browser [8] and the enrichment peaks were visualized by using WashU Epigenome Browser [9]. Used datasets are shown in chapter 10. The cell lines were selected according to *ERBB2* expression levels shown in Figure 6.3A. The mRNA expression levels of the *ERBB2*, *MUC1*, *VIM,* and *GAPDH* genes are also shown in Figure 6.7A-C.

As result, AU565, BT474, HCC-1954, MDA-MB-361 and SKBR3 cell lines showed higher *MUC1* mRNA expression compared to. In contrast, MCF7 and MDA-MB-231 but not MDA-MB-468 cell lines showed higher *VIM* expression compared to AU565, BT474, HCC-1954, MDA-MB-361 and SKBR3 cell lines (Figures 6.7A-C). These results further approve that *ERBB2* expression is correlated positively with epithelial phenotype and negatively with mesenchymal phenotype suggesting that mesenchymal-like breast cancer cells show low *ERBB2* expression.

ChIP-seq data of histone marks showed higher enrichment of H2BK120ub (Figure 6.7E), H3K39me5 (Figure 6.7F) and H3K79me3 (Figure 6.7G) at *ERBB2* gene body in the HER2-high cell lines compared to that in the HER2-low cell lines. Results also showed that enrichment of open/active promoter chromatin marks H3K4me2 (Figure 6.8A) and H3K4me3 (Figure 6.8B) at promoter chromatin of *ERBB2* gene in EHR2-high cell lines were significantly higher than that in HER2-low cell lines. The HER2-high cell lines showed relatively higher enrichment levels of open/active enhancer chromatin histone marks H3K9ac (Figure 6.9A), H3K27ac (Figure 6.9B) and H4K8ac (Figure 6.9C) at enhancer chromatin of *ERBB2* gene when compared with the HER2-low cell lines. In addition, enrichment levels of closed/inactive promoter and enhancer chromatin histone marks H3K9me (Figure 6.9A) and H3K27me3 (Figure 6.9B) at *ERBB2* gene were relatively low in HER2-high as well as in HER2-low cell lines.

Taken together, these results suggest that (1) Chromatin activity of the *ERBB2* gene governs *ERBB2* gene expression in breast cancer. ERBB2 chromatin activity (2) Epithelial-like breast cancer cells express higher levels of *ERBB2* in comparison with mesenchymal-like breast cancer cells due to open chromatin of *ERBB2* promoter and enhancer regions in the epithelial-like breast cancers. *ERBB2* chromatin activity is correlated with the enrichment of epithelial phenotype

transcription factors and open/active chromatin histone modifications. (3) Whereas mesenchymal-like cells show negligible *ERBB2* expression. This is because of closed/inactive *ERBB2* promoter and enhancer chromatin that is correlated with the absence of epithelial phenotype transcription factors as well with higher enrichment of mesenchymal phenotype transcription factors at their *ERBB2* chromatin. (4) Closed/inactive status of *ERBB2* chromatin in mesenchymal-like cells is not due to inactivator histone modifications, but maybe because of the absence of activator histone modifications at *ERBB2* chromatin.

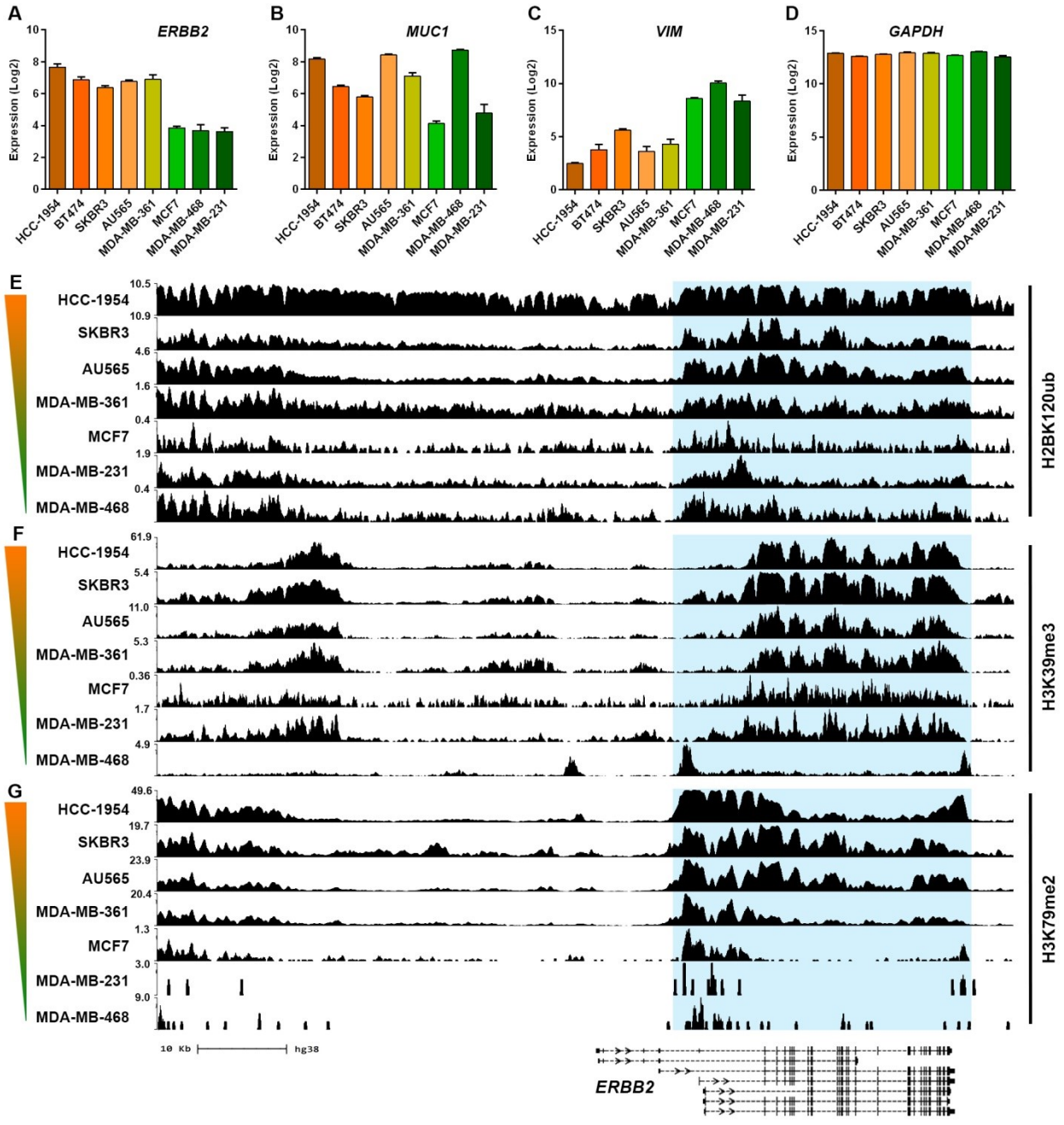

Figure 6.7. mRNA expression and open/active gene body chromatin histone marks of *ERBB2* gene in breast cancer cell lines. (**A-D**) mRNA expression levels of *ERBB2* (**A**), epithelial marker *MUC1* (**B**), mesenchymal marker (*VIM*) and GAPDH in HER2-high (HCC-1954, BT474, SKBR3, AU464, MDA-MB-361) and HER2-low (MCF7, MDA-MB-231, MDA-MB-468) breast cancer cell lines. Data obtained from GEO database with accession numbers GSE50811 [5] and GSE66071 [6]). (**E-G**) ChIP-seq enrichment of open/active gene body chromatin histone modification marks H2BK120ub (**E**), H3K39me3 (**F**), and H3K79me2 (**G**) at the chromatin of *ERBB2* gene and upstream region in the cells lines. Color gradient bars indicate the HER2 expression level. Genomic coordinate: chr17:39643771-39735523 (GRCh38/hg38 assembly).

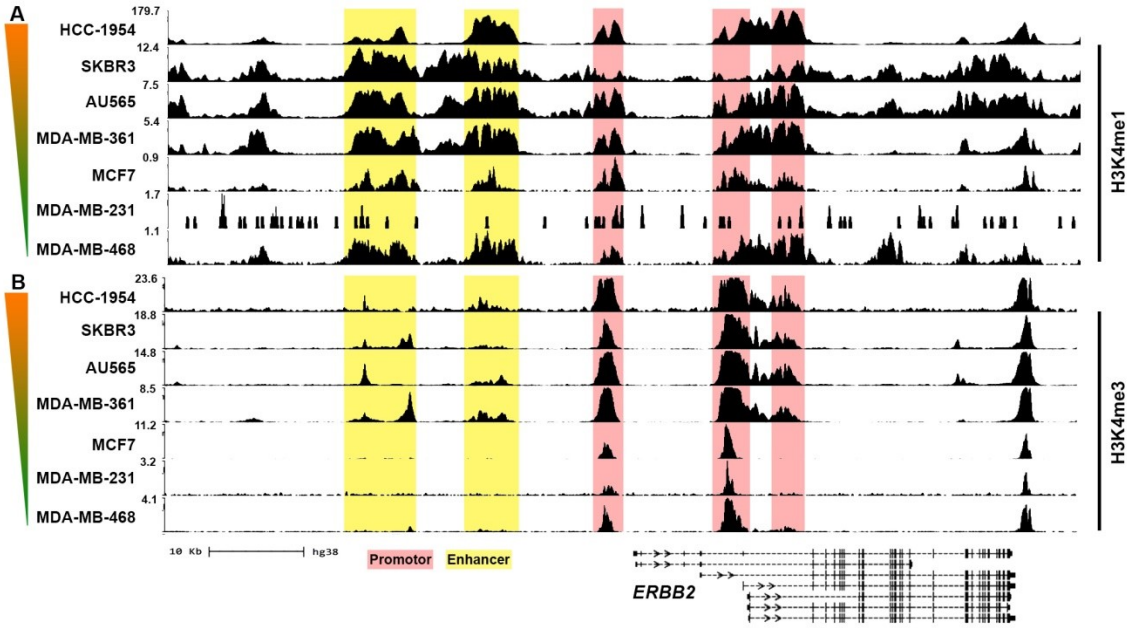

Figure 6.8. Open/active promoter histone marks of *ERBB2* gene in breast cancer cell lines. ChIP-seq enrichment of (**A**) H3K4me1 and (**B**) H3K4me3 at the chromatin of *ERBB2* gene and upstream region in HER2-high (HCC-1954, BT474, SKBR3, AU464, MDA-MB-361) and HER2-low (MCF7, MDA-MB-231, MDA-MB-468) breast cancer cell lines. Color gradient bars indicate the HER2 expression level. Genomic coordinate: chr17:39,643,771-39,735,523 (GRCh38/hg38 assembly).

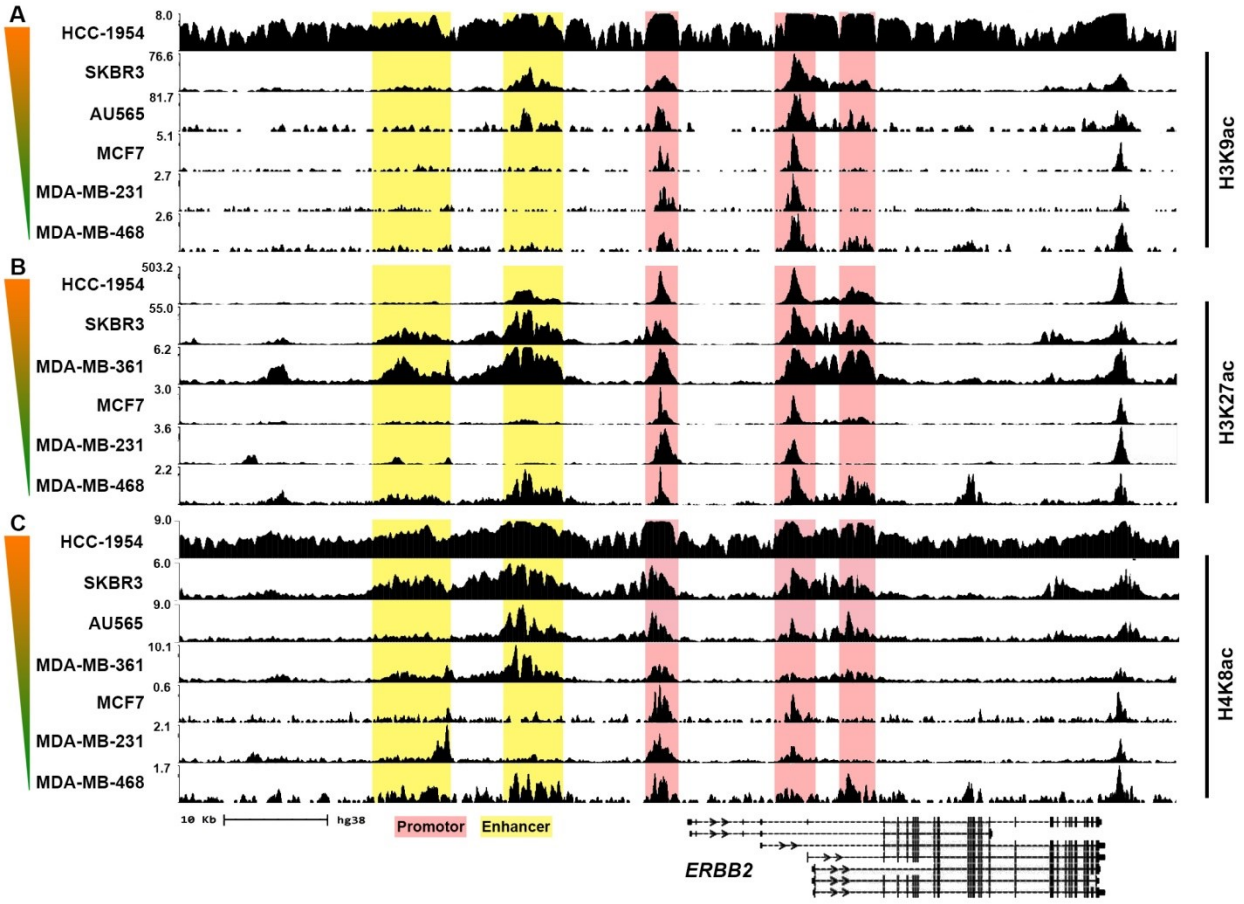

Figure 6.9. Open/active enhancer histone marks of *ERBB2* gene in breast cancer cell lines. ChIP-seq enrichment of (**A**) H3K9ac, (**B**) H3K27ac and (**C**) H4K8ac at the chromatin of *ERBB2* gene and upstream region in HER2-high (HCC-1954, BT474, SKBR3, AU464, MDA-MB-361) and HER2-low (MCF7, MDA-MB-231, MDA-MB-468) breast cancer cell lines. Color gradient bars indicate the HER2 expression level. Genomic coordinate: chr17:39,643,771-39,735,523 (GRCh38/hg38 assembly).

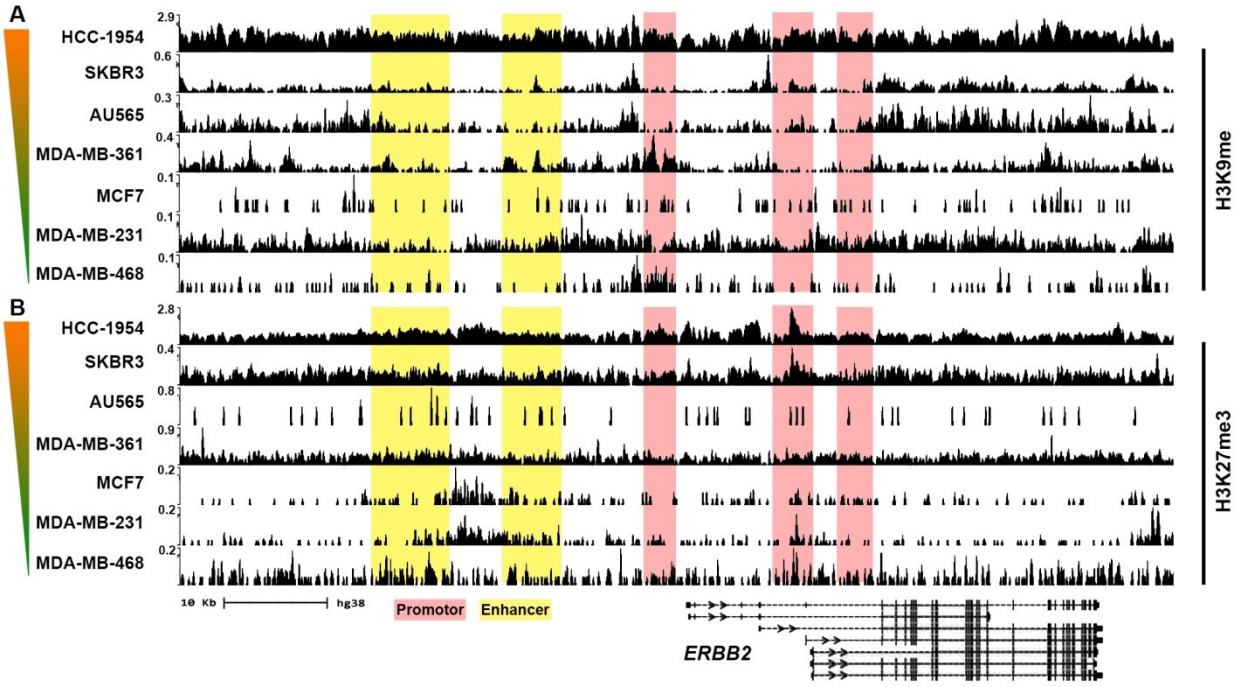

Figure 6.10. Closed/inactive promoter and enhancer histone marks of *ERBB2* gene in breast cancer cell lines. ChIP-seq enrichment of (**A**) H3K9me and (**B**) H3K27me3 at the chromatin of *ERBB2* gene and upstream region in HER2-high (HCC-1954, BT474, SKBR3, AU464, MDA-MB-361) and HER2-low (MCF7, MDA-MB-231, MDA-MB-468) breast cancer cell lines. Color gradient bars indicate the HER2 expression level. Genomic coordinate: chr17:39,643,771-39,735,523 (GRCh38/hg38 assembly).

6.3.5. 3D genome organization of *ERBB2* chromatin in HER2-high and HER2-low breast cancer cell lines

Our ChIP-seq histone modification analysis results revealed that *ERBB2* chromatin in HER2-high epithelial-like breast cancer cell lines is open/active, while HER2-low mesenchymal-like breast cancer cell lines showed closed/inactive *ERBB2* chromatin. HER2-low mesenchymal-like cells also showed lower chromatin-chromatin interactions and lower 3D genome structures including topologically associating domains (TADs). It is expected that the interaction of *ERBB2* promoter with canonical and non-canonical enhancers is lower in HER2-low mesenchymal-like cells. To examine this, we performed a 4D genome organization analysis of *ERBB2* gene by analyzing interactions between *ERBB2* chromatin and upstream and downstream chromatin regions in HCC-1954 and MCF7 cell lines. To this objective, we analyzed experimental IM-PET

(Integrated Methods for Predicting Enhancer Targets) and ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag Sequencing) data from the cell lines by using 4Dgenome database [10]. Results showed the interaction of *ERBB2* promoter with 240 target enhancer regions in HCC-1954 cell line (Figures 6.11A and B). Of 240 target enhancers, 134 were at upstream and 106 were at downstream of *ERBB2* promoter. The chromatin loop size of 106 interactions was found smaller than 50 kb and 18 interactions formed a chromatin loop larger than 500 kb (Figures 6.11A and B). While MCF7 cell line showed the interaction of *ERBB2* promoter with 11 target enhancers which all were at upstream region of *ERBB2* promoter. Of 11 interactions, 10 had a chromatin loop size of fewer than 50 kb, and 1 interaction had a loop size of approximately 244 kb (Figures 6.11C and D).

We also analyzed ChIP-seq H3K27ac enrichment profile of the *ERBB2* interaction sites in HCC-1954 and MCF7 in order to examine whether *ERBB2* promoter interaction with the target enhancer depends on chromatin activity of *ERBB2* promoter or target enhancer. The result showed higher H3K27ac enrichment at *ERBB2* chromatin in HCC-18954 cells in comparison with MCF7 cell lines. Whereas, MCF7 cell line showed higher H3K27ac enrichment at non-*ERBB2* chromatin than that in HCC-1954 cell lines. These results show the association of number and loop size of *ERBB2* chromatin interaction with the H3K27ac signature of ERBB2 chromatin but not non-ERBB2 chromatin. This suggests that ERBB2 promoter interaction with enhancers depends on the accessibility/activity of *ERBB2* promoter chromatin but not target enhancer chromatin. Overall, these results show that the number and size of promoter-enhancer interactions of *ERBB2* gene in HER2-high epithelial-like breast cancer cells are higher than that in HER2-low mesenchymal-like breast cancer cells. This is due to the accessibility/activity of *ERBB2* chromatin in HER2-high epithelial-like cells, and inaccessibility/inactivity of *ERBB2* chromatin in HER2-low mesenchymal-like cells.

For more confirmation. we studied also 3D genome organization of *ERBB2* and flanking chromatin (2 Mb upstream and 2 Mb downstream from *ERBB2* TSS) using HiC data of MCF7 (HER2-low) and MCF10A (HER2-negative) cell lines. *ERBB2* (Chr17:37,844,336-37,884,915) gene was used as bait. The data was analyzed by using 3DIV (3D-Genome Interaction Viewer) database [11]. As result, MCF7 cell line showed higher numbers of chromatin interactions and TADs at the quiry region compared to MCF10A cell line. The MCF7 cell line also showed 5

super-enhancers located at downstream of *ERBB2* TSS, however no super-enhancer was detected at the same chromatin of MCF10A cell line (Figures 6.11E and F). These results show a higher and a lower chromatin organization and topology of *ERBB2* gene and flanking chromatin in MCF7 and MCF10A cell lines respectively. suggesting that different *ERBB2*. These results also further confirm that the *ERBB2* expression levels and *ERBB2* chromatin accessibility/activity idepends to global chromatin architecture of the cells.

Taken together, these results indicate higher 3D chromatin interaction of *ERBB2* gene in the HER2-high breast cancer cells compared to the HER2-low breast cancer cells. HER2-high epithelial-like breast cancer cells show a higher number of chromatin interactions between *ERBB2* promoter and target enhancers. Whereas the number of *ERBB2* promoter-enhancer interactions in HER2-low mesenchymal cells is lower. In addition, accessibility/activity of ERBB2 chromatin was the main factor in the formation of ERBB2 chromatin interaction with distance enhancer regions. This indicates the epigenetic role of breast cancer EMT regulators in the chromatin dynamics of *ERBB2* that controls HER2 expression in breast cancer cells.

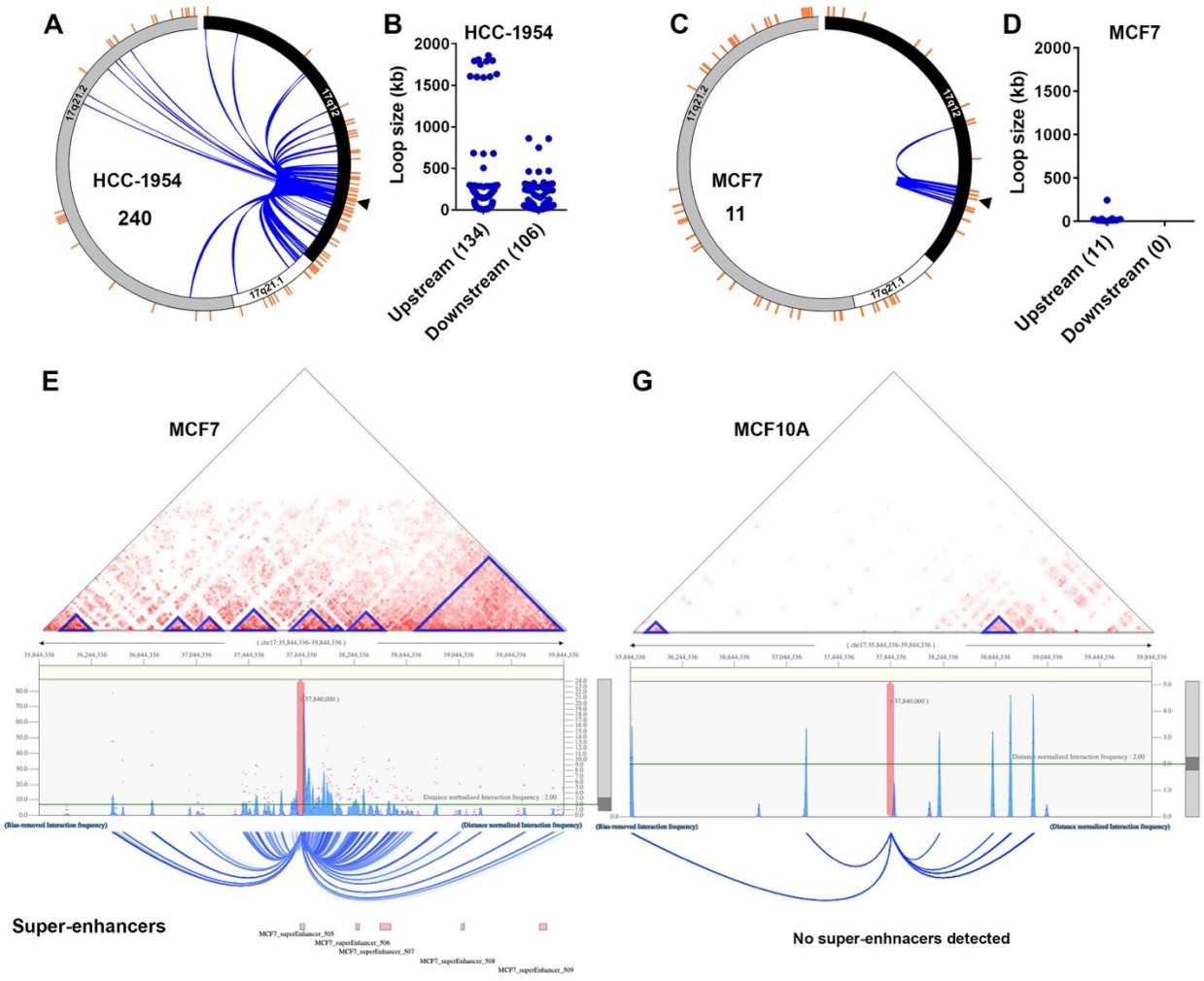

Figure 6.11. 3D genome organization of *ERBB2* gene in breast cancer cell lines. (**A**) Circle plot of IM-PET promoter-enhancer interaction of *ERBB2* gene in HCC-1954 cell lines. (**B**) Scatter plot illustrating chromatin loop size of the *ERBB2* promoter-enhancer interactions divided by location of target enhancers (upstream or downstream from *ERBB2* TSS). (**C**) IM-PET promoter-enhancer interaction of *ERBB2* gene in MCF7 cell lines. (**D**) Scatter plot illustrating chromatin loop size of *ERBB2* promoter-enhancer interactions divided by location of target enhancers (upstream or downstream from *ERBB2* TSS). *ERBB2* TSS is indicated by arrowhead. Data was obtained from 4Dgenome database [10]. (**E** and **F**) HiC heatmap plot and chromatin interactions of *ERBB2* gene in MCF7 (HER2-low; **E**) and MCF10A (HER2-negative; **F**) cell lines. Data were analyzed by using 3DIV database [11]. HiC heatmap resolution: 20 kbp. TAD identification method: TopDom (window size: 20 kb). Genomic coordinate: chr17:35,844,336-39,844,336 (GRCh37/hg19 assembly).

6.3.6. Downregulated *ERBB2* expression and upregulated EMT in lapatinib resistance

To investigate the role of EMT in development of resistance of HER2-positive breast cancer cells to anti-HER2 drugs, we studied mRNA expression of *ERBB2*, epithelial phenotype markers *CDH1*, *ALCAM*, *FOXA1*, *NECTIN2* and *OCLN*, mesenchymal phenotype markers *CDH2*, *FN1*, *FOXC1*, *SNAI2* and *VIM*, as well as matrix metalloproteinase *MMP1*, *MMP2*, *MMP3*, *MMP9*, *MMP10* and *MMP28* in lapatinib sensitive and acquired lapatinib resistant BT474 cells. The array expression profiling data obtained from GEO database (Series GSE16179 [12]).

As result, lapatinib resistant cells showed lower expression levels of *ERBB2* (Figure 6.12A) and epithelial marker genes (Figure 6.12B) and higher expression levels of mesenchymal marker genes (Figure 6.12.C) and MMPs (Figure 6.12D) when compared with the lapatinib sensitive cells. These results indicate that lapatinib sensitive cells are HER2-high and epithelial-like cells, while lapatinib resistance cells are HER2-low and mesenchymal-like cells, suggesting that EMT induces lapatinib resistance via downregulating HER2 expression.

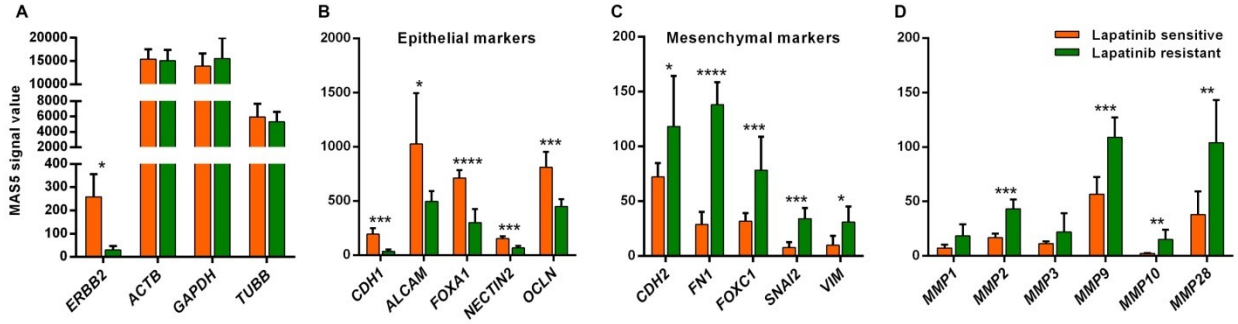

Figure 6.12. Downregulated *ERBB2* expression and upregulated EMT in lapatinib resistance. mRNA expression levels of (**A**) *ERBB2* and housekeeping genes, (**B**) epithelial phenotype marker genes, (**C**) mesenchymal phenotype marker genes and (**D**) matrix metalloproteinases in lapatinib sensitive and resistant BT474 cells. Data obtained from GEO database (Series GSE16179 [12]).

6.3.7. EMT reduces HER2 expression and decreases trastuzumab binding to HER2-positive breast cancer cells

To examine whether induction of EMT in HER2-positive epithelial cell reduces HER2 expression, we analyzed mRNA expression of *ERBB2*, epithelial phenotype markers *CDH1, EPCAM*, *MUC1* and *OCLN*, mesenchymal phenotype markers *CDH2*, *FN1*, *SNAI2* and *VIM*, matrix metalloproteinase *MMP1*, *MMP2*, *MMP3*, *MMP9*, *MMP10* and *MMP28*, as well as *ADAM10*, *ADAM17* and *ADAM19* in A549 cell line (HER2-high human lung cancer epithelial cell line) subjected to EMT induction by treatment with 5 ng/ml TGF-β for 0, 0.5, 1, 2, 4, 8, 16, 24, and 72 hours. The array expression profiling data obtained from GEO database (Series GSE17708 [13]). Results showed significantly decreased mRNA expression of the epithelial marker genes (Figure 6.13B) and increased mRNA expression of the mesenchymal marker genes (Figure 6.13C), MMPs (Figure 6.13D) as well as ADAMs (Figure 6.13E), demonstrating EMT induction in the cells. Downregulated epithelial marker genes and upregulated mesenchymal marker genes were correlated with significant reduction of *ERBB2* expression ($P < 0.001$) at 72 hours after TGF-β treatment started (Figure 6.13A).

To investigate whether EMT reduces trastuzumab binding to HER2 we induced EMT in BT474 by treatment with EMT inducing media supplements cocktail (containing Wnt-5a, TGF-β1, anti-human E-Cadherin antibody, anti-human sFRP1 antibody, and anti-human Dkk1 antibody) for 15 days. Induction of EMT was confirmed by monitoring cell morphology (Figure 6.14.A) and immunofluorescence staining of Vimentin (Figure 6.14B). After the majority of cells gained mesenchymal phenotype, the cells were treated with 10 μg/ml trastuzumab for 1 hour and then trastuzumab was stained by immunofluorescence staining. Results showed lower binding of trastuzumab to HER2 in the cells underwent EMT compared to control cells. These results confirm that EMT downregulates HER2 expression that causes a decreased rate of trastuzumab binding to HER2-positive breast cancer cells.

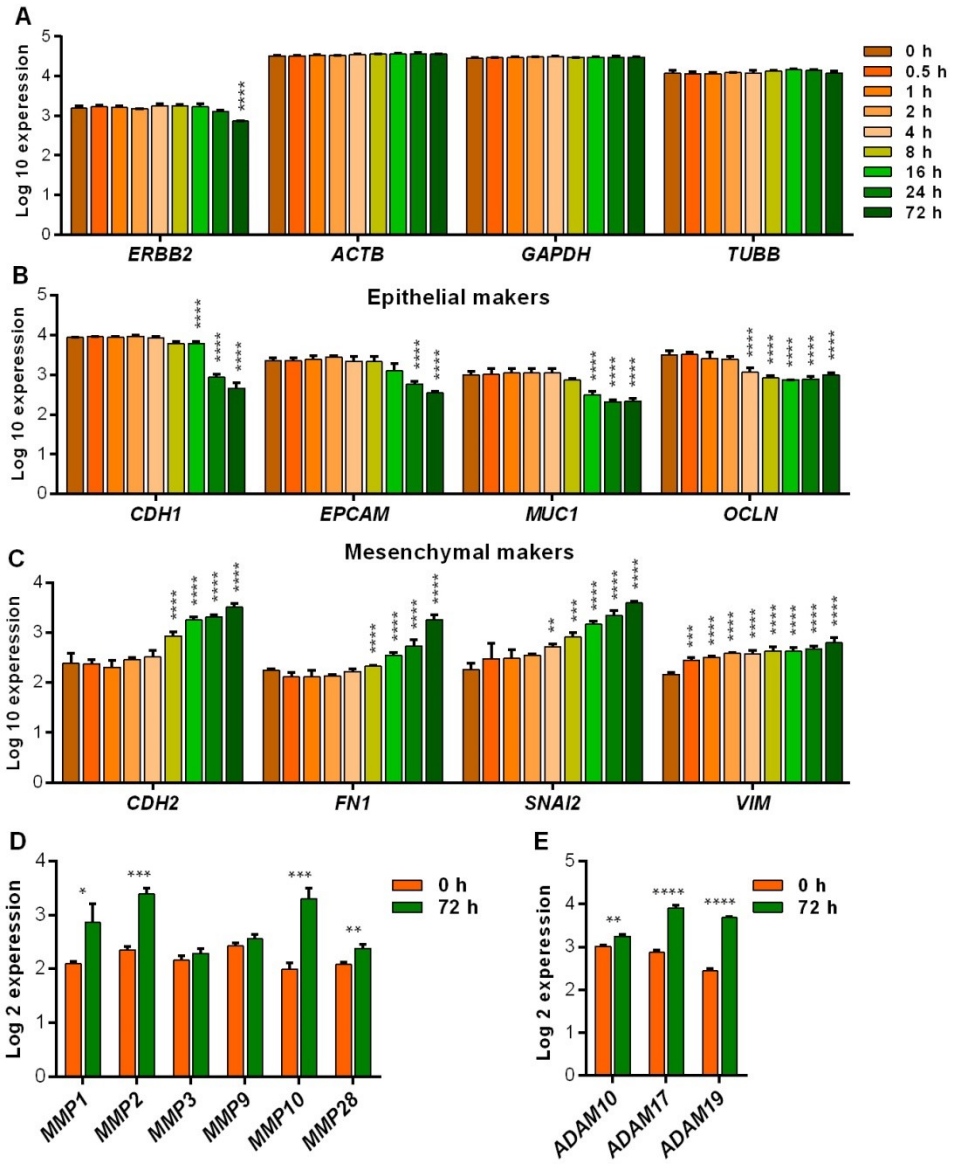

Figure 6.13. EMT reduces *ERBB2* expression. mRNA expression levels of (**A**) *ERBB2* and housekeeping genes, (**B**) epithelial phenotype marker genes, (**C**) mesenchymal phenotype marker genes and (**D**) MMPs and (**E**) ADAMs in TGF-β-mediated EMT-induced A549 cells. Data obtained from GEO database (Series GSE17708 [13]).

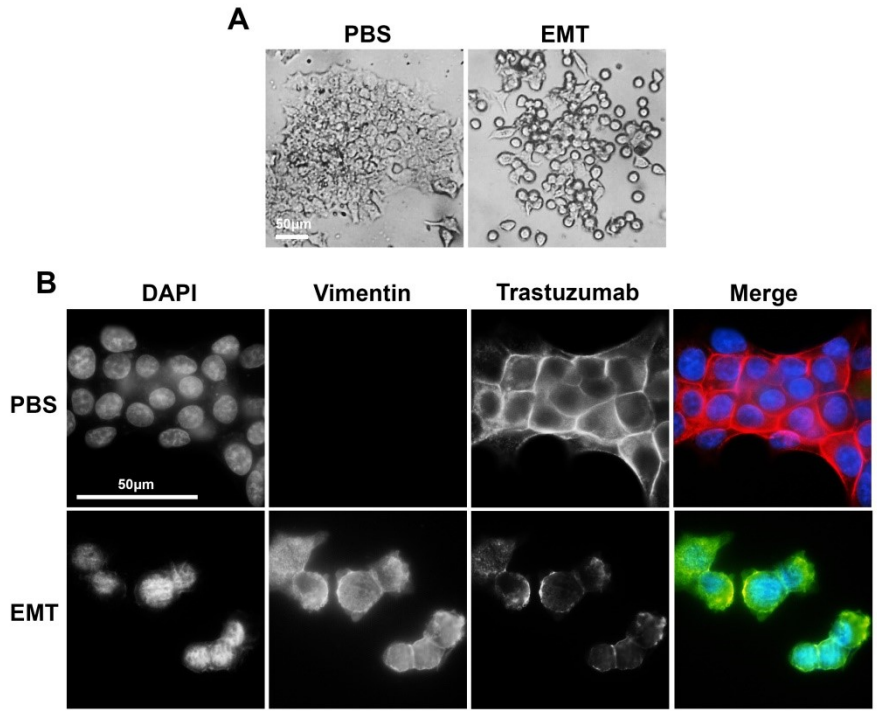

Figure 6.14. EMT decreases trastuzumab binding to HER2. (**A**) Epithelial morphology of PBS-treated BT474 cells and mesenchymal morphology of EMT-induced BT474 cells. (**B**) Immunofluorescence staining of Vimentin and trastuzumab in PBS-treated and EMT-induced BT474 cells treated with trastuzumab (10 μg/ml) for 1 hour.

6.4. DISCUSSION

HER2 is an important target for the treatment of HER2-positive breast cancers. Several HER2-targeting agents such as trastuzumab have been approved by the FDA to treat HER2 positive breast cancer. However, the resistance to these HER2 targeting agents has become a huge obstacle for the treatment of HER2-positive breast cancer patients. It is not clear why many HER2-positive tumors develop resistance to anti-HER2 neoadjuvant trastuzumab. In this chapter, we studied the chromatin signature of *ERBB2* gene in epithelial-like HER2-high and mesenchymal-like HER2-low breast cancer cells and the role of EMT-mediated epigenetic regulation in *ERBB2* chromatin organization by analyzing genomics and epigenomics data from publicly available databases. We found that the expression of EMT marker and inducer is negatively correlated with HER2 expression, and positively correlated with trastuzumab and

lapatinib resistance. HER2 expression in epithelial-like breast cancer cells is significantly higher than that in mesenchymal-like breast cancer cells. This is due to open/active chromatin of *ERBB2* gene in epithelial-like cells, as well as closed/inactive chromatin of *ERBB2* gene in mesenchymal-like breast cancer cells. This figure is also correlated with enrichment levels of EMT regulator transcription factor at the *cis*-regulatory regions of *ERBB2* gene. We also showed downregulated HER2 expression and upregulated EMT in BT474 cells resistance to lapatinib compared to lapatinib-sensitive cells. Induction of EMT in HER2-high epithelial-like breast cancer cells resulted in the downregulation of HER2 and decreased rate of trastuzumab binding to the cells. Our results suggest that EMT of HER2-positive breast cancer cells results in abrogation of HER2 expression by chromatin-base epigenetic silencing of *ERBB2* gene that leads to emergence of resistance to trastuzumab (Figure 6.15).

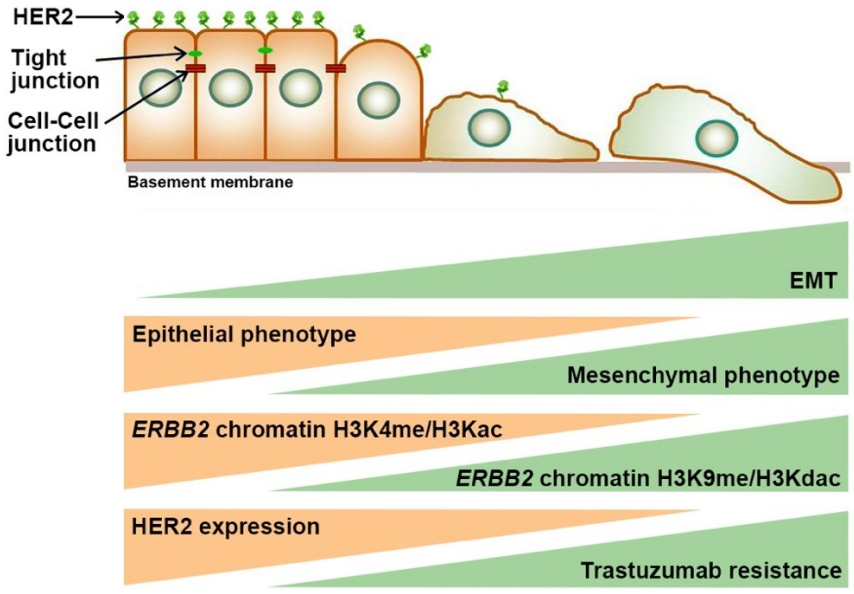

Figure 6.15. Schematic summary of findings described in chapter 6. EMT of HER2-positive breast cancer cells increases trastuzumab resistance by chromatin-based epigenetic downregulation of HER2 expression. Increased EMT and mesenchymal phenotype is correlated with decreased expression of epithelial phenotype (including tight junctions and cell-cell junction proteins) and increased mesenchymal phenotype, decreased enrichment of open/active chromatin marks (H3K4me and H3Kac as examples), increased enrichment of closed/inactive chromatin marks (H3K9me and H3Kdac as examples), decreased HER2 expression and increased trastuzumab resistance.

Liu et al. [92] demonstrated that CD44+/CD24- cells are mesenchymal-like BCSCs that localized at the tumor invasive margins and are correspond to migration and metastasis, whereas ALDH+ cells are defined as epithelial-like BCSCs that are located in deeper sites of the tumors and exhibit more proliferative property. CD44+/CD24- cells were isolated by FACS from non-tumorigenic human mammary epithelial cells that have undergone an induced EMT, exhibited many properties of BCSCs including mammosphere-formation ability [14]. On the other side, CD44+/CD24- BCSCs isolated from breast tumors expressed a low level of E-cadherin, but high levels of EMT markers including N-cadherin, Vimentin, Fibronectin, ZEB1/2, FOXC2, Snail, Slug, and Twist1/2 [14]. Clinical studies revealed that HER2-positive metastatic breast cancers were associated with EMT [15,16]. HER2 signaling in human mammary epithelial cells results in increased expression of Vimentin, N-cadherin, and Integrin-α5, as well as the loss of E-cadherin and Desmoplakin. However, some recent study suggests that loss of E-cadherin is not essential for HER2-induced EMT [17,18]. It is shown recently that overexpression of HER2 in epithelial breast cancer cell line D492 induces EMT and maintains the mesenchymal phenotype in the absence of EGFR [19]. There are also many reports revealing crosstalk of HER2 receptor and its downstream pathways with the stemness signaling pathways to prone mammary epithelial cells towards EMT.

We hypothesized that response to trastuzumab in luminal cells and resistance to trastuzumab in basal/mesenchymal cells may link to EMT. Trastuzumab-resistant tumors are thought to be enriched for EMT features. Basal breast cancer cell line JIMT-1 which is HER2-positive, trastuzumab-refractory, ER-negative, and Vimentin-positive is a best cell model to explain the link between HER2, EMT and trastuzumab resistance. De novo resistance of JIMT-1 cells to trastuzumab can be explained by the emergence of trastuzumab-resistance BCSCs due to the dynamic interaction between HER2 and EMT [1,20–25]. The subpopulation of CD44+/CD24- BCSC in the early culture of the JIMT-1 cell line is reported around 10%. However, this rate was increased to 85% at the late-passages which was also associated with a dramatic decline in HER2 expression levels [1]. Treatment of CD44+/CD24- BCSCs derived from breast tumor tissues treated with formestane, an aromatase inhibitor, resulted in a 16% decrease ($P < 0.01$) in the cell proliferation in response to single-agent trastuzumab and 50% decrease ($P < 0.001$) in response to treatment with trastuzumab combined with formestane. The combined treatment also inhibits the expression of EGFR, HER2, aromatase and Cyclin D1 in

CD44+/CD24- cells, which suggests that targeting HER2 by trastuzumab may inhibit the growth of CD44+/CD24- BCSCs through the inhibition of cell cycle progression [23]. Some other reports suggest that preferential killing of the putative CD44+/CD24- BCSCs might be sufficient to overcome primary resistance to trastuzumab. The CD44+/CD24- BCSCs derived from trastuzumab-refractory JIMT-1 cells were 10-fold more sensitive to cell growth inhibitory effects of metformin than the other cells [25]. JIMT-1 cell line is highly enriched with the mesenchymal phenotype CD44+/CD24- fraction in late passages [1,26,27]. Indeed, treatment of JIMT-1 tumors with trastuzumab failed to exhibit significant reductions in tumor volume but when trastuzumab was combined with metformin, the tumor size was significantly smaller than those of the groups treated with single-agent trastuzumab or metformin [25]. These results suggest that BCSCs inside JIMT-1 tumor escape from trastuzumab effects, that support the hypothesis that mesenchymal cell subpopulation is responsible for resistance of the tumor against trastuzumab.

It is further revealed that trastuzumab-resistant HER2-positive cells show spontaneous EMT and predominant exhibition of CD44+/CD24- phenotype [28]. There is a synchronous increase in CD44 and elements of Wnt/β-catenin signaling and a decrease in CD24 expression in mesenchymal colony clusters of SKBR3 cells. SKBR3 cell line is characterized as HER2-positive, trastuzumab-sensitive liminal cell line. The CD44+/CD24- mesenchymal colonies of SKBR3 also show significantly upregulated EMT markers including Vimentin, N-cadherin, Twist1 and Fibronectin. The colonies were highly resistant to trastuzumab and lapatinib while the luminal/epithelial SKBR3 cells remained trastuzumab-sensitive. Similar to previous reports, HER2 expression levels in mesenchymal colonies were negatively correlated with trastuzumab resistance [28]. Thus, lapatinib which is recently approved to treat trastuzumab-resistant breast cancers, may not be a useful therapeutic option in targeting CD44+/CD24- mesenchymal cell-rich tumors due to the negative regulation of HER2 during EMT.

Furthermore, the expression of the EMT-driving transcription factors Slug, Twist1 and ZEB1 are higher in trastuzumab-refractory basal HER2-positive JIMT-1 cells than that in the trastuzumab-responsive SKBR3 cells. The knockdown of the transcription factors in parental JIMT-1 cells reduced the subpopulation of CD44+/CD24- BCSC by 5, 5 and 2-fold, respectively. Interestingly, depletion of the EMT-driving transcription factors increased the trastuzumab-refractory in JIMT-1 tumors due to sensitized CD44+/CD24- BCSCs inside the bulk

JIMT-1 tumors [24]. HER2-positive basal epithelial BCSCs are susceptible to change in their expression signature during EMT. This phenomenon may explain the resistance of some HER2-positive breast tumors to HER2-targeting agent including trastuzumab. Further, CD44+/CD24- BCSC subpopulation of HER2-positive cell lines or tumors may escape from trastuzumab-mediated ADCC. BCSCs could survive the immunoselection process in breast cancer cells co-cultured with NK cells and trastuzumab. This resistance may be attributed to the reduced HER2 expression levels on their surface [2]. Overall, our results demonstrate that EMT of HER2-positive breast cancer cells causes the emergence of a mesenchymal-like cell subpopulation which are HER2-low/negative and highly resistant to trastuzumab and lapatinib. The significant depletion of HER2 in the mesenchymal-like cells inside the tumor can take place by chromatin-based epigenetic silencing of *ERBB2* gene during EMT. Thus, we suggest that EMT and mesenchymal-like cells can be major mechanism of and responsible for de novo resistance to HER2-targeting therapeutics including trastuzumab and lapatinib and serve as potential effective targets for therapy and to overcome drug resistance in breast cancers.

6.5. REFERENCES

1. Oliveras-Ferraros C, Vazquez-Martin A, Martin-Castillo B, Cufi S, Del Barco S, Lopez-Bonet E, et al. Dynamic emergence of the mesenchymal CD44(pos)CD24(neg/low) phenotype in HER2-gene amplified breast cancer cells with de novo resistance to trastuzumab (Herceptin). Biochem Biophys Res Commun. 2010;397: 27–33. doi:10.1016/j.bbrc.2010.05.041

2. Reim F, Dombrowski Y, Ritter C, Buttmann M, Hausler S, Ossadnik M, et al. Immunoselection of breast and ovarian cancer cells with trastuzumab and natural killer cells: selective escape of CD44high/CD24low/HER2low breast cancer stem cells. Cancer Res. 2009;69: 8058–8066. doi:10.1158/0008-5472.CAN-09-0834

3. Pereira B, Chin S-F, Rueda OM, Vollan H-KM, Provenzano E, Bardwell HA, et al. The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. Nat Commun. 2016;7: 11479. doi:10.1038/ncomms11479

4. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2: 401–404. doi:10.1158/2159-8290.CD-12-0095

5. Dezso Z, Oestreicher J, Weaver A, Santiago S, Agoulnik S, Chow J, et al. Gene expression profiling reveals epithelial mesenchymal transition (EMT) genes can selectively differentiate eribulin sensitive breast cancer cells. PLoS One. 2014;9: e106131. doi:10.1371/journal.pone.0106131

6. Xiao Y, Nimmer P, Sheppard GS, Bruncko M, Hessler P, Lu X, et al. MCL-1 Is a Key Determinant of Breast Cancer Cell Survival: Validation of MCL-1 Dependency Utilizing a Highly Selective Small Molecule Inhibitor. Mol Cancer Ther. 2015;14: 1837–1847. doi:10.1158/1535-7163.MCT-14-0928

7. Di Cello F, Cope L, Li H, Jeschke J, Wang W, Baylin SB, et al. Methylation of the claudin 1 promoter is associated with loss of expression in estrogen receptor positive breast cancer. PLoS One. 2013;8: e68630. doi:10.1371/journal.pone.0068630

8. Mei S, Qin Q, Wu Q, Sun H, Zheng R, Zang C, et al. Cistrome Data Browser: a data portal for ChIP-Seq and chromatin accessibility data in human and mouse. Nucleic Acids Research. 2017. pp. D658-62. doi:10.1093/nar/gkw983

9. Zhou X, Lowdon RF, Li D, Lawson HA, Madden PAF, Costello JF, et al. Exploring long-range genome interactions using the WashU Epigenome Browser. Nature methods. United States; 2013. pp. 375–376. doi:10.1038/nmeth.2440

10. Teng L, He B, Wang J, Tan K. 4DGenome: a comprehensive database of chromatin interactions. Bioinformatics. 2015;31: 2560–2564. doi:10.1093/bioinformatics/btv158

11. Yang D, Jang I, Choi J, Kim M-S, Lee AJ, Kim H, et al. 3DIV: A 3D-genome Interaction Viewer and database. Nucleic Acids Res. 2018;46: D52–D57. doi:10.1093/nar/gkx1017

12. Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. 2009;69: 6871–6878. doi:10.1158/0008-5472.CAN-08-4490

13. Sartor MA, Mahavisno V, Keshamouni VG, Cavalcoli J, Wright Z, Karnovsky A, et al. ConceptGen: a gene set enrichment and gene set relation mapping tool. Bioinformatics. 2010;26: 456–463. doi:10.1093/bioinformatics/btp683

14. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008. pp. 704–715. doi:10.1016/j.cell.2008.03.027

15. Giordano A, Mego M, Lee B, Anfossi S, Parker CA, Alvarez RH, et al. Epithelial-mesenchymal transition in patients with HER2+ metastatic breast cancer. J Clin Oncol. 2011;29: 623.

16. Giordano A, Gao H, Anfossi S, Cohen E, Mego M, Lee B-N, et al. Epithelial-mesenchymal transition and stem cell markers in patients with HER2-positive metastatic breast cancer. Mol Cancer Ther. 2012;11: 2526–2534. doi:10.1158/1535-7163.MCT-12-0460

17. Jenndahl LE, Isakson P, Baeckstrom D. c-erbB2-induced epithelial-mesenchymal transition in mammary epithelial cells is suppressed by cell-cell contact and initiated prior to E-cadherin downregulation. Int J Oncol. 2005;27: 439–448.

18. Nilsson GMA, Akhtar N, Kannius-Janson M, Baeckstrom D. Loss of E-cadherin expression is not a prerequisite for c-erbB2-induced epithelial-mesenchymal transition. Int J Oncol. 2014;45: 82–94. doi:10.3892/ijo.2014.2424

19. Ingthorsson S, Andersen K, Hilmarsdottir B, Maelandsmo GM, Magnusson MK, Gudjonsson T. HER2 induced EMT and tumorigenicity in breast epithelial progenitor cells is inhibited by coexpression of EGFR. Oncogene. 2016;35: 4244–4255. doi:10.1038/onc.2015.489

20. Rennstam K, Jonsson G, Tanner M, Bendahl P-O, Staaf J, Kapanen AI, et al. Cytogenetic characterization and gene expression profiling of the trastuzumab-resistant breast cancer cell line JIMT-1. Cancer Genet Cytogenet. 2007;172: 95–106. doi:10.1016/j.cancergencyto.2006.09.014

21. Barok M, Isola J, Palyi-Krekk Z, Nagy P, Juhasz I, Vereb G, et al. Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Mol Cancer Ther. 2007;6: 2065–2072. doi:10.1158/1535-7163.MCT-06-0766

22. Koninki K, Barok M, Tanner M, Staff S, Pitkanen J, Hemmila P, et al. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. Cancer Lett. 2010;294: 211–219. doi:10.1016/j.canlet.2010.02.002

23. Cavaliere C, Corvigno S, Galgani M, Limite G, Nardone A, Veneziani BM. Combined inhibitory effect of formestane and herceptin on a subpopulation of CD44+/CD24low breast cancer cells. Cancer Sci. 2010;101: 1661–1669. doi:10.1111/j.1349-7006.2010.01593.x

24. Oliveras-Ferraros C, Corominas-Faja B, Cufi S, Vazquez-Martin A, Martin-Castillo B, Iglesias JM, et al. Epithelial-to-mesenchymal transition (EMT) confers primary resistance to trastuzumab (Herceptin). Cell Cycle. 2012;11: 4020–4032. doi:10.4161/cc.22225

25. Cufí S, Corominas-Faja B, Vazquez-Martin A, Oliveras-Ferraros C, Dorca J, Bosch-Barrera J, et al. Metformin-induced preferential killing of breast cancer initiating CD44(+)CD24(−/low) cells is sufficient to overcome primary resistance to trastuzumab in HER2+ human breast cancer xenografts. Oncotarget. 2012. pp. 395–398.

26. Bauerschmitz GJ, Ranki T, Kangasniemi L, Ribacka C, Eriksson M, Porten M, et al. Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. Cancer Res. 2008;68: 5533–5539. doi:10.1158/0008-5472.CAN-07-5288

27. Rennstam K, McMichael N, Berglund P, Honeth G, Hegardt C, Ryden L, et al. Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer. Breast Cancer Res Treat. 2010;122: 315–324. doi:10.1007/s10549-009-0568-x

28. Lesniak D, Sabri S, Xu Y, Graham K, Bhatnagar P, Suresh M, et al. Spontaneous Epithelial-Mesenchymal Transition and Resistance to HER-2-Targeted Therapies in HER-2-Positive Luminal Breast Cancer. Tan M, editor. PLoS ONE. San Francisco, USA; 2013.

doi:10.1371/journal.pone.0071987

Chapter 7. Overall discussion

HER2 (ErbB2/Neu) is a 185 kDa transmembrane receptor belongs to the tyrosine kinase epidermal growth factor receptor family including other receptors EGFR (HER1/ErbB1), HER3 (ErbB3) and HER4 (ErbB4) [1–3] Dimerization of HER receptors leads to activation of their intracellular tyrosine kinase domains leading to the phosphorylation of both receptors [3]. Phosphorylated HER receptor dimers initiate multiple signaling pathways including PI3K/Akt, PLC-ɣ, and MAPK signaling pathways, which promote cell growth, division and motility [3]. Activation of HER2 tyrosine kinase domain takes place after homodimerization and heterodimerization with either EGFR, HER3, or HER4. HER2 is encoded by *ERBB2* gene which is known as an oncogene and amplification causes overexpression of HER2 receptor in the cells. Overexpression of HER2 mostly due to gene amplification is a common oncogenic phenomenon in many cancer types and is associated with poor clinical outcome [4]. HER2 is overexpressed more than 10 times in tumor cells than that in normal cells in 15-30% of all breast cancers [2,5–7], 2-66% of all ovarian cancers [8,9], and 4-35% of all lung adenocarcinoma [10,11]. The cancers with HER2 overexpression which are known as "HER2-positive cancers" grow faster due to more HER2 signaling but are vulnerable to anti-HER2 targeting therapies including trastuzumab and pertuzumab.

Trastuzumab and pertuzumab bind to domains IV and II of the extracellular part of HER2 respectively. Trastuzumab is reported to block the homodimerization of HER2, and to inhibit ligand-independent HER2-mediated signaling as HER2 is an orphan receptor, but could homodimerize when overexpressed [12–14]. Pertuzumab is known to block the heterodimerization of HER2 and to inhibit ligand-dependent HER2-mediated signaling. However, we showed that trastuzumab and pertuzumab have no effect on HER2 homodimerization, phosphorylation and downstream signaling [15]. So far evidence on the exact mode of action of trastuzumab and resistance mechanism still remains controversial.

7.1. INHIBITING p85HER2 AS A MECHANISM OF TRASTUZUMAB ACTION

Trastuzumab binds to domain IV of HER2 and is thought to block binding pocket for receptor homo-dimerization, thereby blocking HER2 homo-dimerization, phosphorylation and consequently inhibition of downstream signaling pathways [16,17]. The following mechanisms have been suggested for the tumor inhibitory effects of trastuzumab. (i) Trastuzumab binding to HER2 suppresses PI3K/Akt and MAPK pathways by inhibition of HER2 activation [13]. In this model, trastuzumab binding to HER2 may prevent tyrosine kinase Src signaling and upregulates the activity of the tumor suppressor PTEN [18,19]. This inhibition also leads to suppression of PI3K/Akt signaling, activation of the tumor suppressor p27 and suppression of CDK2 thus arresting cell cycle and growth in breast tumor cells [20–22]. (ii) Trastuzumab causes endocytosis and degradation of HER2 through blocking the activity of tyrosine kinases [23]. (iii) Preclinical and clinical studies revealed that coating HER2 overexpressed tumor cells by trastuzumab summons more immune cells especially natural killer cells to attack the tumor by ADCC mechanism [24,25]. Many clinical trial studies have demonstrated effectiveness of trastuzumab in combination with docetaxel in metastatic HER2-positive breast cancers [26–29]. However, the exact mode of action and resistance mechanism still remain ambiguous.

In chapters 3 and 5 we showed that trastuzumab induces ADCC of HER-positive cells but does not inhibit HER2 receptor activation and canonical downstream pathways. We showed that trastuzumab blocks proteolytic cleavage, production and nuclear localization of ctHER2 which is a novel molecular mechanism of action of trastuzumab. ctHER2 fragments are truncated HER2 proteins characterized by the lack of extracellular domain, but still possessing tyrosine kinase activity [30–34]. According to previous studies, ctHER2 with an approximately 95 kDa in weight (also known p95HER2) arise by two different mechanisms: (i) proteolytic shedding/cleavage of p185HER2 by zinc-containing metalloproteinases, including ADAMs and MMPs family members [33,35,36]; and (ii) Alternative splicing of *ERBB2* mRNA that initiate protein translation of HER2 from methionines located near the transmembrane domain of the full-length molecule [37]. Breast cancer patients expressing p95HER2 are more likely to develop nodal metastasis [32,38,39] and have worse prognoses than those predominantly expressing the full-length receptor [38]. Our results showed a synergism effect between trastuzumab and metalloproteinase inhibitor in blocking HER2 cleavage and shedding.

Interestingly, the upregulation of the metalloproteinases is a hallmark of EMT and mesenchymal cells [40–43]. Several metalloproteinases including ADAMs 9, 10, 12, 15, 17, 28 [44–49] and also MMPs 1, 2, 7, 9 11, 12, 13, 14 and 16 are reported as overactivated in many breast cancers [50–53]. ADAMs 10 and 17 are the major sheddase enzymes involved in HER2 shedding [54]. Recent studies show that the metalloproteinases are also associated with poorer relapse-free survival in HER2-positive breast cancer patients and the inhibition of metalloproteinases by chemicals overcame trastuzumab resistance in both naïve and trastuzumab-resistant HER2-positive cell lines [55–57]. These data suggest ADAMs 10 and 17 as key drivers of trastuzumab resistance and potential targets to overcome trastuzumab resistance in HER2-positive breast cancers [58]. Moreover, a preclinical study revealed that inhibitors of MMPs 1, 2, 3 and 9 suppress HER2 shedding [59]. Many cohort studies conclusively show the correlation of p95HER2 expression with poor prognosis and trastuzumab resistance in breast cancer, corroborating p95HER2 as a prognostic factor for metastasis and a predictive marker of trastuzumab resistance [38,60–63]. Taking together, HER2 cleavage is a result of increased metalloproteinases activity during EMT that is associated with upregulated stemness signaling pathways.

To understand the biological function of trastuzumab through blocking HER2 cleavage, we studied the consequence of HER2 cleavage and the function of the cleaved form of HER2. Results revealed that after cleavage, HER2 migrates to the nucleus wherein contributes to spliceosome and regulation of RNA processing. It seems that nuclear HER2 promotes tumorigenesis and metastasis of HER2-positive breast cancer cells, as a part of stemness regulation of the cells. We suggest not only by canonical function but also by its nuclear function, HER2 can promote breast cancer stemness. Some limited reports show that trastuzumab reduces the BCSC subpopulation of HER2-positive tumors. A retrospective analysis revealed that chemotherapy combined with trastuzumab reduced cancer relapse in 5 of 18 (27%) patients with BCSC-enriched HER2-positive tumors compared to the patients who received only chemotherapy ($P = 0.019$). This result indicates that trastuzumab therapy reduces metastasis by 2.4-fold in these patients. Trastuzumab also improved the OS rate of patients with BCSC-enriched HER2-positive breast cancer (by 2.9-fold; $P = 0.008$) [64]. Further, trastuzumab decreases the percentage of CD44+/CD24- phenotype, ALDH+ cells, and mammosphere counts in luminal mammary carcinoma cell but not in basal/claudin-low cells. Injection of HER2-

positive BCSCs to NOD/SCID mice generated bigger tumors in a shorter period compared with HER2-negative BCSCs mice group. Interestingly, treating the HER2-positive tumors with single-agent trastuzumab immediately after tumor inoculation (early-treatment), result in a significant decrease in tumor size when compared with administration after the establishment of tumors (late-treatment). These data indicate that trastuzumab may inhibit tumor growth by targeting cancer stem cells [65]. The combination of these results with our mass-spectrometry and GSEA results indicates that nuclear HER2 is a positive factor for breast cancer stemness and ENT. Therefore, we suggest that blocking nuclear HER2 by trastuzumab results in inhibition of breast cancer stemness and consequently, inhibition of metastasis. However, the investigation of this hypothesis is needed.

7.2. WHAT ABOUT PERTUZUMAB?!

As a fully humanized recombinant monoclonal antibody, pertuzumab represents a new class of agents that inhibit HER2 dimerization. Pertuzumab specifically interacts with the subdomain II of HER2 extracellular domain, sterically blocking a binding pocket necessary for receptor dimerization, thus blocking HER2 dimerization mediated by the HER2 dimerization domain [66]. Indeed, the same research showed that pertuzumab blocked heregulin-induced heterodimerization between HER2 and HER3 [66]. Inhibition of dimerization will lead to the blocking of HER2 activation and HER2-mediated downstream signaling [67]. This understanding is mostly based on important early research [68]. This research showed that pertuzumab blocks the association of HER2 and HER3 diminishes ligand-activated HER2 signaling including Erk activation and inhibits the growth of human breast cancer cell lines only in the presence of ligand (heregulin) [68]. This research was conducted with breast cancer cell lines that co-express both HER2 and HER3 in the context of heregulin stimulation. Subsequent brief research suggests the synergistic effect of trastuzumab and pertuzumab on breast cancer survival, but showed that pertuzumab alone is less effective in blocking Akt phosphorylation than trastuzumab and both antibodies have no effect on Erk phosphorylation in BT474 cells [69]. It was further reported that pertuzumab disrupts EGF-induced heterodimerization of HER2 and EGFR in ovarian cancer cells, expressing both EGFR and HER2. Pertuzumab also inhibits in vitro and in vivo growth of the same ovarian cancers [70]. Moreover, pertuzumab can abrogate

the inhibitory effect of HER2 on the degradation of HER3 [71]. A recent study showed that both trastuzumab and pertuzumab inhibit NRF2 function in ovarian cancers and the combination of the antibodies produces more potent effects than a single antibody alone [72]. In summary, while the data regarding the mode of action of pertuzumab is quite limited, the available data mostly support the role of pertuzumab in blocking the heterodimerization of HER2, which in turn blocks the activation of HER2- and HER3-mediated signal transduction pathways leading to cancer cell proliferation and survival.

In chapter 4 we showed that pertuzumab does not inhibit HER2 homodimerization and phosphorylation. Instead, pertuzumab induces phosphorylation of HER2 at Y1127, Y1139 and Y1196 phospho-sites independent of HER2 homodimerization. Moreover, pertuzumab did not block HER2 homodimer-induced cell proliferation. These data suggest that pertuzumab may exert its function by inhibition of HER2 heterodimers, rather than HER2 homodimers. Our data suggest that pertuzumab may suppress HER2 function activated through the non-canonical pathway(s) rather than its canonical downstream pathways (PI3K/Akt and MAPK). One of the candidate oncogenic mechanisms of HER2 independently of PI3K/Akt and MAPK is proteolytic cleavage of HER2 and production of C-terminal HER2 fragments which is able to translocate to the nucleus and act as transcription co-factor [73].

7.3. STEMNESS/EMT AS MECHANISM OF HER2 CLEAVAGE/SILENCING, AND TRASTUZUMAB ACTION/RESISTANCE

Many mechanisms have been suggested for the resistance of HER2-positive breast cancer to trastuzumab and pertuzumab. The resistance may arise due to the altered HER2 expression status of the cancer cells [74,75]. The resistance may also arise due to the alteration of HER2 molecule structures, such as proteolytic truncation of HER2 extracellular domain, which prevents the binding of trastuzumab to the truncated but constitutively activated HER2 [62,76]. Activation of other HER receptors such as EGFR, which compensate the lost HER2 signaling due to trastuzumab inhibition [77,78], or activation of HER2 through a mechanism that is not sensitive to trastuzumab [79,80]. Constitutive activation of downstream signaling pathways due to mutations is also a major mechanism for trastuzumab resistance. The most prominent case is the

constitutive activation of the PI3K/Akt/mTOR pathway due to the gain of function mutation of PI3K, and the loss of function of PTEN [18,81–86]. As HER2/HER3 heterodimer-mediated activation of PI3K/Akt/mTOR has been considered the most important signaling pathway driving the development of breast cancer, combined inhibition of both HER2 and PI3K/Akt/mTOR has been explored to overcome trastuzumab resistance [69,87]. Most research has demonstrated that additional inhibition of PI3K/Akt/mTOR could overcome trastuzumab resistance in HER2-positive breast cancers [87–90]. Some other mechanisms are also reported, including Fcγ receptor polymorphism [91,92], miRNAs [93,94], and Mucin 4 expression induced by TNFα [95].

In chapter 6 we showed that epithelial-like breast cancer cells are HER2-high, while mesenchymal-like breast cancers are HER2-low. This is due to different chromatin regulation of *ERBB2* gene in the two types of cells. The chromatin of *ERBB2* gene which is active in epithelial-like cells can turn to inactive after EMT of the cells. This regulation takes place by the global epigenetic reprogramming during EMT. On the other hand, HER2 overexpression can exert a positive effect on the activation of stemness pathways and thus, induction of EMT in HER2-positive breast cancer cells. This suggests a negative feedback regulation between HER2 and EMT. In Chater 5, we addressed one side of the negative feedback loop showing how EMT can suppress HER2 in HER2-positive breast cancer cells by epigenetic silencing. here we review the other side of the negative feedback loop indicating how HER2 can contribute to the induction of HER2-positive breast cancer cells.

Some evidence suggests that HER2 may be a novel regulator of BCSCs. It is found that ALDH+ BCSC-enriched tumors were associated with HER2 overexpression [96]. Korkaya et al. [97] showed that HER2 overexpression was positively correlated with an increased subpopulation of mammosphere-forming ALDH+ BCSC in breast cancer cell lines as well as xenograft tumors. Overexpression of HER2 is also correlated with the increased expression of stem cell markers Oct3/4, Notch1, Notch2, Jagged1, and Gli1 and with the activation of PI3K/Akt pathway. Moreover, targeting HER2 with trastuzumab led to significant decline in ALDH+ cell subpopulation [97]. Injecting HER2 overexpressing ALDH+ cells into the mammary fat pads of NOD/SCID mice generated tumors with 4-fold more BCSCs than the tumor that developed from ALDH+ cells with normal HER2 expression. In addition, ALDH and

HER2 were found co-expressed in invasive cells of luminal breast tumors [65]. Several clinical and preclinical studies demonstrated that HER2 blockade could reduce CD44+/CD24- and ALDH+ BCSC subpopulation inside the tumors [64,65,98,99]. A preclinical study revealed that HER2 expression in mammosphere-forming breast cancer with high levels of the stem cell markers Oct4 and Bmi1 is 2 to 7-fold higher than other groups of the cells from the same origin. This suggests that high HER2 expression is finally associated with BCSC properties. In parallel with HER2 expression in these cells, they also reported a higher level of Notch signaling in the cells. The depletion of Notch1 led to a significant decrease in HER2 expression in the mammosphere-forming cells. Treatment of mice bearing tumors raised from these cells with trastuzumab resulted in a significant regression in tumor growth. The cells derived from these tumors were unable to generate new tumors in the next in vitro tumor passage [100].

On the other hand, HER2 is also highly expressed in undifferentiated human embryonic stem cells (ESCs). The evidence that HER2 has pleiotropic effects on multiple cell types and organs suggests that HER2 crosstalks with a variety of signaling pathways that are essential in the maintenance and/or differentiation of ESCs [101,102]. Interestingly, HER2 has been suggested to be a positive factor in the development of the normal mammary gland and breast tumor by interaction with stemness signaling pathways. For example, HER2-mediated activation of PI3K/Akt signaling led to the enrichment of ALDH+ BCSCs in breast cancer cell culture and tumor xenografts through upregulating Wnt/β-catenin [97].

7.3.1. HER2 crosstalks with stemness/EMT pathways

HER2 crosstalks with TGF-β pathway

Accumulating evidence also indicates functional crosstalk between HER2 tyrosine kinase and the TGF-β signaling. In HER2-overexpressing breast cancer, this crosstalk results in increased cancer cell proliferation, survival, and invasion, accelerated cancer progression and metastasis in animal models, as well as resistance to chemotherapy and HER2-targeted therapy. HER2 crosstalks with TGF-β pathway by several ways including; (i) suppression of Smad-dependent transcriptional regulation and its downstream target genes by HER2; (ii) activation of HER2 downstream pathways (PI3K/Akt and MAPK pathways) by TGF-β in a Smad-independent manner and (iii) modification of the tumor microenvironment by secretory

mediators that are regulated by both downstream mediators of HER2 and TGF-β receptors [103]. Ueda et al. [104] showed that exogenous TGF-β ligand and ectopic expression of TGF-β type I receptor ALK5 activated TGF-β signaling and induced motility in HER2-overexpressing MCF10A cells. Moreover, inhibition of HER2, PI3K/Akt, MAPK, and Integrin β1 all abrogate TGF-β-induced motility in MCF10A/HER2 cells. In addition, trastuzumab blocks TGF-β-stimulated Rac1 activation in the HER2-overexpressing cells, which suggests that HER2 and TGF-β crosstalk with each other regulate tumor cell motility [104]. Overexpression of either TGF-β1 or ALK5 in HER2-positive breast cancer xenograft tumors reduces apoptosis but increases survival, angiogenesis, local invasion, metastasis [105–107]. The ability of HER2 to cooperate with TGF-β was correlated with higher levels of active Smad2, AKT, MAPK, and p38, as well as Vimentin [106,107]. As TGF-β can activate PI3K/Akt and MAPK pathways independent of Smad, it seems that HER2 and TGF-β utilize common paths to promote tumor cell invasion.

Moreover, the interaction between HER2 and TGF-β regulates DNA repair and the resistance to DNA-damaging chemotherapy in cancer cells. TGF-β/Smad signaling requires p53 to regulate MutS homolog 2 (MSH2), a key component of the DNA mismatch repair (MMR) system. Obviously, this function of TGF-β is impaired in the absence of p53, a frequent mutation in breast cancers. On the other hand, through the PI3K/Akt pathway, HER2 downregulates p53 signaling by inducing nuclear translocation of MDM2, an E3 ubiquitin ligase that targets p53 [108]. Yu et al. [109] reported that HER2 can also abrogate p53-mediated transcriptional regulation of MSH2 in p53-proficient breast cancer cells by increasing the expression level of miR-21 via TGF-β. The blockade of HER2-TGF-β crosstalk may enhance the efficiency of conventional therapies in breast cancer patients with HER2 overexpression [110]. In summary in HER2-overexpressing breast cancer, crosstalk between HER2 and TGF-β results in increased cancer cell proliferation, survival, and invasion, accelerated EMT and metastasis in animal models, resistance to chemotherapy and HER2-targeted therapy and perhaps upregulation of BCSCs.

HER2 crosstalks with Notch pathway

Notch signaling may support breast tumorigenesis by promoting cell growth and survival and inhibiting differentiation. Cooperation of Notch1 and HER2-mediated PI3K/Akt and MAPK

pathways has been demonstrated in the development of breast cancer [111,112]. It seems that Notch upregulates HER2 but HER2 downregulates Notch signaling. Osipo et al. [113] showed that HER2-positive breast cancer cells have low Notch signaling activity and inhibition of HER2 by trastuzumab increases nuclear localization of Notch1 and expression of Notch pathway target genes. The mechanism by which HER2 downregulates Notch signaling is not clear. A study shows that HER2/MAPK pathway suppresses the activity of the γ-secretase complex thus resulting in reduced levels of Notch1 cleavage and NICD1 expression [114]. Pandaya et al. [115] recently showed that HER2 may limit ubiquitinylation of Jagged1, by suppressing the expression of Mindbomb1 (Mib1), an E3 ligase, and by activating Protein kinase C-α (PKCα) that negatively regulates the interaction between Mib1 and Jagged1. Finally, since trastuzumab-resistant cells show a high level of Notch activity, inhibition of Notch pathway by GSIs overcame trastuzumab resistance in the cells [113] may be due to inhibition of HER2 cleavage. Moreover, trastuzumab treatment induces activation of Notch signaling thus a combined inhibition of HER2 and Notch signaling (trastuzumab plus GSIs) has a better outcomes in both trastuzumab-resistant and sensitive HER2-positive breast cancer tumors [113,115,116].

HER2 crosstalks with Wnt/β-catenin pathway

Some reports suggest a friend and foe relationship of HER2 with Wnt/β-catenin signaling pathway in breast tumor cells. Schroeder et al. [117] reported that HER2 makes a complex with both membranous and cytoplasmic β-catenin protein to induce phosphorylation of β-catenin in ductal breast cancer tissues but not in normal mammary tissues. Wang et al. [118] showed that the destabilization of HER2 receptor by HSP90 inhibitor geldanamycin disrupts the association of HER2 with β-catenin and suppresses Wnt/β-catenin signaling pathway. HSP90 is shown as the main chaperon icHER2 translocation into the nucleus. Geldanamycin-mediated inhibition of HER2 also attenuates HER2-positive breast cancer cell proliferation and motility via suppression of Wnt/β-catenin [118]. These suggest nuclear localization of HER2 is a positive factor for Wnt/β-catenin pathway activation. HER2 also influences Wnt/β-catenin signaling through its downstream regulators Akt and MAPK. These regulators can inhibit GSK3 that leads to translocation of β-catenin to the nucleus to promote transcription of β-catenin-TCF target genes [119]. Expression of nucleocytoplasmic β-catenin is significantly abundant in HER2 expressing node-positive breast carcinomas when compared with HER2-low node-positive tumors.

Nucleocytoplasmic β-catenin expression was also higher in transgene HER2-positive murine mammary ductal carcinoma in situ (DCIS) tumors [120].

Wnt3 ligand-mediated activation of the Wnt/β-catenin pathway induces EMT and reduces sensitivity to trastuzumab in HER2-positive breast cancer cells [121]. According to this report, approximately 95% (22 genes) of Wnt/β-catenin signaling genes were regulated in trastuzumab-resistant HER2-positive breast cancer. Of 22 genes, 11 genes were upregulated and 11 genes were downregulated, which suggests that Wnt/β-catenin/TCF axis may drive trastuzumab resistance via regulating EMT [121]. As mentioned above one of the characteristics of EMT is the upregulation of MMPs. Thus, it is likely that the resistance to trastuzumab is due to HER2 cleavage and loss of HER2 extracellular part by EMT-related MMPs. We here suggest that it is also due to the epigenetic silencing of *ERBB2* gene in the result of the activated Wnt/β-catenin pathway. These results suggest a negative feedback loop between HER2 and Wnt/β-catenin pathway through EMT. However, some other studies showed that upregulated expression and localization of β-catenin are more common in HER2-low breast cancer cells compared to HER2-positive cells [122,123].

HER2 crosstalks with JAK/STAT pathway

HER2 regulates STAT-mediated induction of breast cancer EMT and stemness. Olayioye et al. [124] reported that the heterodimerization between HER2 and HER4 leads to the activation of Src kinase, which stimulates JAK/STAT5 signaling pathway. HER2 dimerization induces phosphorylation, dimerization and nuclear translocation of STAT3 in an Src-dependent fashion [125]. HER2 exerts some other functions through JAK/STAT3 signaling. The HER2-mediated activity of Src further activates STAT3 that upregulates the transcriptional expression of $p21^{Cip1}$, a CDK inhibitor [126]. Silencing STAT3 in HER2-positive breast cancer cells reduces tumor invasion suggesting cooperation between HER2 and STAT3 in tumorigenesis [127]. In HER2-positive breast cancer, HER2 increases STAT3 activation and expression of STAT3 target genes including MMPs in an autocrine fashion by inducing IL6 secretion [128]. It is possible that the HER2/IL6/STAT3 signaling axis drives EMT by upregulating MMPs. Phosphorylated STAT3 in HER2-overexpressing breast cancer cell lines promotes the mesenchymal-like cell and EMT phenotype by upregulating Oct4, Sox2, CD44, and Slug. Activation of STAT3 in HER2 overexpression cells also increases the mammosphere-formation efficiency while inhibition of

HER2 and/or STAT3 abolishes BCSC and EMT phenotype. These data suggest the cooperation of HER2 with JAK/STAT signaling in the emergence of trastuzumab-resistant mesenchymal cells [129].

HER2 crosstalks with Hedgehog pathway

It is recently reported that high-level expression of Shh and Gli1 is correlated with HER2 expression. Inhibition of Hedgehog acyltransferase, a key enzyme for Shh synthesis, reduced HER2-positive breast cancer growth [130–132]. The data regarding the crosstalk between HER2 and Hh signaling in breast cancer are very limited. However, It is reported that HER2 downstream pathways PI3K/Akt and MAPK interact with Hh signaling pathway in regulating tumorigenesis and stemness in chronic lymphocytic leukemia [133], ovarian [134], pancreatic [135] and esophageal [136] cancers.

7.3.2. Inhibition of HER2 kinase activity prevents stemness/EMT-mediated trastuzumab resistance

Lapatinib is a small molecule dual inhibitor of the tyrosine kinase activity of HER2 and EGFR. It is used in combination with trastuzumab to treat advanced or metastatic HER2-positive breast cancers and is currently under phase III clinical evaluation [137,138]. Lapatinib inhibits HER2-positive breast cancer growth both in preclinical and clinical studies and improves the survival rate of patients. lapatinib in combination with trastuzumab showed complementary effects of HER2 blockade and improved response in patients with HER2-positive breast cancer [139]. In addition to dual targeting advantage of lapatinib, it can cross the blood-brain barrier, therefore being an effective treatment option for patients with brain metastases [140]. Lapatinib targets cancer stem cells as well. In a study, treatment with lapatinib inhibited mammosphere-formation of CD44+/CD24- BCSCs isolated from HER2-positive breast cancer cell lines [98]. It also decreased the percentage of ALDH+ cells by approximately 10–100-fold. Treatment with a combination of lapatinib and doxorubicin increased the cell death rate from 27.8% at single-agent treatment to 75.1% after combined treatment [98]. Li et al. [99] examined the post-chemotherapeutic CD44+/CD24- BCSC subpopulation in 31 breast cancer patients with HER2-negative tumor who received docetaxel or doxorubicin and cyclophosphamide for 12 weeks at standard doses (group 1) and in 21 patients with locally advanced HER2-positive breast cancer who received lapatinib for 6 weeks followed by docetaxel and trastuzumab for 12 weeks at

standard doses (group 2). Seven of 31 (23%) patients from the first group showed the pathological complete response for conventional chemotherapy, while the pathological complete response rate in the patients from the second group was 62%. The percentage of CD44+/CD24- cancer stem cell in bulk tumor of the first group was increased from a mean of 4.7% at baseline to 13.6% ($P < 0.001$) after 12 weeks of chemotherapy. In addition, an increased mammosphere-formation efficiency (MSFE) from 13.3% at baseline to 53.2% ($P < 0.001$) was observed in the MSFE assay of tumor biopsies from these patients after conventional chemotherapy. Interestingly, the baseline CD44+/CD24- cell BCSC population in the HER2-positive breast cancers was higher than in the HER2-negative tumors (10.0% versus 4.7%). This further approves that HER2 expression is a positive factor for BCSC self-renewal. More interestingly, the post-chemotherapeutic percentage of CD44+/CD24- cancer stem cell in HER2-positive tumors reduced from 10% at baseline to under 8% after 6 weeks of lapatinib therapy [99]. These results demonstrate the role of HER2 in breast tumor invasion and chemoresistance through upregulating EMT inside the tumor and the hypothesis that mesenchymal cells are major responsible to tumor resistance and post-therapeutic cancer relapse.

In a preclinical study, treatment with 2.5 μM lapatinib significantly inhibited mammosphere-forming ability of CD44+/CD24-/Lin- phenotype BCSCs more than 80% ($P < 0.03$) and reduced the subpopulation of the BCSCs from 16% to 3% ($P < 0.002$). In addition, treatment with 1 μM lapatinib dramatically reduced (by 5-fold less; $P < 0.04$) mammosphere-forming frequency of bulk cells in the second passage. In parallel with the inhibitory effect of lapatinib on BCSCs, lapatinib therapy also restrained the growth of xenograft breast tumors in mice. Twice daily oral gavage treatment by lapatinib for 14 days, resulted in a significant decline in tumor progression to 3.5-fold less ($P < 0.001$) in tumor size than vehicle-treated tumors. Moreover, tumors from lapatinib treated mice had 50% less ($P < 0.02$) BCSCs. These mice generated 6-fold less new tumors in secondary *in vivo* transplantation. Lapatinib-mediated reduction of BCSC subpopulation was correlated with the inhibition of phosphorylated HER2 inside the tumors by 40% [141]. Lapatinib also reduced mammosphere-formation and proliferation of BCSCs in both HER2-positive and HER2-normal DCIS cell lines as well as in DCIS cells derived from patient samples. Lapatinib also reduced the acini size of HER2-positive DCIS cells in 3D matrigel culture via suppressing cell proliferation [142]. A recent study reported that a lapatinib-resistant oral squamous cell carcinoma cell line SAS developed sensitivity to lapatinib during sphere-

formation through the activation of HER2/Akt/Cyclin D2 pathway [143]. Induced lapatinib resistance in HER2-positive breast cancer cells also shows an upregulated Snail and Vimentin and downregulated E-cadherin, therefor increasing intrinsic EMT capability [144].

7.4. REFERENCES

1. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, et al. The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature. 1984;312: 513–516.

2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989;244: 707–712.

3. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2: 127–137. doi:10.1038/35052073

4. Yan M, Schwaederle M, Arguello D, Millis SZ, Gatalica Z, Kurzrock R. HER2 expression status in diverse cancers: review of results from 37,992 patients. Cancer Metastasis Rev. 2015;34: 157–164. doi:10.1007/s10555-015-9552-6

5. Loibl S, Gianni L. HER2-positive breast cancer. Lancet (London, England). 2017;389: 2415–2429. doi:10.1016/S0140-6736(16)32417-5

6. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235: 177–182.

7. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/neu oncogene protein and prognosis in breast cancer. J Clin Oncol. 1989;7: 1120–1128. doi:10.1200/jco.1989.7.8.1120

8. Reibenwein J, Krainer M. Targeting signaling pathways in ovarian cancer. Expert Opin Ther Targets. 2008;12: 353–365. doi:10.1517/14728222.12.3.353

9. Serrano-Olvera A, Duenas-Gonzalez A, Gallardo-Rincon D, Candelaria M, De la Garza-Salazar J. Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer. Cancer Treat Rev. 2006;32: 180–190. doi:10.1016/j.ctrv.2006.01.001

10. Garrido-Castro AC, Felip E. HER2 driven non-small cell lung cancer (NSCLC): potential therapeutic approaches. Transl lung cancer Res. 2013;2: 122–127. doi:10.3978/j.issn.2218-6751.2013.02.02

11. Kim EK, Kim KA, Lee CY, Shim HS. The frequency and clinical impact of HER2 alterations in lung adenocarcinoma. PLoS One. 2017;12: e0171280. doi:10.1371/journal.pone.0171280

12. Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, et al. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. 2011;71: 1871–1882. doi:10.1158/0008-5472.CAN-10-1872

13. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell. 2009;15: 429–440. doi:10.1016/j.ccr.2009.03.020

14. Nami B, Maadi H, Wang Z. Mechanisms Underlying the Action and Synergism of Trastuzumab and Pertuzumab in Targeting HER2-Positive Breast Cancer. Cancers (Basel). 2018;10. doi:10.3390/cancers10100342

15. Maadi H, Nami B, Tong J, Li G, Wang Z. The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2. BMC Cancer. 2018;18: 238. doi:10.1186/s12885-018-4143-x

16. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A. 1992;89: 4285–4289.

17. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. 1990;50: 1550–1558.

18. Nagata Y, Lan K-H, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004;6: 117–127. doi:10.1016/j.ccr.2004.06.022

19. Zhang S, Huang W-C, Li P, Guo H, Poh S-B, Brady SW, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat Med. 2011;17: 461–469. doi:10.1038/nm.2309

20. Nahta R, Takahashi T, Ueno NT, Hung M-C, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res. 2004;64: 3981–3986. doi:10.1158/0008-5472.CAN-03-3900

21. Le X-F, Bedrosian I, Mao W, Murray M, Lu Z, Keyomarsi K, et al. Anti-HER2 antibody trastuzumab inhibits CDK2-mediated NPAT and histone H4 expression via the PI3K pathway. Cell Cycle. 2006;5: 1654–1661.

22. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, et al. Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A. 2011;108: 3761–3766. doi:10.1073/pnas.1014835108

23. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. Cancer Res. 2000;60: 3384–3388.

24. Clynes RA, Towers TL, Presta LG, Ravetch J V. Inhibitory Fc receptors modulate in vivo

cytotoxicity against tumor targets. Nat Med. 2000;6: 443–446. doi:10.1038/74704

25. Raab S, Steinbacher J, Schmiedel BJ, Kousis PC, Steinle A, Jung G, et al. Fc-optimized NKG2D-Fc constructs induce NK cell antibody-dependent cellular cytotoxicity against breast cancer cells independently of HER2/neu expression status. J Immunol. 2014;193: 4261–4272. doi:10.4049/jimmunol.1400872

26. Esteva FJ, Valero V, Booser D, Guerra LT, Murray JL, Pusztai L, et al. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20: 1800–1808. doi:10.1200/JCO.2002.07.058

27. Sato N, Sano M, Tabei T, Asaga T, Ando J, Fujii H, et al. Combination docetaxel and trastuzumab treatment for patients with HER-2-overexpressing metastatic breast cancer: a multicenter, phase-II study. Breast Cancer. 2006;13: 166–171.

28. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. J Clin Oncol. 2005;23: 4265–4274. doi:10.1200/JCO.2005.04.173

29. Gianni L, Pienkowski T, Im Y-H, Roman L, Tseng L-M, Liu M-C, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012;13: 25–32. doi:10.1016/S1470-2045(11)70336-9

30. Christianson TA, Doherty JK, Lin YJ, Ramsey EE, Holmes R, Keenan EJ, et al. NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. Cancer Res. 1998;58: 5123–5129.

31. Arribas J, Esselens C. ADAM17 as a therapeutic target in multiple diseases. Curr Pharm Des. 2009;15: 2319–2335. doi:10.2174/138161209788682398

32. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res. 2001;61: 4744–4749.

33. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. Cancer Res. 1999;59: 1196–1201.

34. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28: 803–814. doi:10.1038/onc.2008.432

35. Pedersen K, Angelini P-D, Laos S, Bach-Faig A, Cunningham MP, Ferrer-Ramon C, et al. A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. Mol Cell Biol. 2009;29: 3319–3331. doi:10.1128/MCB.01803-08

36. Yuan C-X, Lasut AL, Wynn R, Neff NT, Hollis GF, Ramaker ML, et al. Purification of Her-2 extracellular domain and identification of its cleavage site. Protein Expr Purif. 2003;29: 217–222.

37. Anido J, Scaltriti M, Bech Serra JJ, Santiago Josefat B, Todo FR, Baselga J, et al. Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. EMBO J. 2006;25: 3234–3244. doi:10.1038/sj.emboj.7601191

38. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, et al. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res. 2006;12: 424–431. doi:10.1158/1078-0432.CCR-05-1807

39. Molina MA, Saez R, Ramsey EE, Garcia-Barchino M-J, Rojo F, Evans AJ, et al. NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. Clin Cancer Res. 2002;8: 347–353.

40. Ruff M, Leyme A, Le Cann F, Bonnier D, Le Seyec J, Chesnel F, et al. The Disintegrin and Metalloprotease ADAM12 Is Associated with TGF-beta-Induced Epithelial to Mesenchymal Transition. PLoS One. 2015;10: e0139179. doi:10.1371/journal.pone.0139179

41. Duhachek-Muggy S, Qi Y, Wise R, Alyahya L, Li H, Hodge J, et al. Metalloprotease-disintegrin ADAM12 actively promotes the stem cell-like phenotype in claudin-low breast cancer. Mol Cancer. 2017;16: 32. doi:10.1186/s12943-017-0599-6

42. Pulyaeva H, Bueno J, Polette M, Birembaut P, Sato H, Seiki M, et al. MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. Clin Exp Metastasis. 1997;15: 111–120.

43. Tester AM, Ruangpanit N, Anderson RL, Thompson EW. MMP-9 secretion and MMP-2 activation distinguish invasive and metastatic sublines of a mouse mammary carcinoma system showing epithelial-mesenchymal transition traits. Clin Exp Metastasis. 2000;18: 553–560.

44. O'Shea C, McKie N, Buggy Y, Duggan C, Hill ADK, McDermott E, et al. Expression of ADAM-9 mRNA and protein in human breast cancer. Int J cancer. 2003;105: 754–761. doi:10.1002/ijc.11161

45. Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA. ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem. 2004;279: 51323–51330. doi:10.1074/jbc.M409565200

46. Lendeckel U, Kohl J, Arndt M, Carl-McGrath S, Donat H, Rocken C. Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. J Cancer Res Clin Oncol. 2005;131: 41–48. doi:10.1007/s00432-004-0619-y

47. Mitsui Y, Mochizuki S, Kodama T, Shimoda M, Ohtsuka T, Shiomi T, et al. ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res. 2006;66: 9913–9920. doi:10.1158/0008-5472.CAN-06-0377

48. McGowan PM, Ryan BM, Hill ADK, McDermott E, O'Higgins N, Duffy MJ. ADAM-17 expression in breast cancer correlates with variables of tumor progression. Clin Cancer Res. 2007;13: 2335–2343. doi:10.1158/1078-0432.CCR-06-2092

49. Mullooly M, McGowan PM, Kennedy SA, Madden SF, Crown J, O' Donovan N, et al. ADAM10: a new player in breast cancer progression? Br J Cancer. 2015;113: 945–951. doi:10.1038/bjc.2015.288

50. Köhrmann A, Kammerer U, Kapp M, Dietl J, Anacker J. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer. 2009. p. 188. doi:10.1186/1471-2407-9-188

51. Benson CS, Babu SD, Radhakrishna S, Selvamurugan N, Ravi Sankar B. Expression of matrix metalloproteinases in human breast cancer tissues. Dis Markers. 2013;34: 395–405. doi:10.3233/DMA-130986

52. Bartsch JE, Staren ED, Appert HE. Matrix metalloproteinase expression in breast cancer. J Surg Res. 2003;110: 383–392.

53. Merdad A, Karim S, Schulten H-J, Dallol A, Buhmeida A, Al-Thubaity F, et al. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res. 2014;34: 1355–1366.

54. Liu PCC, Liu X, Li Y, Covington M, Wynn R, Huber R, et al. Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells. Cancer Biol Ther. 2006;5: 657–664. doi:10.4161/cbt.5.6.2708

55. Feldinger K, Generali D, Kramer-Marek G, Gijsen M, Ng TB, Wong JH, et al. ADAM10 mediates trastuzumab resistance and is correlated with survival in HER2 positive breast cancer. Oncotarget. 2014;5: 6633–6646. doi:10.18632/oncotarget.1955

56. Gijsen M, King P, Perera T, Parker PJ, Harris AL, Larijani B, et al. HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. PLoS Biol. 2010;8: e1000563. doi:10.1371/journal.pbio.1000563

57. Caiazza F, McGowan PM, Mullooly M, Murray A, Synnott N, O'Donovan N, et al. Targeting ADAM-17 with an inhibitory monoclonal antibody has antitumour effects in triple-negative breast cancer cells. Br J Cancer. 2015;112: 1895–1903. doi:10.1038/bjc.2015.163

58. Duffy MJ, Mullooly M, O'Donovan N, Sukor S, Crown J, Pierce A, et al. The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? Clin Proteomics. 2011;8: 9. doi:10.1186/1559-0275-8-9

59. Burns DM, He C, Li Y, Scherle P, Liu X, Marando CA, et al. Conversion of an MMP-potent scaffold to an MMP-selective HER-2 sheddase inhibitor via scaffold hybridization and subtle P1' permutations. Bioorg Med Chem Lett. 2008;18: 560–564. doi:10.1016/j.bmcl.2007.11.086

60. Duchnowska R, Sperinde J, Chenna A, Haddad M, Paquet A, Lie Y, et al. Quantitative

measurements of tumoral p95HER2 protein expression in metastatic breast cancer patients treated with trastuzumab: independent validation of the p95HER2 clinical cutoff. Clin Cancer Res. 2014;20: 2805–2813. doi:10.1158/1078-0432.CCR-13-2782

61. Han S-W, Cha Y, Paquet A, Huang W, Weidler J, Lie Y, et al. Correlation of HER2, p95HER2 and HER3 expression and treatment outcome of lapatinib plus capecitabine in her2-positive metastatic breast cancer. PLoS One. 2012;7: e39943. doi:10.1371/journal.pone.0039943

62. Lipton A, Goodman L, Leitzel K, Cook J, Sperinde J, Haddad M, et al. HER3, p95HER2, and HER2 protein expression levels define multiple subtypes of HER2-positive metastatic breast cancer. Breast Cancer Res Treat. 2013;141: 43–53. doi:10.1007/s10549-013-2665-0

63. Sperinde J, Jin X, Banerjee J, Penuel E, Saha A, Diedrich G, et al. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin Cancer Res. 2010;16: 4226–4235. doi:10.1158/1078-0432.CCR-10-0410

64. Liu JC, Voisin V, Bader GD, Deng T, Pusztai L, Symmans WF, et al. Seventeen-gene signature from enriched Her2/Neu mammary tumor-initiating cells predicts clinical outcome for human HER2(+):ERα(−) breast cancer. Proceedings of the National Academy of Sciences of the United States of America. 2012. pp. 5832–5837. doi:10.1073/pnas.1201105109

65. Ithimakin S, Day KC, Malik F, Zen Q, Dawsey SJ, Bersano-Begey TF, et al. HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: Implications for efficacy of adjuvant trastuzumab. Cancer research. 2013. pp. 1635–1646. doi:10.1158/0008-5472.CAN-12-3349

66. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5: 317–328.

67. De Mattos-Arruda L, Cortes J. Use of pertuzumab for the treatment of HER2-positive metastatic breast cancer. Adv Ther. 2013;30: 645–658. doi:10.1007/s12325-013-0043-2

68. Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell. 2002;2: 127–137.

69. Nahta R, O'Regan RM. Evolving strategies for overcoming resistance to HER2-directed therapy: targeting the PI3K/Akt/mTOR pathway. Clin Breast Cancer. 2010;10 Suppl 3: S72-8. doi:10.3816/CBC.2010.s.015

70. Takai N, Jain A, Kawamata N, Popoviciu LM, Said JW, Whittaker S, et al. 2C4, a monoclonal antibody against HER2, disrupts the HER kinase signaling pathway and inhibits ovarian carcinoma cell growth. Cancer. 2005;104: 2701–2708. doi:10.1002/cncr.21533

71. Sak MM, Szymanska M, Bertelsen V, Hasmann M, Madshus IH, Stang E. Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. Carcinogenesis. 2013;34: 2031–2038. doi:10.1093/carcin/bgt173

72. Khalil HS, Langdon SP, Goltsov A, Soininen T, Harrison DJ, Bown J, et al. A novel mechanism of action of HER2 targeted immunotherapy is explained by inhibition of NRF2 function in ovarian cancer cells. Oncotarget. 2016;7: 75874–75901. doi:10.18632/oncotarget.12425

73. Nami B, Wang Z. HER2 in Breast Cancer Stemness: A Negative Feedback Loop towards Trastuzumab Resistance. Cancers (Basel). 2017;9. doi:10.3390/cancers9050040

74. Niikura N, Liu J, Hayashi N, Mittendorf EA, Gong Y, Palla SL, et al. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. J Clin Oncol. 2012;30: 593–599. doi:10.1200/JCO.2010.33.8889

75. Mittendorf EA, Wu Y, Scaltriti M, Meric-Bernstam F, Hunt KK, Dawood S, et al. Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. Clin Cancer Res. 2009;15: 7381–7388. doi:10.1158/1078-0432.CCR-09-1735

76. Tural D, Akar E, Mutlu H, Kilickap S. P95 HER2 fragments and breast cancer outcome. Expert Rev Anticancer Ther. 2014;14: 1089–1096. doi:10.1586/14737140.2014.929946

77. Yonemori K, Tsuta K, Shimizu C, Hatanaka Y, Hirakawa A, Ono M, et al. Immunohistochemical expression of HER1, HER3, and HER4 in HER2-positive breast cancer patients treated with trastuzumab-containing neoadjuvant chemotherapy. J Surg Oncol. 2010;101: 222–227. doi:10.1002/jso.21486

78. Gallardo A, Lerma E, Escuin D, Tibau A, Munoz J, Ojeda B, et al. Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. Br J Cancer. 2012;106: 1367–1373. doi:10.1038/bjc.2012.85

79. Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res. 2010;70: 299–308. doi:10.1158/0008-5472.CAN-09-1845

80. Joshi JP, Brown NE, Griner SE, Nahta R. Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. Biochem Pharmacol. 2011;82: 1090–1099. doi:10.1016/j.bcp.2011.07.082

81. Jensen JD, Knoop A, Laenkholm A V, Grauslund M, Jensen MB, Santoni-Rugiu E, et al. PIK3CA mutations, PTEN, and pHER2 expression and impact on outcome in HER2-positive early-stage breast cancer patients treated with adjuvant chemotherapy and trastuzumab. Ann Oncol Off J Eur Soc Med Oncol. 2012;23: 2034–2042. doi:10.1093/annonc/mdr546

82. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12: 395–402. doi:10.1016/j.ccr.2007.08.030

83. Kataoka Y, Mukohara T, Shimada H, Saijo N, Hirai M, Minami H. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. Ann Oncol Off J Eur Soc Med Oncol. 2010;21: 255–262. doi:10.1093/annonc/mdp304

84. Razis E, Bobos M, Kotoula V, Eleftheraki AG, Kalofonos HP, Pavlakis K, et al. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast Cancer Res Treat. 2011;128: 447–456. doi:10.1007/s10549-011-1572-5

85. Esteva FJ, Guo H, Zhang S, Santa-Maria C, Stone S, Lanchbury JS, et al. PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. Am J Pathol. 2010;177: 1647–1656. doi:10.2353/ajpath.2010.090885

86. Dave B, Migliaccio I, Gutierrez MC, Wu M-F, Chamness GC, Wong H, et al. Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. J Clin Oncol. 2011;29: 166–173. doi:10.1200/JCO.2009.27.7814

87. Garcia-Garcia C, Ibrahim YH, Serra V, Calvo MT, Guzman M, Grueso J, et al. Dual mTORC1/2 and HER2 blockade results in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy. Clin Cancer Res. 2012;18: 2603–2612. doi:10.1158/1078-0432.CCR-11-2750

88. O'Brien NA, McDonald K, Tong L, von Euw E, Kalous O, Conklin D, et al. Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT. Clin Cancer Res. 2014;20: 3507–3520. doi:10.1158/1078-0432.CCR-13-2769

89. Rexer BN, Chanthaphaychith S, Dahlman K, Arteaga CL. Direct inhibition of PI3K in combination with dual HER2 inhibitors is required for optimal antitumor activity in HER2+ breast cancer cells. Breast Cancer Res. 2014;16: R9. doi:10.1186/bcr3601

90. Elster N, Cremona M, Morgan C, Toomey S, Carr A, O'Grady A, et al. A preclinical evaluation of the PI3K alpha/delta dominant inhibitor BAY 80-6946 in HER2-positive breast cancer models with acquired resistance to the HER2-targeted therapies trastuzumab and lapatinib. Breast Cancer Res Treat. 2015;149: 373–383. doi:10.1007/s10549-014-3239-5

91. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J Clin Oncol. 2008;26: 1789–1796. doi:10.1200/JCO.2007.14.8957

92. Tamura K, Shimizu C, Hojo T, Akashi-Tanaka S, Kinoshita T, Yonemori K, et al. FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. Ann Oncol Off J Eur Soc Med Oncol. 2011;22: 1302–1307. doi:10.1093/annonc/mdq585

93. Gong C, Yao Y, Wang Y, Liu B, Wu W, Chen J, et al. Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. J Biol Chem. 2011;286: 19127–19137. doi:10.1074/jbc.M110.216887

94. Jung ST, Kelton W, Kang TH, Ng DTW, Andersen JT, Sandlie I, et al. Effective phagocytosis of

low Her2 tumor cell lines with engineered, aglycosylated IgG displaying high FcgammaRIIa affinity and selectivity. ACS Chem Biol. 2013;8: 368–375. doi:10.1021/cb300455f

95. Mercogliano MF, De Martino M, Venturutti L, Rivas MA, Proietti CJ, Inurrigarro G, et al. TNFalpha-Induced Mucin 4 Expression Elicits Trastuzumab Resistance in HER2-Positive Breast Cancer. Clin Cancer Res. 2017;23: 636–648. doi:10.1158/1078-0432.CCR-16-0970

96. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell stem cell. 2007. pp. 555–567. doi:10.1016/j.stem.2007.08.014

97. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene. 2008. pp. 6120–6130. doi:10.1038/onc.2008.207

98. Gong C, Yao H, Liu Q, Chen J, Shi J, Su F, et al. Markers of tumor-initiating cells predict chemoresistance in breast cancer. PLoS One. 2010;5: e15630. doi:10.1371/journal.pone.0015630

99. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu M-F, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst. 2008;100: 672–679. doi:10.1093/jnci/djn123

100. Magnifico A, Albano L, Campaner S, Delia D, Castiglioni F, Gasparini P, et al. Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab. Clin Cancer Res. 2009;15: 2010–2021. doi:10.1158/1078-0432.CCR-08-1327

101. Son M-Y, Kim J, Han H-W, Woo S-M, Cho YS, Kang Y-K, et al. Expression profiles of protein tyrosine kinase genes in human embryonic stem cells. Reproduction. 2008;136: 423–432. doi:10.1530/REP-08-0080

102. Wang L, Schulz TC, Sherrer ES, Dauphin DS, Shin S, Nelson AM, et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood. 2007;110: 4111–4119. doi:10.1182/blood-2007-03-082586

103. Wang SE. The Functional Crosstalk between HER2 Tyrosine Kinase and TGF-β Signaling in Breast Cancer Malignancy. Journal of Signal Transduction. 2011. doi:10.1155/2011/804236

104. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y, Arteaga CL. Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. J Biol Chem. 2004;279: 24505–24513. doi:10.1074/jbc.M400081200

105. Siegel PM, Shu W, Cardiff RD, Muller WJ, Massague J. Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. Proc Natl Acad Sci U S A. 2003;100: 8430–8435. doi:10.1073/pnas.0932636100

106. Muraoka RS, Koh Y, Roebuck LR, Sanders ME, Brantley-Sieders D, Gorska AE, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor

beta1. Mol Cell Biol. 2003;23: 8691–8703.

107. Seton-Rogers SE, Lu Y, Hines LM, Koundinya M, LaBaer J, Muthuswamy SK, et al. Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. Proc Natl Acad Sci U S A. 2004;101: 1257–1262. doi:10.1073/pnas.0308090100

108. Zheng L, Ren JQ, Li H, Kong ZL, Zhu HG. Downregulation of wild-type p53 protein by HER-2/neu mediated PI3K pathway activation in human breast cancer cells: its effect on cell proliferation and implication for therapy. Cell Res. 2004;14: 497–506. doi:10.1038/sj.cr.7290253

109. Yu Y, Wang Y, Ren X, Tsuyada A, Li A, Liu LJ, et al. Context-dependent bidirectional regulation of the MutS homolog 2 by transforming growth factor beta contributes to chemoresistance in breast cancer cells. Mol Cancer Res. 2010;8: 1633–1642. doi:10.1158/1541-7786.MCR-10-0362

110. Chow A, Arteaga CL, Wang SE. When Tumor Suppressor TGFβ Meets the HER2 (ERBB2) Oncogene. Journal of Mammary Gland Biology and Neoplasia. 2011. pp. 81–88. doi:10.1007/s10911-011-9206-4

111. Mittal S, Subramanyam D, Dey D, Kumar R V, Rangarajan A. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. Mol Cancer. 2009;8: 128. doi:10.1186/1476-4598-8-128

112. Majumder M, Xin X, Liu L, Tutunea-Fatan E, Rodriguez-Torres M, Vincent K, et al. COX-2 Induces Breast Cancer Stem Cells via EP4/PI3K/AKT/NOTCH/WNT Axis. Stem Cells. 2016;34: 2290–2305. doi:10.1002/stem.2426

113. Osipo C, Patel P, Rizzo P, Clementz AG, Hao L, Golde TE, et al. ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor. Oncogene. 2008;27: 5019–5032. doi:10.1038/onc.2008.149

114. Ju J, Yang W, Oh S, Nam K, Lee K, Noh D, et al. HER2 stabilizes survivin while concomitantly down-regulating survivin gene transcription by suppressing Notch cleavage. Biochem J. 2013;451: 123–134. doi:10.1042/BJ20121716

115. Pandya K, Wyatt D, Gallagher B, Shah D, Baker A, Bloodworth J, et al. PKCα Attenuates Jagged-1-mediated Notch Signaling in ErbB-2 positive Breast Cancer to Reverse Trastuzumab Resistance. Clinical cancer research : an official journal of the American Association for Cancer Research. 2016. pp. 175–186. doi:10.1158/1078-0432.CCR-15-0179

116. Pandya K, Meeke K, Clementz AG, Rogowski A, Roberts J, Miele L, et al. Targeting both Notch and ErbB-2 signalling pathways is required for prevention of ErbB-2-positive breast tumour recurrence. Br J Cancer. 2011;105: 796–806. doi:10.1038/bjc.2011.321

117. Schroeder JA, Adriance MC, McConnell EJ, Thompson MC, Pockaj B, Gendler SJ. ErbB-beta-catenin complexes are associated with human infiltrating ductal breast and murine mammary tumor virus (MMTV)-Wnt-1 and MMTV-c-Neu transgenic carcinomas. J Biol Chem. 2002;277: 22692–22698. doi:10.1074/jbc.M201975200

118. Wang K, Ma Q, Ren Y, He J, Zhang Y, Zhang Y, et al. Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/beta-catenin signaling in HER2 overexpressing human breast cancer cells. Oncol Rep. 2007;17: 89–96.

119. Yamaguchi H, Chang S-S, Hsu JL, Hung M-C. Signaling cross-talk in the resistance to HER family receptor targeted therapy. Oncogene. 2014;33: 1073–1081. doi:10.1038/onc.2013.74

120. Khalil S, Tan GA, Giri DD, Zhou XK, Howe LR. Activation status of Wnt/ss-catenin signaling in normal and neoplastic breast tissues: relationship to HER2/neu expression in human and mouse. PLoS One. 2012;7: e33421. doi:10.1371/journal.pone.0033421

121. Wu Y, Ginther C, Kim J, Mosher N, Chung S, Slamon D, et al. Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. Mol Cancer Res. 2012;10: 1597–1606. doi:10.1158/1541-7786.MCR-12-0155-T

122. Khramtsov AI, Khramtsova GF, Tretiakova M, Huo D, Olopade OI, Goss KH. Wnt/β-Catenin Pathway Activation Is Enriched in Basal-Like Breast Cancers and Predicts Poor Outcome. The American Journal of Pathology. 2010. pp. 2911–2920. doi:10.2353/ajpath.2010.091125

123. Dey N, Young B, Abramovitz M, Bouzyk M, Barwick B, De P, et al. Differential Activation of Wnt-β-Catenin Pathway in Triple Negative Breast Cancer Increases MMP7 in a PTEN Dependent Manner. Burchell JM, editor. PLoS ONE. San Francisco, USA; 2013. doi:10.1371/journal.pone.0077425

124. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J Biol Chem. 1999;274: 17209–17218.

125. DeArmond D, Brattain MG, Jessup JM, Kreisberg J, Malik S, Zhao S, et al. Autocrine-mediated ErbB-2 kinase activation of STAT3 is required for growth factor independence of pancreatic cancer cell lines. Oncogene. 2003;22: 7781–7795. doi:10.1038/sj.onc.1206966

126. Hawthorne VS, Huang W-C, Neal CL, Tseng L-M, Hung M-C, Yu D. ErbB2-mediated Src and STAT3 Activation Leads to Transcriptional Upregulation of p21(Cip1) and Chemoresistance in Breast Cancer Cells. Molecular cancer research : MCR. 2009. pp. 592–600. doi:10.1158/1541-7786.MCR-08-0316

127. Barbieri I, Quaglino E, Maritano D, Pannellini T, Riera L, Cavallo F, et al. Stat3 is required for anchorage-independent growth and metastasis but not for mammary tumor development downstream of the ErbB-2 oncogene. Mol Carcinog. 2010;49: 114–120. doi:10.1002/mc.20605

128. Hartman ZC, Yang X-Y, Glass O, Lei G, Osada T, Dave SS, et al. HER2 overexpression elicits a pro-inflammatory IL-6 autocrine signaling loop that is critical for tumorigenesis. Cancer research. 2011. pp. 4380–4391. doi:10.1158/0008-5472.CAN-11-0308

129. Chung SS, Giehl N, Wu Y, Vadgama J V. STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. Int J Oncol. 2014;44: 403–411. doi:10.3892/ijo.2013.2195

130. Benvenuto M, Masuelli L, De Smaele E, Fantini M, Mattera R, Cucchi D, et al. In vitro and in vivo inhibition of breast cancer cell growth by targeting the Hedgehog/GLI pathway with SMO (GDC-0449) or GLI (GANT-61) inhibitors. Oncotarget. 2016;7: 9250–9270. doi:10.18632/oncotarget.7062

131. Matevossian A, Resh MD. Hedgehog Acyltransferase as a target in estrogen receptor positive, HER2 amplified, and tamoxifen resistant breast cancer cells. Molecular Cancer. London; 2015. doi:10.1186/s12943-015-0345-x

132. Ramaswamy B, Lu Y, Teng K, Nuova G, Li X, Shapiro CL, et al. Hedgehog Signaling Is a Novel Therapeutic Target in Tamoxifen-Resistant Breast Cancer Aberrantly Activated by PI3K/AKT Pathway. 2012. doi:10.1158/0008-5472.CAN-12-1248

133. Kern D, Regl G, Hofbauer SW, Altenhofer P, Achatz G, Dlugosz A, et al. Hedgehog/GLI and PI3K signaling in the initiation and maintenance of chronic lymphocytic leukemia. Oncogene. 2015;34: 5341–5351. doi:10.1038/onc.2014.450

134. Ke Z, Caiping S, Qing Z, Xiaojing W. Sonic hedgehog-Gli1 signals promote epithelial-mesenchymal transition in ovarian cancer by mediating PI3K/AKT pathway. Med Oncol. 2015;32: 368. doi:10.1007/s12032-014-0368-y

135. Sharma N, Nanta R, Sharma J, Gunewardena S, Singh KP, Shankar S, et al. PI3K/AKT/mTOR and sonic hedgehog pathways cooperate together to inhibit human pancreatic cancer stem cell characteristics and tumor growth. Oncotarget. 2015;6: 32039–32060. doi:10.18632/oncotarget.5055

136. Kebenko M, Drenckhan A, Gros SJ, Jucker M, Grabinski N, Ewald F, et al. ErbB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma: identification of novel targets for concerted therapy concepts. Cell Signal. 2015;27: 373–381. doi:10.1016/j.cellsig.2014.11.022

137. Zhou Y, Li S, Hu YP, Wang J, Hauser J, Conway AN, et al. Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma GEO cells to apoptosis. Cancer Res. 2006;66: 404–411. doi:10.1158/0008-5472.CAN-05-2506

138. Pivot X, Manikhas A, Zurawski B, Chmielowska E, Karaszewska B, Allerton R, et al. CEREBEL (EGF111438): A Phase III, Randomized, Open-Label Study of Lapatinib Plus Capecitabine Versus Trastuzumab Plus Capecitabine in Patients With Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer. J Clin Oncol. 2015;33: 1564–1573. doi:10.1200/JCO.2014.57.1794

139. Chen Z-L, Shen Y-W, Li S-T, Li C-L, Zhang L-X, Yang J, et al. The efficiency and safety of trastuzumab and lapatinib added to neoadjuvant chemotherapy in Her2-positive breast cancer patients: a randomized meta-analysis. Onco Targets Ther. 2016;9: 3233–3247. doi:10.2147/OTT.S106055

140. Saleem A, Searle GE, Kenny LM, Huiban M, Kozlowski K, Waldman AD, et al. Lapatinib access into normal brain and brain metastases in patients with Her-2 overexpressing breast cancer. EJNMMI Research. Berlin/Heidelberg; 2015. doi:10.1186/s13550-015-0103-5

141. Lee CY-F, Lin Y, Bratman S V, Feng W, Kuo AH, Scheeren FA, et al. Neuregulin autocrine signaling promotes self-renewal of breast tumor-initiating cells by triggering HER2/HER3 activation. Cancer research. 2014. pp. 341–352. doi:10.1158/0008-5472.CAN-13-1055

142. Farnie G, Johnson RL, Williams KE, Clarke RB, Bundred NJ. Lapatinib inhibits stem/progenitor proliferation in preclinical in vitro models of ductal carcinoma in situ (DCIS). Cell Cycle. 2014;13: 418–425. doi:10.4161/cc.27201

143. Ohnishi Y, Yasui H, Kakudo K, Nozaki M. Lapatinib-resistant cancer cells possessing epithelial cancer stem cell properties develop sensitivity during sphere formation by activation of the ErbB/AKT/cyclin D2 pathway. Oncol Rep. 2016;36: 3058–3064. doi:10.3892/or.2016.5073

144. Liu J, Chen X, Mao Y, Qu Q, Shen K. Association of epithelial-mesenchymal transition with lapatinib resistance through multipe pathways activation in HER2-positive breast cancer. J Clin Oncol , 2014. 2014;32: (suppl; abstr e11579).

Chapter 8. Overall conclusion

We found that overexpression of HER2 in CHO cells did not increase the activation of PI3K/Akt and MAPK pathways, whoever majorly changed the gene expression profile and increased growth of the cells. This shows that HER2 increased the cell growth not by induction of its canonical downstream pathways. We conclude that HER2 exerts an oncogenic effect through a non-canonical pathway(s). Our results also showed neither trastuzumab not pertuzumab had major effect on HER2 receptor dimerization, phosphorylation and PI3K/Akt and MAPK pathways. However, trastuzumab but not pertuzumab abrogated the positive effect of HER2 overexpression on the cell cycle progress and survival and induced apoptotic pathway. Based on these findings, we conclude that anti-cancer effect of trastuzumab is not through blocking the canonical pathway of HER2 including HER2 receptor activation and HER2-mediated PI3K/Akt and MAPK pathways. Instead, it may inhibit a non-canonical pathway of HER2.

As discussed in the previous chapters, proteolytic cleavage of HER2, shedding, production and nuclear localization of ctHER2 are reported as poor prognosis marks and are associated with tumor growth, invasion, reoccurrence, and drug resistance of HER2-positive breast cancers. It was shown that ctHER2 can directly bind to mRNA, translocate to the nucleus and exert a transcription co-factor function. As discussed in chapter 5, we found that trastuzumab blocks proteolytic cleavage of HER2, production and nuclear localization of p85HER2 protein. We also found that After proteolytic cleavage from the IJM region, the p85HER2 translocates to the nucleus and contributes to spliceosome and regulates non-coding small RNA processing, mRNA alternative splicing and maybe regulation of gene expression as a transcription co-factor. Based on previous results we suggest that nuclear HER2 has a positive factor in the activation of breast cancer stemness. In conclusion, we introduce a novel anti-cancer mechanism of trastuzumab by abrogating the nuclear function of p85HER2 via blocking full-length HER2 cleavage and nuclear translocation of p85HER2 (Figure 8.1).

We discussed the hypothesize of a negative feedback between HER2 and stemness signaling pathways and EMT that leads to trastuzumab resistance. According to our hypothesize, HER2

receptor indirectly induces EMT of HER2-positive breast cancer cells through crossrtalks and activation of stemness signaling pathways. The stemness pathways increases the expression of proteinases and shesdase that are essential for cleavage of adhesion and cell-cell junction proteins and progression of EMT processes. The proteinases are also able to cleave HER2 receptors and shedding of extracellular and intracellular parts of HER2, the processes that leads to trastuzumab Resistance. In addition, during EMT, the cells undergo global epigenetic reprogramming including chromatin remodelling that causes inactivation of *ERBB2* gene chromatin and *ERBB2* gene silencing resulting in development of resistance to trastuzumab (Figure 8.1).

As overall conclusion, we found that HER2 overexpression can induce cell cycle progression and increase survival pathways via a non-RTK pathway, probably via the nuclear function of p85HER2. In this thesis we discovered a novel mechanism of action of trastuzumab in inhibition of HER2-positive breast cancer growth. Contrary to previous reports, trastuzumab does not inhibit HER2 receptor activation and its downstream RTK pathways. Instead, we found that trastuzumab abrogates the effect of HER2 overexpression of cell cycle progression and survival via inhibiting the non-canonical pathways of HER2. Trastuzumab binding to HER2 blocks HER2 cleavage and nuclear localization of p85HER2 (Figure 8.1). We also demonstrated that a negative feedback loop between HER2 and EMT explains the de novo resistance of HER2-positive breast cancer to trastuzumab and lapatinib (Figure 8.1). According to our finding EMT is the main mechanism of HER2 cleavage, p85HER2 proportion and downregulation of HER2 via two mechanism including cleavage/shedding and gene silencing, that all results in more growth, invasion and resistance of HER2-positive breast cancer to HER2 targeting therapy. We suggest targeting EMT and cancer stem cells in the general level and targeting HER2 cleavage and chromatin regulators involving in epigenetic reprogramming of mesenchymal-like cells in specific level, can be a promising approach to inhibit tumor growth and prevent drug resistance.

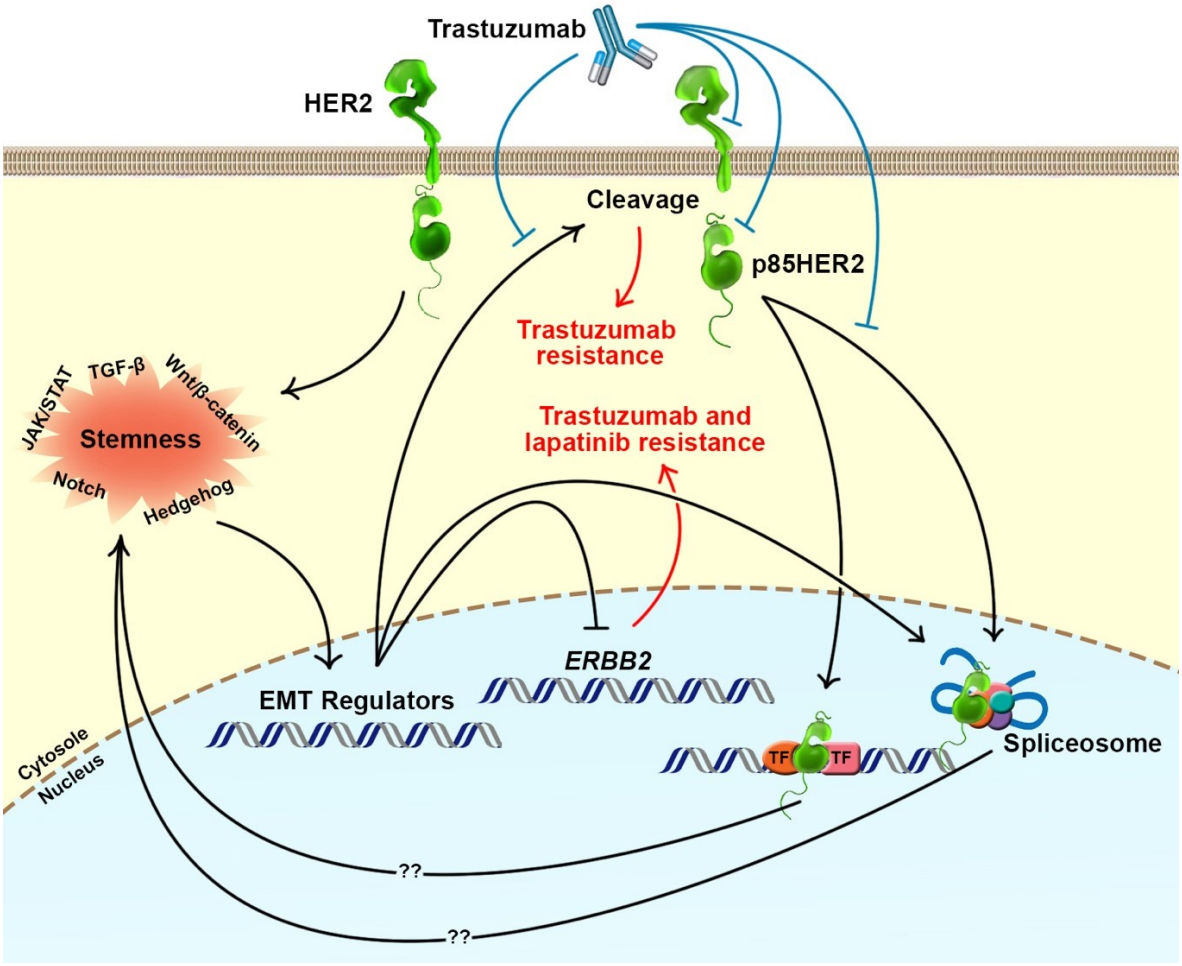

Figure 8.1. Schematic pathways illustrating the mechanism of action of trastuzumab in blocking p85HER2 pathway and the mechanims of trastuzumab resistance. In HER2-positive breast cancer cell, HER2 crosslinking with the stemness pathways (TGF-β, Wnt/β-catenin, JAK/STAT, Notch and Hedgehog pathways) which induce the expression of EMT regulators including stemness mesenchymal transcription factors, mesenchymal cytoskeleton, and proteinases. The proteinases cleave plasma membrane HER2 that causes shedding and profuction p85HER2of. HER2 shedding leads to trastuzumab resistance. The p85HER2 translocates into the nucleus and contributes in spliceosome, RNA processing and probably transcriptional regulation. The p85HER2-mediated regulation may upregulate the stemness pathways in a positive feedback fasion. The EMT-mediated transcription regulators also induces the expression of nuclear p85HER2 client proteins, and inhibits *ERBB2* chromatin activity that results in *ERBB2* gene silencing, abrogation of HER2 expression and development of resistance to trastuzumab and lapatinib. Trastuzumab binding to HER2 blocks

proteolytic cleavage of plasma membrane HER2, intracellular shedding and nuclear localization of p85HER2.

Chapter 9. Limitations and future directions

9.1. LIMITATIONS

We here used CHO cell lines stably overexpressing human HER2. CHO-K6 cells showed high HER2 dimerization and phosphorylation as expected due to HER2 overexpression, however, HER2 overexpression and receptor activation had no significant effect on the activation of downstream PI3K/Akt and MAPK pathways. The CHO cell line is intrinsically negative for the expression of HER receptors. Although this property provides CHO cell line a good cell model to investigate the dynamics of each HER receptor independently, it seems that the cells do not use HER-mediated RTK signaling. This is a limitation of this study. CHO cell line is non-human and non-cancerous cell line and has different molecular biology than that of human breast cancer cells. Our results showed that CHO-K6 cells are a good model for studying HER2 receptor homo-dimerization and phosphorylation, but it is not an appropriate model to assess HER2 kinase activity and HER2-mediated downstream RTK pathway. To overcome this limitation, we tested trastuzumab on HER2 receptor activation and downstream RTK pathways in breast cancer cell lines.

Another limitation is that the crosslinking assay for studying dimerization is not specific and sensitive enough to detect HER2 dimerization in the presence of trastuzumab. Since trastuzumab strongly binds to HER2, the crosslinker reagent links also trastuzumab to HER2 that increases the molecular weight of HER2 homodimers in blotting. In addition, crosslinking assay is not able to distinguish HER2 homodimers form heterodimers in using breast cancer cell lines. For example, SKBR3 cell line which is HER2-positive expresses also high levels of EGFR, HER3, and HER4. In addition to HER2, BT474 cell line expresses EGFR and HER3. In the case of using SKBR3 cells, the crosslinking assay can detect all HER2 homodimer, HER2-EGFR, HER2-HER3, and HER2-HER4 heterodimers in the same size even using a specific antibody against HER2 in blotting. However, this method is reliable to assess HER2 homodimer in CHO-K6 cells, since CHO-K6 cells express only HER2. To overcome this limitation, we studied HER2 heterodimerization by co-IP method.

We studied the effect of trastuzumab binding to HER2 on ADAM17-mediated HER2 cleavage from EJM region by computational simulation. Results showed that docking trastuzumab fab to domain IV of extracellular HER2 blocks appropriate docking and enzymatic attack of ADAM17 to the cleavage site at HER2 EJM. In this model, HER2 cleavage results in shedding of the extracellular part of HER2 and production of p95HER2. Due to several limitations, we could not study HER2 cleavage from the IJM region. One of the limitations is that there is no crystal structure of full-length HER2 at the PDB database. Thus, we were able to use the crystallography structures currently available from the databases. In addition, we were not able to study HER2 cleavage by experimental crystallography analysis due to financial restrictions. However, the current bioinformatic simulation gives general information on the mechanism of trastuzumab in blocking proteolytic cleavage of HER2 by metalloproteinases.

Finally, to investigate the function of nuclear p85HER2 we performed proteomic analysis to determine the client proteins of p85HER2. These experiments revealed the contribution of p85HER2 in spliceosome, RNA splicing and regulation of transcription. Although, this evidence gives reliable information about the nuclear function of p85HER2, further investigations focusing on each function of p85HER2 are needed as future directions.

9.2. FUTURE DIRECTIONS

Previous studies and we here found that HER2 cleavage is an important phenomenon for HER2 nuclear function. HER2 cleavage is shown to be associated with tumor growth, invasion and poor response to therapy. We found that trastuzumab blocks HER2 cleavage and inhibits cell growth. Investigating the molecular mechanism of HER2 cleavage and identifying which proteinases are responsible for HER2 cleavage is necessary and worth to be investigated in future studies. Knocking-down the proteinases by shRNA, and then monitoring the production of p85HER2 is a good approach to finding candidate proteinase. We recommend analyzing the conformation change of full-length HER2 in complex with trastuzumab by crystallography can provide valuable information to understand how trastuzumab blocks HER2 cleavage. This information will also allow us to design small molecules targeting HER2 and mimicking

trastuzumab function on blocking HER2 cleavage as an alternative therapy for trastuzumab to use for HER2-positive breast cancer therapy in combination with trastuzumab and lapatinib. Small chemical inhibitors have several advantages including lower side effects and ability to pass through the blood-brain barrier and reach metastatic tumors in the brain. Another advantage of small chemical inhibitors is lower production cost that makes affordable availability of the drug for patients. In addition to HER2 targeting small molecules, testing proteinase inhibitors in combination with trastuzumab and lapatinib would be a promising approach for treatment of HER2-positive breast cancer and to prevent development of trastuzumab resistance.

We also strongly suggest further studying the oncogenic function of HER2 through regulation of coding and non-coding RNA processing as well as transcription co-factor function of p85HER2 in breast cancers. Most importantly, the interaction of p85HER2 with the proteins detected by this study is to be validated by experimental ChIP analysis. Since nuclear p85HER2 is found to interact with transcription factors and contributed to RNA processing, the interaction of nuclear HER2 with target RNA and DNA is to be investigated by electrophoretic mobility shift assay (EMSA) and ChIP-seq (by using a HER2 antibody). Wang et al. [1] identified that HER2 forms a complex at a specific sequence of the *PTGS2* gene (coding for COX2) promoter (ATAAACTTCAAATTTCAGTA) and is able to stimulate its transcription. As future direction, studying transcriptional regulation function of p85HER2, identifying further p85HER2 client transcription factor and target genes is highly recommended. We here recommend to validate interaction of p85HER2 with the client protein by co-IP. In addition, we suggest that investigating RNA binding activity of p85HER2, the mode of interaction of p85HER2 with spliceosome, client RNAs, the role of p85HER2 in processing of non-coding RNA including shRNAs and microRNAs and oncogenic consequences of the regulations. These experiments will provide very precious pieces of evidence to understand the non-canonical oncogenic pathway(s) of HER2 in breast cancer. Furthermore, our results showed that phosphorylation of HER2 at Y1005 and Y1139 of HER2 seems to be specific for nuclear p85HER2. Investigation of HER2 regulatory phospho-sites and their roles in nuclear function of p85HER2 is also important to understand the non-canonical function of HER2.

It has been demonstrated that HER2 itself induces EMT of breast cancer cells via upregulating stemness pathways. This suggests a negative feedback loop between HER2 and

EMT that gives important clue about the trastuzumab-responsive HER2-positive breast cancer develops resistance to trastuzumab. Our results also suggest that *ERBB2* gene silencing by epigenetic regulation during EMT is an authentic mechanism of downregulated HER2 in the mesenchymal-like cells and the main mechanism of resistance of HER2-positive breast cancer cells to trastuzumab and lapatinib. As a future direction, we recommend investigating that mechanism of the negative feedback loop to understand how HER2 overexpression induces EMT, how EMT causes *ERBB2* gene silencing, and the whether p95HER2 induces stemness.

Finally, we strongly recommend validating all our results, and testing the suggested investigation as future directions, particularly testing small molecule inhibitors in rodent models of breast tumors.

9.3. REFERENCES

1. Wang S-C, Lien H-C, Xia W, Chen I-F, Lo H-W, Wang Z, et al. Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell. 2004;6: 251–261. doi:10.1016/j.ccr.2004.07.012

Chapter 10. Materials and methods

10.1. BUFFERS AND SOLUTIONS

1% agarose gel: 1 g agarose in 100 ml TAE buffer.

10 % running SDS-PAGE gel: 1.25 ml 1.5 M Tris-HCl (pH 8.8), 0.025 ml 20% SDS, 1.25 ml 40% acrylamide, 0.05 ml ammonium persulfate, 0.005 ml TEMED, 2.50 ml dH_2O.

4% stacking SDS-PAGE gel: 1.25 ml 1.5 M Tris-HCl (pH 8.8), 0.025 ml 20% SDS, 0.50 ml 40% acrylamide, 0.025 ml ammonium persulfate, 0.005 ml TEMED, 3.57 ml dH_2O.

7.5% running SDS-PAGE gel: 1.25 ml 1.5 M Tris-HCl (pH 8.8), 0.94 ml 40% acrylamide, 0.05 ml ammonium persulfate, 0.005 ml TEMED, 2.81 ml dH_2O.

Acetonitrile solution: 2% acetonitrile, 1% formic acid, 97% dH_2O.

Buffer EB (DNA elution buffer): 10 mM Tris-HCl, pH 8.0.

Buffer N3 (neutralization buffer for DNA binding): 4.2 M guanidine hydrochloride (GuHCl), 0.9 M potassium acetate, pH 4.8.

Buffer P1 (resuspension buffer): 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 (25ºC), 50-100 µg/ml RNase A.

Buffer P2 (lysis buffer): 200 mM NaOH, 1% SDS.

Burffer PE (wash buffer): (100ml for making 500ml 1x PE Buffer) 80 mM NaCl, 8 mM Tris-HCl, pH 7.5, ethanol added to 80% before use.

Coverslip blocking buffer: 1% BSA in TBS.

DNA loading buffer (6x): 30% (v:v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF.

HBS (2x): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0.

Laemmli Buffer (4x; Protein loading buffer), 4.4 ml 0.5 M Tris base (pH 6.8), 4.4 ml Glycerol, 2.2 ml 20% SDS, 0.5 ml 1% Bromophenol Blue , 0.5 ml 2-mercaptoethanol.

Nitrocellulose blocking Buffer: 5% BSA or 5% skimmed milk in TBS.

NP40 cell lysis solution: 20 mM HEPES-KOH (pH 7.9), 0.42 M KCl, 25% glycerol, 0.1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 0.2% NP40.

PBS: 8 g NaCl, 0.2 KCl, 1.44 g Na_2HPO4, 0.24 g KH_2PO4 in 1 L dH_2O, pH 7.4.

Protein running Buffer: 3 g Tris base, 14.4 g Glycine, 1 g SDS in 1 L dH_2O (pH 8.3).

Protein transfer Buffer: 3 g Tris base, 14.4 g Glycine, 200 ml Methanol in 1 L dH_2O.

SDS-PAGE gel coomassie destaining solution: 40% (v:v) methanol, 10% (v:v) glacial acetic acid, 50% (v:v) dH_2O.

SDS-PAGE gel protein coomassie staining solution: 0.1% (w:v) Coomassie Brilliant Blue, 50% (v:v) isopropanol, 10% (v:v) acetic acid, 40% (v:v) dH_2O.

SDS-PAGE gel protein fixation solution: 50% (v:v) methanol, 10% (v:v) glacial acetic acid, 40% (v:v) dH_2O.

SDS-PAGE gel protein storage solution: 5% (v:v) glacial acetic acid, 95% (v:v) dH_2O

TAE buffer (DNA running buffer): 40 mM Tris base, 20 mM glacial acetic acid, 1mM g EDTA.$2dH_2O$, pH 8.0.

TBS: 1.21 g Tris base, 8.77 g NaCl in 1 L dH_2O, pH 7.4.

TBST: 1.21 g Tris base, 8.77g NaCl, 1 ml Tween-20 in 1 L dH_2O, pH 7.4.

10.2. CHEMICAL INHIBITORS AND ANTIBODIES

Paclitaxel (cat# T7402), Vinorelbine tartrate (cat# V2264), TAPI-2 (cat# SML0420), and recombinant human EGF (cat# E5036), goat anti-mouse IgG-agarose antibody (cat# A6531), isotype human IgG (cat# 56834) other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). CP-724714 (cat# S1167) was purchased from Selleckchem (Houston, TX, USA). Pertuzumab (Perjeta®) and trastuzumab (Herceptin®) were purchased from Roche (Basel, Switzerland). Mouse monoclonal antibodies against HER2 (9G6; cat# sc-08), HER2 (A2; cat# sc-393712), EGFR (A-10; cat# sc-373746), HER3 (RTJ.2; cat# sc-415), Na^+/K^+-ATPase-α (H-3: cat# sc-48345), Vimentin (V9; cat# sc-6260), α-tubulin (B-7; cat# sc-5286), GFP (B-2; cat# sc-9996), rabbit polyclonal antibodies against HER2 (C18; cat# sc-284), pY1248 HER2 (cat# sc-12352-R), Akt1/2 (H-136; cat# sc-8312), Erk1 (k-23; cat# SC-94), pT202/Y204 Erk1/2 (cat# sc-16982), Lamin A (H-102; cat# 20680) and anti-histidine probe antibody (H-15; cat# sc-803) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Rabbit polyclonal anti-human pY1005, pY1112, pY1127, pY1139, and pY1196 and

pY1248 HER2 were purchased from FroggaBio (Toronto, ON, Canada). Rabbit monoclonal pS473 Akt (cat# 4060) and mouse monoclonal anti-human pY1221/1222 HER2 (6B12) (cat# 2243) were from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse RDye® 800CW and RDye® 650 secondary antibodies were purchased from LI-COR biotechnology Inc. (Lincoln, NE, USA). Isotype human IgG and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

10.3. CELL LINES AND CULTURE

COS7, MCF7, MDA-MB-231, SKBR3, BT474 cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). 293T and CHO (CHO-K1) cell lines were obtained as gifts from Dr. Luc Berthiaume (University of Alberta). CHO-K6 cells (stably overexpressing human HER2) [1], CHO-K13 (stably expressing human HER2) [1], and CHO-HER3 (stably expressing human HER3) [2] were obtained as gifts from Drs. Marry Hitt and Holger Buchholz (University of Alberta). CHO-EGFR (stably expressing human EGFR) was previously generated [3]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics including penicillin (100 U/ml) and streptomycin (100 μg/ml) and were maintained at 5% CO2 atmosphere at 37°C. The transgenic selection was maintained by adding G418 (200 μg/ml) for CHO-K6, CHO-K13, and CHO-EGFR, and hygromycin (200 μg/ml) for CHO-HER3 to the culture medium. The cells were starved overnight (16 hours) at DMEM containing 1% FBS before the treatments.

10.4. EMT INDUCTION

Induction of EMT in BT474 was done by culturing the cells for 15 days in the presence of 1x StemXVivo EMT inducing media supplement containing recombinant human Wnt-5a, recombinant human TGF-β1, anti-human E-cadherin, anti-human sFRP-1 and anti-human Dkk-1 antibodies (cat# CCM017; R&D Systems; Minneapolis, MN, USA). Successful EMT induction was studied by investigating cell morphology and Vimentin expression.

10.5. MTT PROLIFERATION ASSAY

A number of 10^4 cells were seeded as per 96-well plates and cultured in 100 µl DMEM medium containing 10% FBS. After 24 hours of culture, the medium was replaced with fresh DMEM containing 1% FBS and the cells were left to starve overnight. The starvation medium then replaced with medium supplemented with 10% FBS and containing treatment agent. The cells were incubated for 3 or 5 days. At the end of the treatment time, 10 µl of 12 mM MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the wells and then the plates were incubated at 37°C for 4 hours until the blue formazan crystals form. Afterward, the medium was removed and replaced with 50 µl DMSO. The plates then were incubated at 37°C for 10 minutes until the crystals dissolve and blue color develops. The color intensity was measured at 540 nm wavelength using a microplate reader. The absorbance values were normalized to those of blank wells.

10.6. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) ASSAY

ADCC was determined by using ADCC Reporter Bioassay kit (cat# G7015; Promega; Madison, WI, USA) according to Manufacturer's instruction. Cultured cells were plated at the density of 15,000 cells per well in complete culture medium overnight before bioassay. On the day of bioassay, the series of concentrations of trastuzumab was added to the cells, followed by the addition of ADCC Bioassay Effector Cells by 5:1 ratio. After 6 hours of induction at 37 °C, Bio-Glo™ Luciferase Assay Reagent was added and then luminescence signals were determined.

10.7. TOTAL PROTEIN EXTRACTION

The cells were washed by adding 10 cm pre-chilled PBS and 0.5 ml (for 10 cm plate culture) NP40 cell lysis solution added to cells and the cells were incubated on ice for 5 minutes. The cells were then collected into an ice-cold microcentrifuge tube and were incubated at 4°C with rocking for 15 minutes. Afterward, the lysate was centrifuged at 14,000 *g* for 15 minutes at 4°C,

and then the supernatant was collected into a new ice-cold microcentrifuge tube and stored at -80°C.

10.8. SUBCELLULAR PROTEIN FRACTIONATION

Proteins from membrane, cytosol and nuclear fractions were isolated by using Subcellular Protein Fractionation Kit (cat# NBP2-47659; Novus Biologicals, Centennial, CO, USA) following the manufacturer's instruction. The culture medium was discarded and the cells (5-10 × 10^6) were washed with PBS and trypsinized. The cells collected into a 15 ml tube pelleted by centrifugation at 200 *g* for 5 minutes. The cell pellet was washed with ice-cold PBS and pelleted again by centrifugation at 200 *g* for 5 minutes. The pellet resuspended in 400 µl of ice-cold Cytosol Extraction Buffer-Mix (CEB-Mix) containing 2 mM dithiothreitol (DTT) and protease inhibitor cocktail by gentle pipetting and incubated at 4°C with rocking for 20 minutes. The cells were then centrifuged at 700 *g* for 10 minutes. The supernatant was collected to a new tube as cytosolic protein fraction. The pellet was resuspended in 400 µl of ice-cold Membrane Extraction Buffer-A Mix (MEB-A Mix) containing 2 mM DTT and protease inhibitor cocktail by vigorous vortexing for 20 seconds. Then, 22 µl of Membrane Extraction Buffer-B was added to the mixture and mixed by vortexing. The mixture was incubated on ice for 1 minute and then centrifuged at 1000 *g* for 10 minutes. The supernatant was collected to a new tube as membrane protein fraction and stored at -80°C. The pellet was resuspended in 200 µl of ice-cold Nuclear Extraction Buffer Mix (NEB-Mix) containing 2 mM DTT and protease inhibitor cocktail by vortexing and was incubated at 4°C with rocking for 40 minutes. Afterward, the mixture was centrifuged at 14,000 *g* for 10 minutes and then the supernatant was collected into a new tube as nuclear protein fraction and stored at -80°C. All centrifugations were done at 4°C.

10.9. TOTAL RNA EXTRACTION

The cells were washed by PBS and 3 ml (for 10 cm plate culture) TRizol® (guanidinium thiocyanate; cat# 15596018; Thermo Fisher Scientific; Waltham, MA, USA) was added to the cells and then the cells were collected to a new microcentrifuge tube and were lysed by vigorous

vortexing. Then, 1.5 ml chloroform added to the mixture and mixed by inverting several times. The mixture was incubated for 5 minutes at room temperature and then centrifuged at 12,000 *g* for 10 minutes at 4°C. Afterward, the upper aqueous phase was collected into a new ice-cold tube and the RNA was precipitated by adding 1.5 ml (70% of aqueous phase volume) isopropanol and incubation at room temperature for 10 minutes. The mixture was then centrifuged at 12,000 *g* for 15 minutes at 4°C. The supernatant was discarded, and the RNA pellet resuspended in 1 ml of 70% ethanol. Then, the RNA mixture was centrifuged at 14,000 *g* for 5 minutes at 4°C. The supernatant was discarded, and the RNA pellet was left to air-dry. The RNA was eluted in 50 µl RNase-free DEPC dH_2O and stored at -80°C.

10.10. PLASMID EXTRACTION

Bacterial culture suspension was centrifuged at 8,000 *g* for 5 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended in 250 µl (for 5 ml culture) Buffer P1 by vigorous vortexing. Then, 250 µl Buffer P2 was added to the mixture and mixed by inverting the tube 5 times, and then 350 µl Buffer N3 was mixed in the mixture by inverting the tube 5 times. The bacterial mixture was centrifuged at 18,000 *g* for 10 minutes and then the supernatant was applied to QIAprep 2.0 spin column (QIAGEN, Hilden, Germany). The column was centrifuged at 8,000 *g* for 1 minute. The flow-through solution was discarded, and the column was washed by adding 0.7 ml Buffer PE and centrifugation at 8,000 *g* for 1 minute. Then, 50 µl buffer EB was added and the column and plasmid were collected into a new microcentrifuge tube by centrifugation at 8,000 *g* for 1 minute and stored at -20°C.

10.11. PLASMID CONSTRUCTION

Truncated HER2 cDNA ORFs were amplified by PCR using previously constructed EGFP-N3-ERBB2 as a PCR template. The sequences of the PCR overhang oligonucleotide primers are shown in Table 8.1. PCR reaction was done using ACCUZYME™ DNA Polymerase kit (cat# BIO-21052; Bioline; Memphis, TN, USA) following the manufacturer's instruction. The PCR cycling was as shown in Table 8.2. The PCR products were blunt-end cloned into pDrive cloning

vector by using QIAGEN PCR Cloning Kit (cat# 231124; Hilden, Germany). The ligation mixes were then transformed into competent E. coli by the heat shock method. The bacteria were cultured overnight on agar plates containing ampicillin. Twenty colonies were separately expanded by culturing in Lysogeny broth (LB) culture, and then the plasmid contents were extracted and run in 1% agar gel by DNA electrophoresis. The colonies possessing pDrive-ERBB2 plasmids were expanded and high volume pDrive-ERBB2 plasmid was extracted.

ERBB2 ORFs were sub-cloned from pDrive-ERBB2 plasmids into pcDNA3.1/*Myc*-HisA(-) and pEGFP-N3 vectors. For this, the empty target plasmids and pDrive-ERBB2 plasmids were digested by Xho1 (cat# R0146S) and HindIII (cat# R0104S) restriction enzymes to provide sticky ends. The digested pDrive-ERBB2 (containing sticky ended *ERBB2* ORFs) mixture were mixed with digested pcDNA3.1/*Myc*-HisA(-) mixtures or empty digested pEGFP-N3 plasmids and T4 DNA ligase (cat# M0202S) was added to the mixture. All enzymes were purchased from New England Biolab; (Ipswich, MA, USA). The ligation mixtures were transformed into competent E. coli and the bacteria were cultured overnight on an agar plate containing ampicillin (for pcDNA3.1/*Myc*-HisA(-) vector) or kanamycin (for pEGFP-N3 vector). After the culture, 20 colonies from each transformation were separately expanded by culture in LB overnight and the plasmids were extracted. Successful colonies were selected based on plasmid size after electrophoresis run into 1% agarose gel and were expanded by culture to extract a high volume of pcDNA-ERBB2 and pEGFP-ERBB2 plasmids. Successful cloning was confirmed by PCR amplification of *ERBB2* ORFs as described above.

Table 10.1. The sequence of used oligonucleotide primer for HER2 ORFs. RE: restriction enzyme. Restriction enzyme recognition sequences as underlined. Start codons are shown as lower case.

Oligo	Strand	Sequence	RE
TM+IC	Sense	3’-AAACTCGAGAAAatgGCCAGCCCTCTGACGTCCA-5’	XhoI
IC	Sense	3’-AAACTCGAGAACatgGGGATCCTCATCAAGCGACGG-5’	XhoI
IC-ΔNLS	Sense	3’-AAACTCGAGAAAatgGAAACGGAGCTGGTGGAGCC-5’	XhoI
R_HER2	Antisense	3’-CCCAAGCTTCACTGGCACGTCCAGACCC3’-5’	HindIII

Table 10.2. PCR cycling protocol.

Step	Temperature	Time	Cycle
Initial denaturation	97°C	1 minutes	1
Denaturation	97°C	15 seconds	35
Annealing	66°C	15 seconds	
Extension	72°C	3 minutes	
Complementary extension	72°C	10 minutes	1

10.12. PLASMID TRANSFECTION (LIPOFECTAMINE® 2000)

A number of 10^6 COS7 cells were plated 24 hours prior to transfection. Three hours prior transfection the culture medium was replaced with 0.5 ml (for 24-well culture) or 7.5 ml (for 10 cm plate culture) of antibiotic-free Opti-MEM medium. Amount of 0.5 µl (for 24-well culture) or 5 µl (for 10 cm plate culture) Lipofectamine® 2000 (cat# 11668027; Thermo Fisher Scientific) were mixed in 50 µl (for 24-well culture) or 750 µl (for 10 cm culture) of Opti-MEM medium and the lipofectamine solution were incubated in room temperature for 5 minutes. Approximately, 1 µg (for 24-well culture) or 10 µg (for 10 cm culture) plasmid DNA were mixed in 50 µl (for 24-well culture) or 750 µl (for 10 cm culture) of Opti-MEM medium. Then, the DNA solution was added to the lipofectamine solution by drop-wise. The transfection mixture was then mixed by pipetting and was left to incubation at room temperature for 30 minutes. Afterward, the transfection mixture was added to the cells by drop-wise and the cells left to incubation for 6 hours at culture condition. After incubation, the medium was replced with fresh DMEM medium supplemented with 10% FBS and antibiotics including penicillin (100 U/ml) and streptomycin (100 µg/ml), and the cells were left to culture for overnight.

10.13. PLASMID TRANSFECTION (CALCIUM PHOSPHATE METHOD)

A number of 10^6 cells were cultured 24 hours prior to transfection. Three hours prior to transfection, the culture medium was replaced with 350 µl (for 24-well culture) or 10 ml (for 10 cm plate culture) of fresh antibiotic-free culture medium. Plasmid solution was prepared by mixing 2 µg plasmid, 2.4 µl 2M $CaCl_2$ and dH_2O (up to 10 ul) for 24-well culture, or 20 µg

plasmid, 72 µl 2M $CaCl_2$ and dH_2O (up to 600 ul) for 10 cm plate culture. The plasmid solution was added to 10 µl (for 24-well culture) or 600 µl (for 10 cm plate culture) of 2x HSB by drop-wise and the solution was mixed by pipetting and incubated at room temperature for 30 minutes. The transfection mixture was then added to the cell cultures by drop-wise, and after mixing by gentle rocking, the cells were incubated for 24 hours at standard culture condition.

10.14. DNA AGAROSE GEL ELECTROPHORESIS

To make 100 ml of 1% agarose gel, 1 g agarose powder was dissolved and boiled in 100 ml of TAE buffer using a microwave oven. After the liquid gel chilled-down to about 50-60°C, 10 µl (1 µl per 10 ml gel) SYBR™ Safe DNA gel stain (cat# S33102; Thermo Fisher Scientific) was mixed to the liquid gel. The gel was then poured into a cassette and left for solidifying at room temperature. Afterward, the gel placed in the electrophoresis tank filled by TAE buffer. Approximately 1 µg DNA was mixed with 6x DNA loading buffer by 3:1 ration and then, the mixture loaded to well. The DNA sample was run into the gel at an electric current of 120 V electric potential for 1 hour. After running the gel was monitored under UV using a gel imaging system.

10.15. PROTEIN SDS-PAGE GEL ELECTROPHORESIS

The SDS-PAGE gel was placed in a vertical electrophoresis tank filled by protein running buffer. Protein samples were boiled in 4x Laemmli Buffer by 1:1 ratio for 5 minutes and then loaded into the SDS-PAGE gel wells. The protein samples were then run into the gels at an electric current of 120 V electric potential for 1 hour.

10.16. COOMASSIE BLUE PROTEIN STAINING

The protein samples were run into SDS-PAGE as described above. The gel was removed from the cassette and rinsed with dH_2O 3 times gently. The gel was then incubated in protein fixation solution for 1 hour at room temperature with gentle agitation. The gel was washed with

dH_2O 3 times and was then incubated in coomassie blue solution at room temperature for 1 hour with gentle agitation. Afterward, the gel was destained by overnight incubation in destaining solution at room temperature with gentle agitation until the background color is removed and the protein bands have appeared.

10.17. WESTERN BLOTTING

Protein samples were run into SDS-PAGE gel as described above. The protein was then transferred onto nitrocellulose membrane at 15 V electric potential for 90 minutes using a semi-dry protein transfer system (Bio-Rad Laboratories, Berkeley, CA, USA). The membrane was blocked by incubation in 5% BSA (RDye® antibodies) in TBS or 5% skimmed milk (HRP-conjugated antibodies) in TBS solution for 60 minutes and was then incubated overnight in 0.2 µg/ml primary antibody solution. After washing with TBST, the membranes were incubated in 25 ng/ml RDye® infrared fluorescent dye-conjugated secondary antibody solution or 0.2 µg/ml HRP-conjugated secondary antibody solution both in protein blocking buffer for 60 minutes. After washing with TBST, the BSA blocked membranes were blotted by RDye® dye for 1 hour and monitored using Odyssey® CLx imaging system (LI-COR biotechnology Inc., Lincoln, NE, USA). The membranes blocked by skimmed milk were blotted with an HRP-conjugated antibody for 1 hour and were treated with enhanced chemiluminescence (ECL) substrate solution (cat# 32106; Thermo Fisher Scientific) for 5 minutes. The membranes were then monitored by development signals on X-ray film using an X-ray developer machine. Query protein band's intensity was quantified and normalized to the intensity of relevant loading control protein bands.

10.18. IMMUNOFLUORESCENCE STAINING ASSAY

The indirect double-immunofluorescence staining was done as described previously [4]. Cell coverslips were washed with ice-cold PBS and the cells were fixed by incubation in -2ºC methanol for 5 minutes. The coverslips were then washed with TBS and blocked in coverslip blocking buffer (1% BSA solution in TBS) for 1 hour. After blocking, the coverslips (except those treated with isotype human IgG, pertuzumab and trastuzumab) were incubated in 2 µg/ml

primary antibody solution for 1 hour. The coverslips were washed and then incubated in 1 µg/ml FITC-conjugated and/or 1 µg/ml TRITC-conjugated secondary antibodies solutions for 1 hour in dark. Afterward, the coverslips were washed with TBS and were then incubated in 1 µg/ml DAPI solution for 5 minutes. The coverslips were mounted on microscope slides, sealed by nail polish and observed under a fluorescence microscope.

10.19. RECEPTOR DIMERIZATION ASSAY

HER2 receptor dimerization assay was done by using a cross-linking reagent as described previously [5]. The cells were cultured in standard culture condition for 24 hours and were then starved overnight in 1% FBS culture medium. After treatment, the cells were collected and suspended in 0.5 ml of 1 mM bissulfosuccinimidyl suberate (BS^3) solution in PBS. The cells were then incubated on ice for 2 hours for a cross-linking reaction. To terminate the reaction 5 µl of 10 mM Tris solution (pH 7.5) was added, and the mixture was incubated on ice for 15 minutes. After centrifugation at 200 *g* for 3 minutes, the cross-linking solution was removed, and the cells were lysed by adding NP40 lysis buffer and incubating on ice for 1 hour. The lysate was run on 4% polyacrylamide gel and HER2 monomer and homodimer were analyzed by western blotting as described above.

10.20. PROTEIN IMMUNOPRECIPITATION ASSAY

Eight µg (20 µl) anti-mouse goat IgG-agarose beads were transferred into a microcentrifuge tube and washed by dissolving in 1 ml ice-cold PBS and centrifugation at 14,000 *g* for 15 minutes at 4ºC. The supernatant was discarded, and the IgG-agarose bead pellet was stored at 4ºC. Two µg (10 µl) primary antibody (anti-HER2 mouse monoclonal IgG) was added to 400 µl of total protein lysate and the mixture was incubated overnight at 4ºC with rocking. The antibody-lysate mixture was then added to the IgG-agarose beads and the IP mixture was incubated overnight at 4ºC with rocking. Afterward, the IP mixture was centrifuged at 14,000 *g* for 15 minutes at 4ºC and the supernatant was collected. The IP pellet was washed by dissolving in 0.5 ml ice-cold lysis buffer followed by centrifugation at 14,000 *g* for 15 minutes at 4ºC. The

washing step was repeated 5 times by centrifugation at 14,000 *g* for 15 minutes at 4ºC. The IP pellet was resuspended in 50 µl lysis buffer and stored at -80ºC.

10.21. MICROARRAY cDNA PROFILING ASSAY

Total RNA from cells was isolated using TRIzol® reagent as described above. Samples for microarray hybridization were prepared according to the Affymetrix Manual Target Preparation for GeneChip® Whole Transcript Expression Arrays (Affymetrix Inc., Santa Clara, CA, USA). An amount of 100 ng of total RNA was used to make double-stranded cDNA following cRNA synthesis. After purification, 15 µg cRNA was subjected to reverse transcription into sense-strand (SS) cDNA when unnatural dUTP residues were incorporated. SScDNA was purified and 5.5 µg of each SScDNA was fragmented using uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE1) at the unnatural dUTP which breaks the DNA strand. Fragmented SScDNA were terminal labeled with biotin and 3 µg from each sample was hybridized to Affymetrix GeneChip® CHO Gene 2.0 ST array (format 100) for 16 hours at 45ºC with rotation at 60 rpm in an Affymetrix GeneChip® Hybridization Oven 645. After hybridization, the arrays were washed and stained in an Affymetrix GeneChip® Fluidics Station FS450. The fluorescent signals were measured with an Affymetrix GeneChip® Scanner 3000 7G. Row data was analyzed by Affymetrix Transcriptome Analysis Console (TAC) 3.0 software (Affymetrix Inc., Santa Clara, CA, USA) using GeneChip® CHO Gene 2.1 ST Array annotation library (GEO platform ID GPL24076). Normalized microarray cDNA expression values uploaded to GEO database by accession number GSE110189 for public use.

10.22. MASS SPECTROMETRY

Mass spectrometry experimental analysis of proteins in SDS-PAGE gel was done by Alberta Proteomics and Mass Spectrometry Facility at the University of Alberta. In-gel trypsin digestion was performed on the samples. Briefly, excised gel bands were destained twice in 100 mM ammonium bicarbonate/acetonitrile (50:50). The samples were then reduced (10 mM 2-Mercaptoethanol in 100 mM bicarbonate) and alkylated (55 mM iodoacetamide in 0.1 M

bicarbonate). After dehydration, enough trypsin (6 ng/µl, Promega Sequencing grade) was added to just cover the gel pieces and the digestion was allowed to proceed overnight (16 hours) at room temperature. Tryptic peptides were first extracted from the gel using acetonitrile solution followed by a second extraction using 50% of the first extraction buffer and 50% acetonitrile.

Fractions containing tryptic peptides were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Fisher Scientific) coupled to an LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 µm inner diameter (300Å, 5µm; New Objective Woburn, MA, USA). Peptide mixtures were injected onto the column at a flow rate of 3000 nl/min and resolved at 500 nl/min using a 60 minutes linear gradient from 0 to 35% v:v aqueous acetonitrile (ACN) in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 30,000 and m/z range of 400-2000. The fourteen most intense multiply charged ions were sequentially fragmented by using collision-induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 1 minute. Data were processed using Proteome Discoverer 1.4 (Thermo Fisher Scientific) and a human proteome database (UniProt) available at https://uniprot.org/ was searched using tandem mass spectrometry data analysis program SEQUEST. Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

10.23. BIOINFORMATIC ANALYSIS

10.23.1. Protein docking analysis

Crystal structures of the extracellular region of rat HER2 (PDB ID: 1N8Y) [6], extracellular domain of human HER2 complexed with Herceptin Fab (PDB ID: 1N8Z) [6] and catalytic domain of ADAM17 (PDB ID: 1BKC) [7] were obtained from Protein Data Bank (PDB)

available at https://rcsb.org. Docking of 1BKC to 1N8Y and to 1N8Z was done using ZDOCK online software [8] available at http://zdock.umassmed.edu.

10.23.2. Gene set enrichment analysis

Gene set enrichment analysis, gene ontology molecular and biological functional analysis and KEGG pathway analysis were done by using Enrichr web server [9] available at https://amp.pharm.mssm.edu/Enrichr/#.

10.23.3. cBioPortal cancer genomics database

RNA-seq and expression Z-scores of 1,904 breast cancer tumors studied by METABRIC study were obtained from and analyzed using cBioPortal cancer genomics database [10,11] available at http://cbioportal.org/index.do.

10.23.4. Gene Expression Omnibus (GEO)

All mRNA expression and methylation data from cell lines were obtained from GEO database available at https://www.ncbi.nlm.nih.gov/geo. GEO series and samples accession IDs of analyzed data are shown in Table 6.3.

10.23.5. ChIP-seq chromatin enrichment

ChIP-seq data were obtained from Cistrome Data Browser [12] available at http://cistrome.org/db/# and GEO database. ChIP-seq data were visualized by using WashU Epigenome Browser [13] available at "https://epigenomegateway.wustl.edu". Cistrome DB and GEO samples accession IDs of analyzed ChIP-seq data are shown in Table 6.4.

10.23.6. 3D and 4D genome data

IM-PET promoter-enhancer interaction data were obtained from 4Dgenome database [14] available at https://4dgenome.research.chop.edu. HiC data were obtained from and virtualized by 3DIV (3D-Genome Interaction Viewer) database [15] available at http://kobic.kr/3div.

10.23.7. Statistical analysis and data visualization

GEO array expression data were analyzed by Affymetrix Transcriptome Analysis Console (TAC) 3.0 software (Affymetrix Inc., Santa Clara, CA, USA). Heatmap and circus plots created by Heatmapper online tool [16] and Circa software respectively. Digitally imaged data were quantified using ImageJ software. Word could diagrams were generated using https://wordart.com web tool. All figure layouts were prepared using Adobe Photoshop CS6 (San Jose, CA, USA). Data were statistically analyzed by two-tailed student's t-test and analysis of variance (ANOVA) using Prism v.6 software (GraphPad Software, La Jolla, CA, USA). Data were presented as mean and SD. $P < 0.050$ was considered as statistically significant.

Table 10.3. GEO series and samples accession IDs of expression and methylation arrays data analyzed in this thesis.

GEO series	GEO Samples	Data type	Reference
GSE110189	GSM2981936	Untreated CHO-K1 cells	[17]
	GSM2981937	CHO-K1 cells treated with trastuzumab+pertuzumab	
	GSM2981938	Untreated CHO-K6	
	GSM2981939	CHO-K6 treated with trastuzumab	
	GSM2981940	CHO-K6 treated with pertuzumab	
	GSM2981941	CHO-K6 treated with trastuzumab+ pertuzumab	
GSE50811	GSM1229992, GSM1229993, GSM1229994, GSM1230001, GSM1230002, GSM1230003, GSM1230010, GSM1230011, GSM1230012, GSM1230019, GSM1230020, GSM1230021, GSM1230028, GSM1230029, GSM1230030, GSM1230037, GSM1230038, GSM1230045, GSM1230046, GSM1230047, GSM1230054, GSM1230055, GSM1230056, GSM1230063, GSM1230064, GSM1230065, GSM1230072, GSM1230073, GSM1230074, GSM1230081, GSM1230082, GSM1230083, GSM1230090, GSM1230091, GSM1230092, GSM1230099, GSM1230100, GSM1230101, GSM1230108, GSM1230109, GSM12301, GSM1230117, GSM1230118, GSM123011910, GSM1230126, GSM1230127, GSM1230134,	Expression profiling of breast cancer cell lines by array	[18]

	GSM1230135, GSM1230136, GSM1230143, GSM1230144, GSM1230145, GSM1230152, GSM1230153, GSM1230154, GSM1230161, GSM1230162, GSM1230163, GSM1230170, GSM1230171, GSM1230172, GSM1230179, GSM1230180, GSM1230181, GSM1230188, GSM1230189, GSM1230190, GSM1230197, GSM1230198, GSM1230199, GSM1230206, GSM1230207, GSM1230208, GSM1230215, GSM1230216, GSM1230217, GSM1230224, GSM1230225, GSM1230226		
GSE44838	GSM1092241, GSM1092242, GSM1092251, GSM1092253, GSM1092255, GSM1092259, GSM1092260, GSM1092261, GSM1092263, GSM1092264,	Expression profiling of breast cancer cell lines by array	[19]
	GSM1092267, GSM1092268, GSM1092277, GSM1092279, GSM1092281, GSM1092285, GSM1092286, GSM1092287, GSM1092289, GSM1092290	Methylation profiling of breast cancer cell lines by genome tiling array	
GSE16179	GSM799168, GSM799169, GSM799170	Lapatinib-sensitive BT474 cell lines	[20]
	GSM799174, GSM799175, GSM799176	Lapatinib-resistant BT474 cell lines	
GSE17708	GSM442026, GSM442027, GSM442028	Untreated A549	[21]
	GSM442029, GSM442030, GSM442031	A549 treated with TGFβ1 for 0.5 hour	
	GSM442032, GSM442033, GSM442034	A549 treated with TGFβ1 for 1 hour	
	GSM442035, GSM442036	A549 treated with TGFβ1 for 2 hours	
	GSM442037, GSM442038, GSM442039	A549 treated with TGFβ1 for 4 hours	
	GSM442040, GSM442041, GSM442042	A549 treated with TGFβ1 for 8 hours	
	GSM442043, GSM442044, GSM442045	A549 treated with TGFβ1 for 16 hours	
	GSM442046, GSM442047, GSM442048	A549 treated with TGFβ1 for 24 hours	

	GSM442049, GSM442050, GSM442051	A549 treated with TGFβ1 for 72 hours

Table 10.4. Cistrome DB and GEO accession IDs of ChIP-seq data analyzed in this thesis.

Factor	X-seq	Cell line	Cistrome DB ID	GEO sample ID	Reference
Tn5	ATAC-seq	MCF7	79676	GSM2714245	[22]
Tn5	ATAC-seq	MDA-MB-231	65718	GSM2439559	[23]
DNase	DNase-seq	MCF7	40995	GSM1008581	[24]
DNase	DNase-seq	MDA-MB-231	78267	GSM2242137	[25]
FOXA1	ChIP-seq	MDA-MB-453	36842	GSM1099031	[26]
FOXA1	ChIP-seq	MCF7	2320	GSM659787	[27]
E2F1	ChIP-seq	MCF7	2281	GSM699986	[28]
E2F1	ChIP-seq	MDA-MB-231	75043	GSM2501567	[25]
H2BK120ub	ChIP-seq	HCC-1954	85603	GSM2258929	[29]
H2BK120ub	ChIP-seq	SKBR3	82286	GSM2258950	[29]
H2BK120ub	ChIP-seq	AU565	85597	GSM2258923	[29]
H2BK120ub	ChIP-seq	MDA-MB-361	81479	GSM2258935	[29]
H2BK120ub	ChIP-seq	MCF7	86405	GSM2258947	[29]
H2BK120ub	ChIP-seq	MDA-MB-231	81472	GSM2258932	[29]
H2BK120ub	ChIP-seq	MDA-MB-468	86411	GSM2258941	[29]
H3K39me3	ChIP-seq	HCC-1954	88403	GSM2258835	[29]
H3K39me3	ChIP-seq	SKBR3	87169	GSM2258798	[29]
H3K39me3	ChIP-seq	AU565	88152	GSM2258816	[29]
H3K39me3	ChIP-seq	MDA-MB-361	81935	GSM2258762	[29]
H3K39me3	ChIP-seq	MCF7	74303	GSM2483406	[29]

H3K39me3	ChIP-seq	MDA-MB-231	2408	GSM425485	[30]
H3K39me3	ChIP-seq	MDA-MB-468	82822	GSM2258889	[29]
K3K79me2	ChIP-seq	HCC-1954	85976	GSM2258841	[29]
K3K79me2	ChIP-seq	SKBR3	84023	GSM2258805	[29]
K3K79me2	ChIP-seq	AU565	84313	GSM2258823	[29]
K3K79me2	ChIP-seq	MDA-MB-361	81942	GSM2258769	[29]
K3K79me2	ChIP-seq	MCF7	86291	GSM2258733	[29]
K3K79me2	ChIP-seq	MDA-MB-231	88654	GSM2258858	[29]
K3K79me2	ChIP-seq	MDA-MB-468	87026	GSM2258894	[29]
H3K4me1	ChIP-seq	HCC-1954	88402	GSM2258836	[29]
H3K4me1	ChIP-seq	SKBR3	84019	GSM2258801	[29]
H3K4me1	ChIP-seq	AU565	88154	GSM2258818	[29]
H3K4me1	ChIP-seq	MDA-MB-361	81941	GSM2258764	[29]
H3K4me1	ChIP-seq	MCF7	82432	GSM2258728	[29]
H3K4me1	ChIP-seq	MDA-MB-231	68383	GSM2036932	[29]
H3K4me1	ChIP-seq	MDA-MB-468	87022	GSM2258890	[29]
H3K4me3	ChIP-seq	HCC-1954	8399	GSM721134	[31]
H3K4me3	ChIP-seq	SKBR3	82540	GSM2258803	[29]
H3K4me3	ChIP-seq	AU565	84311	GSM2258821	[29]
H3K4me3	ChIP-seq	MDA-MB-361	81939	GSM2258766	[29]
H3K4me3	ChIP-seq	MCF7	86289	GSM2258731	[29]
H3K4me3	ChIP-seq	MDA-MB-231	68122	GSM1700393	[32]
H3K4me3	ChIP-seq	MDA-MB-468	54526	GSM1429760	[33]
H3K9ac	ChIP-seq	HCC-1954	84601	GSM2258842	[29]
H3K9ac	ChIP-seq	SKBR3	84020	GSM2258806	[29]

H3K9ac	ChIP-seq	AU565	84254	GSM2258824	[29]
H3K9ac	ChIP-seq	MCF7	86292	GSM2258734	[29]
H3K9ac	ChIP-seq	MDA-MB-231	58110	GSM1619768	[34]
H3K9ac	ChIP-seq	MDA-MB-468	87027	GSM2258897	[29]
H3K27ac	ChIP-seq	HCC-1954	88400	GSM2258830	[29]
H3K27ac	ChIP-seq	SKBR3	87173	GSM2258794	[29]
H3K27ac	ChIP-seq	MDA-MB-361	85478	GSM2258758	[29]
H3K27ac	ChIP-seq	MCF7	82438	GSM2258722	[29]
H3K27ac	ChIP-seq	MDA-MB-231	61914	GSM1855992	[35]
H3K27ac	ChIP-seq	MDA-MB-468	82828	GSM2258884	[29]
H4K8ac	ChIP-seq	HCC-1954	81475	GSM2258931	[29]
H4K8ac	ChIP-seq	SKBR3	82288	GSM2258952	[29]
H4K8ac	ChIP-seq	AU565	85599	GSM2258925	[29]
H4K8ac	ChIP-seq	MDA-MB-361	81477	GSM2258937	[29]
H4K8ac	ChIP-seq	MCF7	86413	GSM2258949	[29]
H4K8ac	ChIP-seq	MDA-MB-231	81478	GSM2258934	[29]
H4K8ac	ChIP-seq	MDA-MB-468	86409	GSM2258943	[29]
H3K9me3	ChIP-seq	HCC-1954	84600	GSM2258845	[29]
H3K9me3	ChIP-seq	SKBR3	84024	GSM2258808	[29]
H3K9me3	ChIP-seq	AU565	84308	GSM2258826	[29]
H3K9me3	ChIP-seq	MDA-MB-361	84978	GSM2258772	[29]
H3K9me3	ChIP-seq	MCF7	86294	GSM2258736	[29]
H3K9me2	ChIP-seq	MDA-MB-231	58111	GSM1619769	[34]
H3K9me3	ChIP-seq	MDA-MB-468	87019	GSM2258898	[29]
H3K27me3	ChIP-seq	HCC-1954	88398	GSM2258832	[29]

H3K27me3	ChIP-seq	SKBR3	87171	GSM2258796	[29]
H3K27me3	ChIP-seq	AU565	88150	GSM2258814	[29]
H3K27me3	ChIP-seq	MDA-MB-361	81936	GSM2258761	[29]
H3K27me3	ChIP-seq	MCF7	82436	GSM2258724	[29]
H3K27me3	ChIP-seq	MDA-MB-231	88660	GSM2258850	[29]
H3K27me3	ChIP-seq	MDA-MB-468	82827	GSM2258887	[29]

10.24. REFERENCES

1. Munch RC, Muhlebach MD, Schaser T, Kneissl S, Jost C, Pluckthun A, et al. DARPins: an efficient targeting domain for lentiviral vectors. Mol Ther. 2011;19: 686–693. doi:10.1038/mt.2010.298

2. Mendrola JM, Berger MB, King MC, Lemmon MA. The single transmembrane domains of ErbB receptors self-associate in cell membranes. J Biol Chem. 2002;277: 4704–4712. doi:10.1074/jbc.M108681200

3. Wang Q, Zhu F, Wang Z. Identification of EGF receptor C-terminal sequences 1005-1017 and di-leucine motif 1010LL1011 as essential in EGF receptor endocytosis. Exp Cell Res. 2007;313: 3349–3363. doi:10.1016/j.yexcr.2007.06.014

4. Nami B, Wang Z. Application of Immunofluorescence Staining to Study ErbB Family of Receptor Tyrosine Kinases. Methods Mol Biol. 2017;1652: 109–116. doi:10.1007/978-1-4939-7219-7_7

5. Maadi H, Nami B, Wang Z. Dimerization Assessment of Epithelial Growth Factor Family of Receptor Tyrosine Kinases by Using Cross-Linking Reagent. Methods Mol Biol. 2017;1652: 101–108. doi:10.1007/978-1-4939-7219-7_6

6. Cho H-S, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DWJ, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421: 756–760. doi:10.1038/nature01392

7. Maskos K, Fernandez-Catalan C, Huber R, Bourenkov GP, Bartunik H, Ellestad GA, et al. Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. Proc Natl Acad Sci U S A. 1998;95: 3408–3412. doi:10.1073/pnas.95.7.3408

8. Pierce BG, Wiehe K, Hwang H, Kim B-H, Vreven T, Weng Z. ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. Bioinformatics. 2014;30: 1771–1773. doi:10.1093/bioinformatics/btu097

9. Kuleshov M V, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44: W90-7. doi:10.1093/nar/gkw377

10. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013;6: pl1. doi:10.1126/scisignal.2004088

11. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2: 401–404. doi:10.1158/2159-8290.CD-12-0095

12. Mei S, Qin Q, Wu Q, Sun H, Zheng R, Zang C, et al. Cistrome Data Browser: a data portal for ChIP-Seq and chromatin accessibility data in human and mouse. Nucleic Acids Research. 2017. pp. D658-62. doi:10.1093/nar/gkw983

13. Zhou X, Lowdon RF, Li D, Lawson HA, Madden PAF, Costello JF, et al. Exploring long-range genome interactions using the WashU Epigenome Browser. Nature methods. United States; 2013. pp. 375–376. doi:10.1038/nmeth.2440

14. Teng L, He B, Wang J, Tan K. 4DGenome: a comprehensive database of chromatin interactions. Bioinformatics. 2015;31: 2560–2564. doi:10.1093/bioinformatics/btv158

15. Yang D, Jang I, Choi J, Kim M-S, Lee AJ, Kim H, et al. 3DIV: A 3D-genome Interaction Viewer and database. Nucleic Acids Res. 2018;46: D52–D57. doi:10.1093/nar/gkx1017

16. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016;44: W147-53. doi:10.1093/nar/gkw419

17. Nami B, Maadi H, Wang Z. The Effects of Pertuzumab and Its Combination with Trastuzumab on HER2 Homodimerization and Phosphorylation. Cancers (Basel). 2019;11. doi:10.3390/cancers11030375

18. Dezso Z, Oestreicher J, Weaver A, Santiago S, Agoulnik S, Chow J, et al. Gene expression profiling reveals epithelial mesenchymal transition (EMT) genes can selectively differentiate eribulin sensitive breast cancer cells. PLoS One. 2014;9: e106131. doi:10.1371/journal.pone.0106131

19. Di Cello F, Cope L, Li H, Jeschke J, Wang W, Baylin SB, et al. Methylation of the claudin 1 promoter is associated with loss of expression in estrogen receptor positive

breast cancer. PLoS One. 2013;8: e68630. doi:10.1371/journal.pone.0068630

20. Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, et al. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. 2009;69: 6871–6878. doi:10.1158/0008-5472.CAN-08-4490

21. Sartor MA, Mahavisno V, Keshamouni VG, Cavalcoli J, Wright Z, Karnovsky A, et al. ConceptGen: a gene set enrichment and gene set relation mapping tool. Bioinformatics. 2010;26: 456–463. doi:10.1093/bioinformatics/btp683

22. Porter JR, Fisher BE, Baranello L, Liu JC, Kambach DM, Nie Z, et al. Global Inhibition with Specific Activation: How p53 and MYC Redistribute the Transcriptome in the DNA Double-Strand Break Response. Mol Cell. 2017;67: 1013-1025.e9. doi:10.1016/j.molcel.2017.07.028

23. Bustos MA, Salomon MP, Nelson N, Hsu SC, DiNome ML, Hoon DSB, et al. Genome-wide chromatin accessibility, DNA methylation and gene expression analysis of histone deacetylase inhibition in triple-negative breast cancer. Genomics data. 2017;12: 14–16. doi:10.1016/j.gdata.2017.01.002

24. Thurman RE, Rynes E, Humbert R, Vierstra J, Maurano MT, Haugen E, et al. The accessible chromatin landscape of the human genome. Nature. 2012;489: 75–82. doi:10.1038/nature11232

25. Gallenne T, Ross KN, Visser NL, Salony, Desmet CJ, Wittner BS, et al. Systematic functional perturbations uncover a prognostic genetic network driving human breast cancer. Oncotarget. 2017;8: 20572–20587. doi:10.18632/oncotarget.16244

26. Ni M, Chen Y, Fei T, Li D, Lim E, Liu XS, et al. Amplitude modulation of androgen signaling by c-MYC. Genes Dev. 2013;27: 734–748. doi:10.1101/gad.209569.112

27. Joseph R, Orlov YL, Huss M, Sun W, Kong SL, Ukil L, et al. Integrative model of genomic factors for determining binding site selection by estrogen receptor-alpha. Mol Syst Biol. 2010;6: 456. doi:10.1038/msb.2010.109

28. Cao AR, Rabinovich R, Xu M, Xu X, Jin VX, Farnham PJ. Genome-wide analysis of transcription factor E2F1 mutant proteins reveals that N- and C-terminal protein interaction domains do not participate in targeting E2F1 to the human genome. J Biol Chem. 2011;286: 11985–11996. doi:10.1074/jbc.M110.217158

29. Franco HL, Nagari A, Malladi VS, Li W, Xi Y, Richardson D, et al. Enhancer transcription reveals subtype-specific gene expression programs controlling breast cancer pathogenesis. Genome Res. 2018;28: 159–170. doi:10.1101/gr.226019.117

30. Hodges E, Smith AD, Kendall J, Xuan Z, Ravi K, Rooks M, et al. High definition

profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res. 2009;19: 1593–1605. doi:10.1101/gr.095190.109

31. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, et al. Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. 2012;22: 246–258. doi:10.1101/gr.125872.111

32. Messier TL, Gordon JAR, Boyd JR, Tye CE, Browne G, Stein JL, et al. Histone H3 lysine 4 acetylation and methylation dynamics define breast cancer subtypes. Oncotarget. 2016;7: 5094–5109. doi:10.18632/oncotarget.6922

33. Zhu J, Sammons MA, Donahue G, Dou Z, Vedadi M, Getlik M, et al. Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. Nature. 2015;525: 206–211. doi:10.1038/nature15251

34. Vasudevan D, Hickok JR, Bovee RC, Pham V, Mantell LL, Bahroos N, et al. Nitric Oxide Regulates Gene Expression in Cancers by Controlling Histone Posttranslational Modifications. Cancer Res. 2015;75: 5299–5308. doi:10.1158/0008-5472.CAN-15-1582

35. Takaku M, Grimm SA, Shimbo T, Perera L, Menafra R, Stunnenberg HG, et al. GATA3-dependent cellular reprogramming requires activation-domain dependent recruitment of a chromatin remodeler. Genome Biol. 2016;17: 36. doi:10.1186/s13059-016-0897-0

Bibliography

1. Abraham, B.K., Fritz, P., McClellan, M., Hauptvogel, P., Athelogou, M., and Brauch, H. (2005). Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. Clin. Cancer Res. *11*, 1154–1159.
2. Aertgeerts, K., Skene, R., Yano, J., Sang, B.-C., Zou, H., Snell, G., Jennings, A., Iwamoto, K., Habuka, N., Hirokawa, A., et al. (2011). Structural analysis of the mechanism of inhibition and allosteric activation of the kinase domain of HER2 protein. J. Biol. Chem. *286*, 18756–18765.
3. Agus, D.B., Akita, R.W., Fox, W.D., Lewis, G.D., Higgins, B., Pisacane, P.I., Lofgren, J.A., Tindell, C., Evans, D.P., Maiese, K., et al. (2002). Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell *2*, 127–137.
4. Aigner, S., Sthoeger, Z.M., Fogel, M., Weber, E., Zarn, J., Ruppert, M., Zeller, Y., Vestweber, D., Stahel, R., Sammar, M., et al. (1997). CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood *89*, 3385–3395.
5. Aktas, B., Tewes, M., Fehm, T., Hauch, S., Kimmig, R., and Kasimir-Bauer, S. (2009). Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. Breast Cancer Res. *11*, R46.
6. Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U. S. A. *100*, 3983–3988.
7. Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J.E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell *86*, 221–232.
8. Allen, B.L., and Taatjes, D.J. (2015). The Mediator complex: a central integrator of transcription. Nat. Rev. Mol. Cell Biol. *16*, 155–166.
9. van Amerongen, R., Bowman, A.N., and Nusse, R. (2012). Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. Cell Stem Cell *11*, 387–400.
10. Anido, J., Scaltriti, M., Bech Serra, J.J., Santiago Josefat, B., Todo, F.R., Baselga, J., and Arribas, J. (2006). Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. EMBO J. *25*, 3234–3244.

11. Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N., and Carter-Su, C. (1993). Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell *74*, 237–244.
12. Arnould, L., Gelly, M., Penault-Llorca, F., Benoit, L., Bonnetain, F., Migeon, C., Cabaret, V., Fermeaux, V., Bertheau, P., Garnier, J., et al. (2006). Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br. J. Cancer *94*, 259–267.
13. Arribas, J., and Esselens, C. (2009). ADAM17 as a therapeutic target in multiple diseases. Curr. Pharm. Des. *15*, 2319–2335.
14. Arteaga, C.L., and Engelman, J.A. (2014). ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. Cancer Cell *25*, 282–303.
15. Asiedu, M.K., Ingle, J.N., Behrens, M.D., Radisky, D.C., and Knutson, K.L. (2011). TGFβ/TNFα-Mediated Epithelial-Mesenchymal Transition Generates Breast Cancer Stem Cells with a Claudin-Low Phenotype. Cancer Res. *71*, 4707–4719.
16. Austin, C.D., De Maziere, A.M., Pisacane, P.I., van Dijk, S.M., Eigenbrot, C., Sliwkowski, M.X., Klumperman, J., and Scheller, R.H. (2004). Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol. Biol. Cell *15*, 5268–5282.
17. Azzam, D.J., Zhao, D., Sun, J., Minn, A.J., Ranganathan, P., Drews-Elger, K., Han, X., Picon-Ruiz, M., Gilbert, C.A., Wander, S.A., et al. (2013). Triple negative breast cancer initiating cell subsets differ in functional and molecular characteristics and in γ-secretase inhibitor drug responses. EMBO Mol. Med. *5*, 1502–1522.
18. Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J.R., Maciejewski, A., and Wishart, D.S. (2016). Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. *44*, W147-53.
19. Bachelder, R.E., Yoon, S.-O., Franci, C., de Herreros, A.G., and Mercurio, A.M. (2005). Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. J. Cell Biol. *168*, 29–33.
20. Baker, A.T., Zlobin, A., and Osipo, C. (2014). Notch-EGFR/HER2 Bidirectional Crosstalk in Breast Cancer. Front. Oncol. *4*, 360.
21. Bakin, A. V, Tomlinson, A.K., Bhowmick, N.A., Moses, H.L., and Arteaga, C.L. (2000).

Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem. *275*, 36803–36810.

22. Balic, M., Lin, H., Young, L., Hawes, D., Giuliano, A., McNamara, G., Datar, R.H., and Cote, R.J. (2006). Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. Clin. Cancer Res. *12*, 5615–5621.
23. Banerji, J., Rusconi, S., and Schaffner, W. (1981). Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. Cell *27*, 299–308.
24. Barbieri, I., Quaglino, E., Maritano, D., Pannellini, T., Riera, L., Cavallo, F., Forni, G., Musiani, P., Chiarle, R., and Poli, V. (2010). Stat3 is required for anchorage-independent growth and metastasis but not for mammary tumor development downstream of the ErbB-2 oncogene. Mol. Carcinog. *49*, 114–120.
25. Barok, M., Isola, J., Palyi-Krekk, Z., Nagy, P., Juhasz, I., Vereb, G., Kauraniemi, P., Kapanen, A., Tanner, M., Vereb, G., et al. (2007). Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Mol. Cancer Ther. *6*, 2065–2072.
26. Bartis, D., Mise, N., Mahida, R.Y., Eickelberg, O., and Thickett, D.R. (2014). Epithelial-mesenchymal transition in lung development and disease: does it exist and is it important? Thorax *69*, 760–765.
27. Bartsch, J.E., Staren, E.D., and Appert, H.E. (2003). Matrix metalloproteinase expression in breast cancer. J. Surg. Res. *110*, 383–392.
28. Baselga, J. (2002). A new anti-ErbB2 strategy in the treatment of cancer: prevention of ligand-dependent ErbB2 receptor heterodimerization. Cancer Cell *2*, 93–95.
29. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C.C., Dantis, L., Sklarin, N.T., Seidman, A.D., Hudis, C.A., Moore, J., et al. (1996). Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J. Clin. Oncol. *14*, 737–744.
30. Baselga, J., Cortes, J., Kim, S.-B., Im, S.-A., Hegg, R., Im, Y.-H., Roman, L., Pedrini, J.L., Pienkowski, T., Knott, A., et al. (2012). Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N. Engl. J. Med. *366*, 109–119.

31. Bauerschmitz, G.J., Ranki, T., Kangasniemi, L., Ribacka, C., Eriksson, M., Porten, M., Herrmann, I., Ristimaki, A., Virkkunen, P., Tarkkanen, M., et al. (2008). Tissue-specific promoters active in CD44+CD24-/low breast cancer cells. Cancer Res. *68*, 5533–5539.
32. Baumann, P., Cremers, N., Kroese, F., Orend, G., Chiquet-Ehrismann, R., Uede, T., Yagita, H., and Sleeman, J.P. (2005). CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res. *65*, 10783–10793.
33. Beevers, A.J., and Kukol, A. (2006). The transmembrane domain of the oncogenic mutant ErbB-2 receptor: a structure obtained from site-specific infrared dichroism and molecular dynamics. J. Mol. Biol. *361*, 945–953.
34. Beguelin, W., Diaz Flaque, M.C., Proietti, C.J., Cayrol, F., Rivas, M.A., Tkach, M., Rosemblit, C., Tocci, J.M., Charreau, E.H., Schillaci, R., et al. (2010). Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. Mol. Cell. Biol. *30*, 5456–5472.
35. Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science *280*, 596–599.
36. Benson, C.S., Babu, S.D., Radhakrishna, S., Selvamurugan, N., and Ravi Sankar, B. (2013). Expression of matrix metalloproteinases in human breast cancer tissues. Dis. Markers *34*, 395–405.
37. Benvenuto, M., Masuelli, L., De Smaele, E., Fantini, M., Mattera, R., Cucchi, D., Bonanno, E., Di Stefano, E., Frajese, G.V., Orlandi, A., et al. (2016). In vitro and in vivo inhibition of breast cancer cell growth by targeting the Hedgehog/GLI pathway with SMO (GDC-0449) or GLI (GANT-61) inhibitors. Oncotarget *7*, 9250–9270.
38. Berns, K., Horlings, H.M., Hennessy, B.T., Madiredjo, M., Hijmans, E.M., Beelen, K., Linn, S.C., Gonzalez-Angulo, A.M., Stemke-Hale, K., Hauptmann, M., et al. (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell *12*, 395–402.
39. Bhowmick, N.A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C.A., Engel, M.E., Arteaga, C.L., and Moses, H.L. (2001). Transforming growth factor-beta1 mediates

epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol. Biol. Cell *12*, 27–36.

40. Bose, R., Kavuri, S.M., Searleman, A.C., Shen, W., Shen, D., Koboldt, D.C., Monsey, J., Goel, N., Aronson, A.B., Li, S., et al. (2013). Activating HER2 mutations in HER2 gene amplification negative breast cancer. Cancer Discov. *3*, 224–237.
41. Bouras, T., Pal, B., Vaillant, F., Harburg, G., Asselin-Labat, M.-L., Oakes, S.R., Lindeman, G.J., and Visvader, J.E. (2008). Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. Cell Stem Cell *3*, 429–441.
42. Bourguignon, L.Y., Zhu, H., Chu, A., Iida, N., Zhang, L., and Hung, M.C. (1997). Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. J. Biol. Chem. *272*, 27913–27918.
43. Bourguignon, L.Y., Zhu, H., Zhou, B., Diedrich, F., Singleton, P.A., and Hung, M.C. (2001). Hyaluronan promotes CD44v3-Vav2 interaction with Grb2-p185(HER2) and induces Rac1 and Ras signaling during ovarian tumor cell migration and growth. J. Biol. Chem. *276*, 48679–48692.
44. Bragin, P.E., Mineev, K.S., Bocharova, O. V, Volynsky, P.E., Bocharov, E. V, and Arseniev, A.S. (2016). HER2 Transmembrane Domain Dimerization Coupled with Self-Association of Membrane-Embedded Cytoplasmic Juxtamembrane Regions. J. Mol. Biol. *428*, 52–61.
45. Brannon, M., Gomperts, M., Sumoy, L., Moon, R.T., and Kimelman, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. *11*, 2359–2370.
46. Brennan, K., McSherry, E.A., Hudson, L., Kay, E.W., Hill, A.D.K., Young, L.S., and Hopkins, A.M. (2013). Junctional adhesion molecule-A is co-expressed with HER2 in breast tumors and acts as a novel regulator of HER2 protein degradation and signaling. Oncogene *32*, 2799–2804.
47. Bretz, N., Noske, A., Keller, S., Erbe-Hofmann, N., Schlange, T., Salnikov, A. V, Moldenhauer, G., Kristiansen, G., and Altevogt, P. (2012). CD24 promotes tumor cell invasion by suppressing tissue factor pathway inhibitor-2 (TFPI-2) in a c-Src-dependent fashion. Clin. Exp. Metastasis *29*, 27–38.
48. Brockhoff, G., Heckel, B., Schmidt-Bruecken, E., Plander, M., Hofstaedter, F., Vollmann,

A., and Diermeier, S. (2007). Differential impact of Cetuximab, Pertuzumab and Trastuzumab on BT474 and SK-BR-3 breast cancer cell proliferation. Cell Prolif. *40*, 488–507.

49. Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A., and Israel, A. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol. Cell *5*, 207–216.
50. Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature *385*, 829–833.
51. Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., and Greenleaf, W.J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat. Methods *10*, 1213–1218.
52. Buijs, J.T., van der Horst, G., van den Hoogen, C., Cheung, H., de Rooij, B., Kroon, J., Petersen, M., van Overveld, P.G.M., Pelger, R.C.M., and van der Pluijm, G. (2012). The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. Oncogene *31*, 2164–2174.
53. Burgering, B.M., and Coffer, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599–602.
54. Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., and Brabletz, T. (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. *9*, 582–589.
55. Burns, D.M., He, C., Li, Y., Scherle, P., Liu, X., Marando, C.A., Covington, M.B., Yang, G., Pan, M., Turner, S., et al. (2008). Conversion of an MMP-potent scaffold to an MMP-selective HER-2 sheddase inhibitor via scaffold hybridization and subtle P1' permutations. Bioorg. Med. Chem. Lett. *18*, 560–564.
56. Bustos, M.A., Salomon, M.P., Nelson, N., Hsu, S.C., DiNome, M.L., Hoon, D.S.B., and Marzese, D.M. (2017). Genome-wide chromatin accessibility, DNA methylation and gene expression analysis of histone deacetylase inhibition in triple-negative breast cancer. Genomics Data *12*, 14–16.
57. Caiazza, F., McGowan, P.M., Mullooly, M., Murray, A., Synnott, N., O'Donovan, N., Flanagan, L., Tape, C.J., Murphy, G., Crown, J., et al. (2015). Targeting ADAM-17 with

an inhibitory monoclonal antibody has antitumour effects in triple-negative breast cancer cells. Br. J. Cancer *112*, 1895–1903.

58. Calo, E., and Wysocka, J. (2013). Modification of enhancer chromatin: what, how, and why? Mol. Cell *49*, 825–837.
59. Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., and Nieto, M.A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. *2*, 76–83.
60. Cao, A.R., Rabinovich, R., Xu, M., Xu, X., Jin, V.X., and Farnham, P.J. (2011). Genome-wide analysis of transcription factor E2F1 mutant proteins reveals that N- and C-terminal protein interaction domains do not participate in targeting E2F1 to the human genome. J. Biol. Chem. *286*, 11985–11996.
61. Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Annu. Rev. Biochem. *56*, 881–914.
62. Carpenter, G., Lembach, K.J., Morrison, M.M., and Cohen, S. (1975). Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. J. Biol. Chem. *250*, 4297–4304.
63. Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., and Shepard, H.M. (1992). Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. U. S. A. *89*, 4285–4289.
64. Castellano, E., and Downward, J. (2011). RAS Interaction with PI3K: More Than Just Another Effector Pathway. Genes Cancer *2*, 261–274.
65. Cavaliere, C., Corvigno, S., Galgani, M., Limite, G., Nardone, A., and Veneziani, B.M. (2010). Combined inhibitory effect of formestane and herceptin on a subpopulation of CD44+/CD24low breast cancer cells. Cancer Sci. *101*, 1661–1669.
66. Di Cello, F., Cope, L., Li, H., Jeschke, J., Wang, W., Baylin, S.B., and Zahnow, C.A. (2013). Methylation of the claudin 1 promoter is associated with loss of expression in estrogen receptor positive breast cancer. PLoS One *8*, e68630.
67. Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. *2*,

401–404.

68. Chandarlapaty, S., Scaltriti, M., Angelini, P., Ye, Q., Guzman, M., Hudis, C.A., Norton, L., Solit, D.B., Arribas, J., Baselga, J., et al. (2010). Inhibitors of HSP90 block p95-HER2 signaling in Trastuzumab-resistant tumors and suppress their growth. Oncogene *29*, 325–334.

69. Chang, Y.-W., Su, Y.-J., Hsiao, M., Wei, K.-C., Lin, W.-H., Liang, C.-L., Chen, S.-C., and Lee, J.-L. (2015). Diverse Targets of beta-Catenin during the Epithelial-Mesenchymal Transition Define Cancer Stem Cells and Predict Disease Relapse. Cancer Res. *75*, 3398–3410.

70. Chaudhury, A., Hussey, G.S., Ray, P.S., Jin, G., Fox, P.L., and Howe, P.H. (2010). TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. Nat. Cell Biol. *12*, 286–293.

71. Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., and Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics *14*, 128.

72. Chen, M.-H., Wilson, C.W., Li, Y.-J., Law, K.K. Lo, Lu, C.-S., Gacayan, R., Zhang, X., Hui, C., and Chuang, P.-T. (2009). Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes Dev. *23*, 1910–1928.

73. Chen, Q.Q., Chen, X.Y., Jiang, Y.Y., and Liu, J. (2005). Identification of novel nuclear localization signal within the ErbB-2 protein. Cell Res. *15*, 504–510.

74. Chen, Z.-L., Shen, Y.-W., Li, S.-T., Li, C.-L., Zhang, L.-X., Yang, J., Lv, M., Lin, Y.-Y., Wang, X., and Yang, J. (2016). The efficiency and safety of trastuzumab and lapatinib added to neoadjuvant chemotherapy in Her2-positive breast cancer patients: a randomized meta-analysis. Onco. Targets. Ther. *9*, 3233–3247.

75. Cheung, H.O.-L., Zhang, X., Ribeiro, A., Mo, R., Makino, S., Puviindran, V., Law, K.K.L., Briscoe, J., and Hui, C.-C. (2009). The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. Sci. Signal. *2*, ra29.

76. Chipumuro, E., Marco, E., Christensen, C.L., Kwiatkowski, N., Zhang, T., Hatheway, C.M., Abraham, B.J., Sharma, B., Yeung, C., Altabef, A., et al. (2014). CDK7 inhibition suppresses super-enhancer-linked oncogenic transcription in MYCN-driven cancer. Cell *159*, 1126–1139.

77. Cho, H.-S., Mason, K., Ramyar, K.X., Stanley, A.M., Gabelli, S.B., Denney, D.W.J., and Leahy, D.J. (2003). Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature *421*, 756–760.

78. Cho, S.H., Park, Y.S., Kim, H.J., Kim, C.H., Lim, S.W., Huh, J.W., Lee, J.H., and Kim, H.R. (2012). CD44 enhances the epithelial-mesenchymal transition in association with colon cancer invasion. Int. J. Oncol. *41*, 211–218.

79. Chow, A., Arteaga, C.L., and Wang, S.E. (2011). When Tumor Suppressor TGFβ Meets the HER2 (ERBB2) Oncogene. J. Mammary Gland Biol. Neoplasia *16*, 81–88.

80. Christianson, T.A., Doherty, J.K., Lin, Y.J., Ramsey, E.E., Holmes, R., Keenan, E.J., and Clinton, G.M. (1998). NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. Cancer Res. *58*, 5123–5129.

81. Chu, E.Y., Hens, J., Andl, T., Kairo, A., Yamaguchi, T.P., Brisken, C., Glick, A., Wysolmerski, J.J., and Millar, S.E. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development *131*, 4819–4829.

82. Chung, S.S., Giehl, N., Wu, Y., and Vadgama, J. V (2014). STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. Int. J. Oncol. *44*, 403–411.

83. Citri, A., and Yarden, Y. (2006). EGF-ERBB signalling: towards the systems level. Nat. Rev. Mol. Cell Biol. *7*, 505–516.

84. Clynes, R.A., Towers, T.L., Presta, L.G., and Ravetch, J. V (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat. Med. *6*, 443–446.

85. Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J.M., Paton, V., Shak, S., Lieberman, G., et al. (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J. Clin. Oncol. *17*, 2639–2648.

86. Cochrane, C.R., Szczepny, A., Watkins, D.N., and Cain, J.E. (2015). Hedgehog Signaling in the Maintenance of Cancer Stem Cells. Cancers (Basel). *7*, 1554–1585.

87. Codony-Servat, J., Albanell, J., Lopez-Talavera, J.C., Arribas, J., and Baselga, J. (1999).

Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. Cancer Res. *59*, 1196–1201.

88. Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D., and van Roy, F. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell *7*, 1267–1278.
89. Corbit, K.C., Aanstad, P., Singla, V., Norman, A.R., Stainier, D.Y.R., and Reiter, J.F. (2005). Vertebrate Smoothened functions at the primary cilium. Nature *437*, 1018–1021.
90. Cordo Russo, R.I., Beguelin, W., Diaz Flaque, M.C., Proietti, C.J., Venturutti, L., Galigniana, N., Tkach, M., Guzman, P., Roa, J.C., O'Brien, N.A., et al. (2015). Targeting ErbB-2 nuclear localization and function inhibits breast cancer growth and overcomes trastuzumab resistance. Oncogene *34*, 3413–3428.
91. Cossu-Rocca, P., Muroni, M.R., Sanges, F., Sotgiu, G., Asunis, A., Tanca, L., Onnis, D., Pira, G., Manca, A., Dore, S., et al. (2016). EGFR kinase-dependent and kinase-independent roles in clear cell renal cell carcinoma. Am. J. Cancer Res. *6*, 71–83.
92. Creighton, C.J., Li, X., Landis, M., Dixon, J.M., Neumeister, V.M., Sjolund, A., Rimm, D.L., Wong, H., Rodriguez, A., Herschkowitz, J.I., et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc. Natl. Acad. Sci. U. S. A. *106*, 13820–13825.
93. Croker, A.K., Goodale, D., Chu, J., Postenka, C., Hedley, B.D., Hess, D.A., and Allan, A.L. (2009). High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J. Cell. Mol. Med. *13*, 2236–2252.
94. Cuello, M., Ettenberg, S.A., Clark, A.S., Keane, M.M., Posner, R.H., Nau, M.M., Dennis, P.A., and Lipkowitz, S. (2001). Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. Cancer Res. *61*, 4892–4900.
95. Cufí, S., Corominas-Faja, B., Vazquez-Martin, A., Oliveras-Ferraros, C., Dorca, J., Bosch-Barrera, J., Martin-Castillo, B., and Menendez, J.A. (2012). Metformin-induced

preferential killing of breast cancer initiating CD44(+)CD24(−/low) cells is sufficient to overcome primary resistance to trastuzumab in HER2+ human breast cancer xenografts. Oncotarget *3*, 395–398.

96. Cymer, F., and Schneider, D. (2010). Transmembrane helix-helix interactions involved in ErbB receptor signaling. Cell Adh. Migr. *4*, 299–312.
97. Dahabreh, I.J., Linardou, H., Siannis, F., Fountzilas, G., and Murray, S. (2008). Trastuzumab in the adjuvant treatment of early-stage breast cancer: a systematic review and meta-analysis of randomized controlled trials. Oncologist *13*, 620–630.
98. Dankort, D., Maslikowski, B., Warner, N., Kanno, N., Kim, H., Wang, Z., Moran, M.F., Oshima, R.G., Cardiff, R.D., and Muller, W.J. (2001). Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. Mol. Cell. Biol. *21*, 1540–1551.
99. Dave, B., Migliaccio, I., Gutierrez, M.C., Wu, M.-F., Chamness, G.C., Wong, H., Narasanna, A., Chakrabarty, A., Hilsenbeck, S.G., Huang, J., et al. (2011). Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. J. Clin. Oncol. *29*, 166–173.
100. Dave, B., Landis, M.D., Tweardy, D.J., Chang, J.C., Dobrolecki, L.E., Wu, M.-F., Zhang, X., Westbrook, T.F., Hilsenbeck, S.G., Liu, D., et al. (2012). Selective small molecule Stat3 inhibitor reduces breast cancer tumor-initiating cells and improves recurrence free survival in a human-xenograft model. PLoS One *7*, e30207.
101. Davis, F.M., Azimi, I., Faville, R.A., Peters, A.A., Jalink, K., Putney, J.W.J., Goodhill, G.J., Thompson, E.W., Roberts-Thomson, S.J., and Monteith, G.R. (2014). Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. Oncogene *33*, 2307–2316.
102. Davoli, A., Hocevar, B.A., and Brown, T.L. (2010). Progression and treatment of HER2-positive breast cancer. Cancer Chemother. Pharmacol. *65*, 611–623.
103. DeArmond, D., Brattain, M.G., Jessup, J.M., Kreisberg, J., Malik, S., Zhao, S., and Freeman, J.W. (2003). Autocrine-mediated ErbB-2 kinase activation of STAT3 is required for growth factor independence of pancreatic cancer cell lines. Oncogene *22*, 7781–7795.
104. Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome

conformation. Science *295*, 1306–1311.

105. Derynck, R., and Zhang, Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature *425*, 577–584.
106. Derynck, R., Rhee, L., Chen, E.Y., and Van Tilburg, A. (1987). Intron-exon structure of the human transforming growth factor-beta precursor gene. Nucleic Acids Res. *15*, 3188–3189.
107. Dey, N., Young, B., Abramovitz, M., Bouzyk, M., Barwick, B., De, P., and Leyland-Jones, B. (2013). Differential Activation of Wnt-β-Catenin Pathway in Triple Negative Breast Cancer Increases MMP7 in a PTEN Dependent Manner. PLoS One *8*.
108. Dezso, Z., Oestreicher, J., Weaver, A., Santiago, S., Agoulnik, S., Chow, J., Oda, Y., and Funahashi, Y. (2014). Gene expression profiling reveals epithelial mesenchymal transition (EMT) genes can selectively differentiate eribulin sensitive breast cancer cells. PLoS One *9*, e106131.
109. Dickel, D.E. (2014). Function-based Identification of Mammalian Enhancers Using Site-Specific Integration. *11*, 566–571.
110. Dickinson, M.E., Kobrin, M.S., Silan, C.M., Kingsley, D.M., Justice, M.J., Miller, D.A., Ceci, J.D., Lock, L.F., Lee, A., and Buchberg, A.M. (1990). Chromosomal localization of seven members of the murine TGF-beta superfamily suggests close linkage to several morphogenetic mutant loci. Genomics *6*, 505–520.
111. Diermeier-Daucher, S., Hasmann, M., and Brockhoff, G. (2008). Flow cytometric FRET analysis of erbB receptor interaction on a cell-by-cell basis. Ann. N. Y. Acad. Sci. *1130*, 280–286.
112. Diermeier-Daucher, S., Breindl, S., Buchholz, S., Ortmann, O., and Brockhoff, G. (2011). Modular anti-EGFR and anti-Her2 targeting of SK-BR-3 and BT474 breast cancer cell lines in the presence of ErbB receptor-specific growth factors. Cytometry. A *79*, 684–693.
113. Diessner, J., Bruttel, V., Becker, K., Pawlik, M., Stein, R., Hausler, S., Dietl, J., Wischhusen, J., and Honig, A. (2013). Targeting breast cancer stem cells with HER2-specific antibodies and natural killer cells. Am. J. Cancer Res. *3*, 211–220.
114. Doherty, M.R., Smigiel, J.M., Junk, D.J., and Jackson, M.W. (2016). Cancer Stem Cell Plasticity Drives Therapeutic Resistance. Cancers (Basel). *8*.
115. Dokmanovic, M., Wu, Y., Shen, Y., Chen, J., Hirsch, D.S., and Wu, W.J. (2014).

Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition. Cancer Biol. Ther. *15*, 1029–1041.

116. Dontu, G., Jackson, K.W., McNicholas, E., Kawamura, M.J., Abdallah, W.M., and Wicha, M.S. (2004). Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res. *6*, R605-15.
117. Draffin, J.E., McFarlane, S., Hill, A., Johnston, P.G., and Waugh, D.J.J. (2004). CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. Cancer Res. *64*, 5702–5711.
118. Duchnowska, R., Sperinde, J., Chenna, A., Haddad, M., Paquet, A., Lie, Y., Weidler, J.M., Huang, W., Winslow, J., Jankowski, T., et al. (2014). Quantitative measurements of tumoral p95HER2 protein expression in metastatic breast cancer patients treated with trastuzumab: independent validation of the p95HER2 clinical cutoff. Clin. Cancer Res. *20*, 2805–2813.
119. Duffy, M.J., Mullooly, M., O'Donovan, N., Sukor, S., Crown, J., Pierce, A., and McGowan, P.M. (2011). The ADAMs family of proteases: new biomarkers and therapeutic targets for cancer? Clin. Proteomics *8*, 9.
120. Duhachek-Muggy, S., Qi, Y., Wise, R., Alyahya, L., Li, H., Hodge, J., and Zolkiewska, A. (2017). Metalloprotease-disintegrin ADAM12 actively promotes the stem cell-like phenotype in claudin-low breast cancer. Mol. Cancer *16*, 32.
121. Duneau, J.-P., Vegh, A.P., and Sturgis, J.N. (2007). A dimerization hierarchy in the transmembrane domains of the HER receptor family. Biochemistry *46*, 2010–2019.
122. Duong, M.N., Cleret, A., Matera, E.-L., Chettab, K., Mathe, D., Valsesia-Wittmann, S., Clemenceau, B., and Dumontet, C. (2015). Adipose cells promote resistance of breast cancer cells to trastuzumab-mediated antibody-dependent cellular cytotoxicity. Breast Cancer Res. *17*, 57.
123. Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., and McMahon, A.P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell *75*, 1417–1430.
124. Eger, A., Stockinger, A., Schaffhauser, B., Beug, H., and Foisner, R. (2000). Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear

translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J. Cell Biol. *148*, 173–188.

125. Eiermann, W. (2001). Trastuzumab combined with chemotherapy for the treatment of HER2-positive metastatic breast cancer: pivotal trial data. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *12 Suppl 1*, S57-62.
126. Elias, S.G., Adams, A., Wisner, D.J., Esserman, L.J., van't Veer, L.J., Mali, W.P.T.M., Gilhuijs, K.G.A., and Hylton, N.M. (2014). Imaging features of HER2 overexpression in breast cancer: a systematic review and meta-analysis. Cancer Epidemiol. Biomarkers Prev. *23*, 1464–1483.
127. Elster, N., Cremona, M., Morgan, C., Toomey, S., Carr, A., O'Grady, A., Hennessy, B.T., and Eustace, A.J. (2015). A preclinical evaluation of the PI3K alpha/delta dominant inhibitor BAY 80-6946 in HER2-positive breast cancer models with acquired resistance to the HER2-targeted therapies trastuzumab and lapatinib. Breast Cancer Res. Treat. *149*, 373–383.
128. ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. Nature *489*, 57–74.
129. Escher, C., Cymer, F., and Schneider, D. (2009). Two GxxxG-like motifs facilitate promiscuous interactions of the human ErbB transmembrane domains. J. Mol. Biol. *389*, 10–16.
130. Espinoza, I., Pochampally, R., Xing, F., Watabe, K., and Miele, L. (2013). Notch signaling: targeting cancer stem cells and epithelial-to-mesenchymal transition. Onco. Targets. Ther. *6*, 1249–1259.
131. Esteva, F.J., Valero, V., Booser, D., Guerra, L.T., Murray, J.L., Pusztai, L., Cristofanilli, M., Arun, B., Esmaeli, B., Fritsche, H.A., et al. (2002). Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J. Clin. Oncol. *20*, 1800–1808.
132. Esteva, F.J., Guo, H., Zhang, S., Santa-Maria, C., Stone, S., Lanchbury, J.S., Sahin, A.A., Hortobagyi, G.N., and Yu, D. (2010). PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. Am. J. Pathol. *177*, 1647–1656.
133. Farnie, G., Clarke, R.B., Spence, K., Pinnock, N., Brennan, K., Anderson, N.G., and

Bundred, N.J. (2007). Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways. J. Natl. Cancer Inst. *99*, 616–627.

134. Farnie, G., Johnson, R.L., Williams, K.E., Clarke, R.B., and Bundred, N.J. (2014). Lapatinib inhibits stem/progenitor proliferation in preclinical in vitro models of ductal carcinoma in situ (DCIS). Cell Cycle *13*, 418–425.
135. Feldinger, K., Generali, D., Kramer-Marek, G., Gijsen, M., Ng, T.B., Wong, J.H., Strina, C., Cappelletti, M., Andreis, D., Li, J.-L., et al. (2014). ADAM10 mediates trastuzumab resistance and is correlated with survival in HER2 positive breast cancer. Oncotarget *5*, 6633–6646.
136. Fendly, B.M., Winget, M., Hudziak, R.M., Lipari, M.T., Napier, M.A., and Ullrich, A. (1990). Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res. *50*, 1550–1558.
137. Ferguson, K.M. (2008). Structure-based view of epidermal growth factor receptor regulation. Annu. Rev. Biophys. *37*, 353–373.
138. Fiszman, G.L., and Jasnis, M.A. (2011). Molecular Mechanisms of Trastuzumab Resistance in HER2 Overexpressing Breast Cancer. Int. J. Breast Cancer *2011*, 352182.
139. Fitzgerald, K., and Greenwald, I. (1995). Interchangeability of Caenorhabditis elegans DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. Development *121*, 4275–4282.
140. Fleeman, N., Bagust, A., Beale, S., Dwan, K., Dickson, R., Proudlove, C., and Dundar, Y. (2015). Pertuzumab in combination with trastuzumab and docetaxel for the treatment of HER2-positive metastatic or locally recurrent unresectable breast cancer. Pharmacoeconomics *33*, 13–23.
141. Franco, H.L., Nagari, A., Malladi, V.S., Li, W., Xi, Y., Richardson, D., Allton, K.L., Tanaka, K., Li, J., Murakami, S., et al. (2018). Enhancer transcription reveals subtype-specific gene expression programs controlling breast cancer pathogenesis. Genome Res. *28*, 159–170.
142. Franklin, M.C., Carey, K.D., Vajdos, F.F., Leahy, D.J., de Vos, A.M., and Sliwkowski, M.X. (2004). Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell *5*, 317–328.

143. Friederichs, J., Zeller, Y., Hafezi-Moghadam, A., Grone, H.J., Ley, K., and Altevogt, P. (2000). The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res. *60*, 6714–6722.

144. Fu, Y.-Z., Yan, Y.-Y., He, M., Xiao, Q.-H., Yao, W.-F., Zhao, L., Wu, H.-Z., Yu, Z.-J., Zhou, M.-Y., Lv, M.-T., et al. (2016). Salinomycin induces selective cytotoxicity to MCF-7 mammosphere cells through targeting the Hedgehog signaling pathway. Oncol. Rep. *35*, 912–922.

145. Fujisawa, T., and Filippakopoulos, P. (2017). Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. Nat. Rev. Mol. Cell Biol. *18*, 246–262.

146. Fullwood, M.J., Liu, M.H., Pan, Y.F., Liu, J., Xu, H., Mohamed, Y. Bin, Orlov, Y.L., Velkov, S., Ho, A., Mei, P.H., et al. (2009). An oestrogen-receptor-alpha-bound human chromatin interactome. Nature *462*, 58–64.

147. Gallardo, A., Lerma, E., Escuin, D., Tibau, A., Munoz, J., Ojeda, B., Barnadas, A., Adrover, E., Sanchez-Tejada, L., Giner, D., et al. (2012). Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. Br. J. Cancer *106*, 1367–1373.

148. Gallenne, T., Ross, K.N., Visser, N.L., Salony, Desmet, C.J., Wittner, B.S., Wessels, L.F.A., Ramaswamy, S., and Peeper, D.S. (2017). Systematic functional perturbations uncover a prognostic genetic network driving human breast cancer. Oncotarget *8*, 20572–20587.

149. Gao, A.C., Lou, W., Dong, J.T., and Isaacs, J.T. (1997). CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. Cancer Res. *57*, 846–849.

150. Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., et al. (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci. Signal. *6*, pl1.

151. Gao, Z.-H., Seeling, J.M., Hill, V., Yochum, A., and Virshup, D.M. (2002). Casein kinase I phosphorylates and destabilizes the beta-catenin degradation complex. Proc. Natl. Acad. Sci. U. S. A. *99*, 1182–1187.

152. Garcia-Garcia, C., Ibrahim, Y.H., Serra, V., Calvo, M.T., Guzman, M., Grueso, J., Aura, C., Perez, J., Jessen, K., Liu, Y., et al. (2012). Dual mTORC1/2 and HER2 blockade results in antitumor activity in preclinical models of breast cancer resistant to anti-HER2 therapy.

Clin. Cancer Res. *18*, 2603–2612.

153. Garrett, T.P.J., McKern, N.M., Lou, M., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Kofler, M., Jorissen, R.N., Nice, E.C., Burgess, A.W., et al. (2003). The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol. Cell *11*, 495–505.
154. Garrido-Castro, A.C., and Felip, E. (2013). HER2 driven non-small cell lung cancer (NSCLC): potential therapeutic approaches. Transl. Lung Cancer Res. *2*, 122–127.
155. Gerber, D., Sal-Man, N., and Shai, Y. (2004). Two motifs within a transmembrane domain, one for homodimerization and the other for heterodimerization. J. Biol. Chem. *279*, 21177–21182.
156. Ghosh, R., Narasanna, A., Wang, S.E., Liu, S., Chakrabarty, A., Balko, J.M., Gonzalez-Angulo, A.M., Mills, G.B., Penuel, E., Winslow, J., et al. (2011). Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. *71*, 1871–1882.
157. Gianni, L., Pienkowski, T., Im, Y.-H., Roman, L., Tseng, L.-M., Liu, M.-C., Lluch, A., Staroslawska, E., de la Haba-Rodriguez, J., Im, S.-A., et al. (2012). Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet. Oncol. *13*, 25–32.
158. Gijsen, M., King, P., Perera, T., Parker, P.J., Harris, A.L., Larijani, B., and Kong, A. (2010). HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer. PLoS Biol. *8*, e1000563.
159. Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C., Liu, S., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell *1*, 555–567.
160. Giordano, A., Mego, M., Lee, B., Anfossi, S., Parker, C.A., Alvarez, R.H., Ueno, N.T., Valero, V., Cristofanilli, M., and Reuben, J.M. (2011). Epithelial-mesenchymal transition in patients with HER2+ metastatic breast cancer. J. Clin. Oncol. *29*, 623.
161. Giordano, A., Gao, H., Anfossi, S., Cohen, E., Mego, M., Lee, B.-N., Tin, S., De Laurentiis, M., Parker, C.A., Alvarez, R.H., et al. (2012). Epithelial-mesenchymal

transition and stem cell markers in patients with HER2-positive metastatic breast cancer. Mol. Cancer Ther. *11*, 2526–2534.

162. Gong, C., Yao, H., Liu, Q., Chen, J., Shi, J., Su, F., and Song, E. (2010). Markers of tumor-initiating cells predict chemoresistance in breast cancer. PLoS One *5*, e15630.
163. Gong, C., Yao, Y., Wang, Y., Liu, B., Wu, W., Chen, J., Su, F., Yao, H., and Song, E. (2011). Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. J. Biol. Chem. *286*, 19127–19137.
164. Gong, Y., Lazaris, C., Sakellaropoulos, T., Lozano, A., Kambadur, P., Ntziachristos, P., Aifantis, I., and Tsirigos, A. (2018). Stratification of TAD boundaries reveals preferential insulation of super-enhancers by strong boundaries. Nat. Commun. *9*, 542.
165. Gorbatenko, A., Olesen, C.W., Loebl, N., Sigurdsson, H.H., Bianchi, C., Pedraz-Cuesta, E., Christiansen, J., and Pedersen, S.F. (2016). Oncogenic p95HER2 regulates Na+-HCO3-cotransporter NBCn1 mRNA stability in breast cancer cells via 3'UTR-dependent processes. Biochem. J. *473*, 4027–4044.
166. Gregory, P.A., Bert, A.G., Paterson, E.L., Barry, S.C., Tsykin, A., Farshid, G., Vadas, M.A., Khew-Goodall, Y., and Goodall, G.J. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. *10*, 593–601.
167. Groschel, S., Sanders, M.A., Hoogenboezem, R., de Wit, E., Bouwman, B.A.M., Erpelinck, C., van der Velden, V.H.J., Havermans, M., Avellino, R., van Lom, K., et al. (2014). A single oncogenic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in leukemia. Cell *157*, 369–381.
168. Gross, D.S., and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. *57*, 159–197.
169. Grudzien, P., Lo, S., Albain, K.S., Robinson, P., Rajan, P., Strack, P.R., Golde, T.E., Miele, L., and Foreman, K.E. (2010). Inhibition of Notch signaling reduces the stem-like population of breast cancer cells and prevents mammosphere formation. Anticancer Res. *30*, 3853–3867.
170. Guo, L., Chen, C., Shi, M., Wang, F., Chen, X., Diao, D., Hu, M., Yu, M., Qian, L., and Guo, N. (2013). Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition. Oncogene *32*,

5272–5282.

171. Han, B., Qu, Y., Yu-Rice, Y., Johnson, J., and Cui, X. (2016). FOXC1-induced Gli2 activation: A non-canonical pathway contributing to stemness and anti-Hedgehog resistance in basal-like breast cancer. Mol. Cell. Oncol. *3*, e1131668.
172. Han, S.-W., Cha, Y., Paquet, A., Huang, W., Weidler, J., Lie, Y., Sherwood, T., Bates, M., Haddad, M., Park, I.H., et al. (2012). Correlation of HER2, p95HER2 and HER3 expression and treatment outcome of lapatinib plus capecitabine in her2-positive metastatic breast cancer. PLoS One *7*, e39943.
173. Hanna, W., Barnes, P., Berendt, R., Chang, M., Magliocco, A., Mulligan, A.M., Rees, H., Miller, N., Elavathil, L., Gilks, B., et al. (2012). Testing for her2 in breast cancer: current pathology challenges faced in Canada. Curr. Oncol. *19*, 315–323.
174. Harbeck, N., Beckmann, M.W., Rody, A., Schneeweiss, A., Muller, V., Fehm, T., Marschner, N., Gluz, O., Schrader, I., Heinrich, G., et al. (2013). HER2 Dimerization Inhibitor Pertuzumab - Mode of Action and Clinical Data in Breast Cancer. Breast Care (Basel). *8*, 49–55.
175. Harrison, H., Farnie, G., Howell, S.J., Rock, R.E., Stylianou, S., Brennan, K.R., Bundred, N.J., and Clarke, R.B. (2010). Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. Cancer Res. *70*, 709–718.
176. Hartman, Z.C., Yang, X.-Y., Glass, O., Lei, G., Osada, T., Dave, S.S., Morse, M.A., Clay, T.M., and Lyerly, H.K. (2011). HER2 overexpression elicits a pro-inflammatory IL-6 autocrine signaling loop that is critical for tumorigenesis. Cancer Res. *71*, 4380–4391.
177. Hawthorne, V.S., Huang, W.-C., Neal, C.L., Tseng, L.-M., Hung, M.-C., and Yu, D. (2009). ErbB2-mediated Src and STAT3 Activation Leads to Transcriptional Upregulation of p21(Cip1) and Chemoresistance in Breast Cancer Cells. Mol. Cancer Res. *7*, 592–600.
178. Hazan, R., Margolis, B., Dombalagian, M., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1990). Identification of autophosphorylation sites of HER2/neu. Cell Growth Differ. *1*, 3–7.
179. He, M., Fu, Y., Yan, Y., Xiao, Q., Wu, H., Yao, W., Zhao, H., Zhao, L., Jiang, Q., Yu, Z., et al. (2015). The Hedgehog signalling pathway mediates drug response of MCF-7 mammosphere cells in breast cancer patients. Clin. Sci. (Lond). *129*, 809–822.
180. Heim, M.H., Kerr, I.M., Stark, G.R., and Darnell, J.E.J. (1995). Contribution of STAT SH2

groups to specific interferon signaling by the Jak-STAT pathway. Science *267*, 1347–1349.

181. Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, L.K., Stuart, R.K., Ching, C.W., et al. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature *459*, 108–112.
182. Henderson, S.T., Gao, D., Christensen, S., and Kimble, J. (1997). Functional domains of LAG-2, a putative signaling ligand for LIN-12 and GLP-1 receptors in Caenorhabditis elegans. Mol. Biol. Cell *8*, 1751–1762.
183. Hernandez-Vargas, H., Ouzounova, M., Le Calvez-Kelm, F., Lambert, M.-P., McKay-Chopin, S., Tavtigian, S. V, Puisieux, A., Matar, C., and Herceg, Z. (2011). Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. Epigenetics *6*, 428–439.
184. Herranz, D., Ambesi-Impiombato, A., Palomero, T., Schnell, S.A., Belver, L., Wendorff, A.A., Xu, L., Castillo-Martin, M., Llobet-Navas, D., Cordon-Cardo, C., et al. (2014). A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. Nat. Med. *20*, 1130–1137.
185. Hnisz, D., Abraham, B.J., Lee, T.I., Lau, A., Saint-André, V., Sigova, A.A., Hoke, H., and Young, R.A. (2013). Transcriptional super-enhancers connected to cell identity and disease. Cell *155*.
186. Hnisz, D., Schuijers, J., Lin, C.Y., Weintraub, A.S., Abraham, B.J., Lee, T.I., Bradner, J.E., and Young, R.A. (2015). Convergence of developmental and oncogenic signaling pathways at transcriptional super-enhancers. Mol. Cell *58*, 362–370.
187. Hodges, E., Smith, A.D., Kendall, J., Xuan, Z., Ravi, K., Rooks, M., Zhang, M.Q., Ye, K., Bhattacharjee, A., Brizuela, L., et al. (2009). High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res. *19*, 1593–1605.
188. Hon, G.C., Hawkins, R.D., Caballero, O.L., Lo, C., Lister, R., Pelizzola, M., Valsesia, A., Ye, Z., Kuan, S., Edsall, L.E., et al. (2012). Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. *22*, 246–258.
189. Honeth, G., Bendahl, P.-O., Ringner, M., Saal, L.H., Gruvberger-Saal, S.K., Lovgren, K.,

Grabau, D., Ferno, M., Borg, A., and Hegardt, C. (2008). The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res. *10*, R53.

190. Horiguchi, K., Shirakihara, T., Nakano, A., Imamura, T., Miyazono, K., and Saitoh, M. (2009). Role of Ras signaling in the induction of snail by transforming growth factor-beta. J. Biol. Chem. *284*, 245–253.
191. Hsieh, J.-C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M.E., Rosenquist, T., and Holdener, B.C. (2003). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell *112*, 355–367.
192. Hsu, L.C., Chang, W.C., and Yoshida, A. (1989). Genomic structure of the human cytosolic aldehyde dehydrogenase gene. Genomics *5*, 857–865.
193. Hu, S., Sun, Y., Meng, Y., Wang, X., Yang, W., Fu, W., Guo, H., Qian, W., Hou, S., Li, B., et al. (2015). Molecular architecture of the ErbB2 extracellular domain homodimer. Oncotarget *6*, 1695–1706.
194. Hughes, J.B., Berger, C., Rodland, M.S., Hasmann, M., Stang, E., and Madshus, I.H. (2009). Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization. Mol. Cancer Ther. *8*, 1885–1892.
195. Hynes, N.E., and Lane, H.A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. Nat. Rev. Cancer *5*, 341–354.
196. Hynes, N.E., and MacDonald, G. (2009). ErbB receptors and signaling pathways in cancer. Curr. Opin. Cell Biol. *21*, 177–184.
197. Ingthorsson, S., Andersen, K., Hilmarsdottir, B., Maelandsmo, G.M., Magnusson, M.K., and Gudjonsson, T. (2016). HER2 induced EMT and tumorigenicity in breast epithelial progenitor cells is inhibited by coexpression of EGFR. Oncogene *35*, 4244–4255.
198. Ithimakin, S., Day, K.C., Malik, F., Zen, Q., Dawsey, S.J., Bersano-Begey, T.F., Quraishi, A.A., Ignatoski, K.W., Daignault, S., Davis, A., et al. (2013). HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: Implications for efficacy of adjuvant trastuzumab. Cancer Res. *73*, 1635–1646.
199. Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D., and Jain, R.K. (2002). Tumour biology: herceptin acts as an anti-angiogenic cocktail. Nature *416*, 279–280.
200. Jackson, B., Brocker, C., Thompson, D.C., Black, W., Vasiliou, K., Nebert, D.W., and

Vasiliou, V. (2011). Update on the aldehyde dehydrogenase gene (ALDH) superfamily. Hum. Genomics *5*, 283–303.

201. Jenndahl, L.E., Isakson, P., and Baeckstrom, D. (2005). c-erbB2-induced epithelial-mesenchymal transition in mammary epithelial cells is suppressed by cell-cell contact and initiated prior to E-cadherin downregulation. Int. J. Oncol. *27*, 439–448.
202. Jensen, J.D., Knoop, A., Laenkholm, A. V, Grauslund, M., Jensen, M.B., Santoni-Rugiu, E., Andersson, M., and Ewertz, M. (2012). PIK3CA mutations, PTEN, and pHER2 expression and impact on outcome in HER2-positive early-stage breast cancer patients treated with adjuvant chemotherapy and trastuzumab. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *23*, 2034–2042.
203. Jeon, M., Lee, J., Nam, S.J., Shin, I., Lee, J.E., and Kim, S. (2015). Induction of fibronectin by HER2 overexpression triggers adhesion and invasion of breast cancer cells. Exp. Cell Res. *333*, 116–126.
204. Jeong, H., Kim, J., Lee, Y., Seo, J.H., Hong, S.R., and Kim, A. (2014). Neuregulin-1 induces cancer stem cell characteristics in breast cancer cell lines. Oncol. Rep. *32*, 1218–1224.
205. Jin, C., Zang, C., Wei, G., Cui, K., Peng, W., Zhao, K., and Felsenfeld, G. (2009). H3.3/H2A.Z double variant-containing nucleosomes mark “nucleosome-free regions” of active promoters and other regulatory regions. Nat. Genet. *41*, 941–945.
206. Joseph, R., Orlov, Y.L., Huss, M., Sun, W., Kong, S.L., Ukil, L., Pan, Y.F., Li, G., Lim, M., Thomsen, J.S., et al. (2010). Integrative model of genomic factors for determining binding site selection by estrogen receptor-alpha. Mol. Syst. Biol. *6*, 456.
207. Joshi, J.P., Brown, N.E., Griner, S.E., and Nahta, R. (2011). Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. Biochem. Pharmacol. *82*, 1090–1099.
208. Ju, J., Jang, K., Lee, K.-M., Kim, M., Kim, J., Yi, J.Y., Noh, D.-Y., and Shin, I. (2011). CD24 enhances DNA damage-induced apoptosis by modulating NF-kappaB signaling in CD44-expressing breast cancer cells. Carcinogenesis *32*, 1474–1483.
209. Ju, J., Yang, W., Oh, S., Nam, K., Lee, K., Noh, D., and Shin, I. (2013). HER2 stabilizes survivin while concomitantly down-regulating survivin gene transcription by suppressing Notch cleavage. Biochem. J. *451*, 123–134.

210. Jung, S.T., Kelton, W., Kang, T.H., Ng, D.T.W., Andersen, J.T., Sandlie, I., Sarkar, C.A., and Georgiou, G. (2013). Effective phagocytosis of low Her2 tumor cell lines with engineered, aglycosylated IgG displaying high FcgammaRIIa affinity and selectivity. ACS Chem. Biol. *8*, 368–375.

211. Jung, Y.H., Sauria, M.E.G., Lyu, X., Cheema, M.S., Ausio, J., Taylor, J., and Corces, V.G. (2017). Chromatin States in Mouse Sperm Correlate with Embryonic and Adult Regulatory Landscapes. Cell Rep. *18*, 1366–1382.

212. Junttila, T.T., Akita, R.W., Parsons, K., Fields, C., Lewis Phillips, G.D., Friedman, L.S., Sampath, D., and Sliwkowski, M.X. (2009). Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell *15*, 429–440.

213. Kalluri, R., and Weinberg, R.A. (2009). The basics of epithelial-mesenchymal transition. J. Clin. Invest. *119*, 1420–1428.

214. Kalous, O., Conklin, D., Desai, A.J., O'Brien, N.A., Ginther, C., Anderson, L., Cohen, D.J., Britten, C.D., Taylor, I., Christensen, J.G., et al. (2012). Dacomitinib (PF-00299804), an irreversible Pan-HER inhibitor, inhibits proliferation of HER2-amplified breast cancer cell lines resistant to trastuzumab and lapatinib. Mol. Cancer Ther. *11*, 1978–1987.

215. Kataoka, Y., Mukohara, T., Shimada, H., Saijo, N., Hirai, M., and Minami, H. (2010). Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *21*, 255–262.

216. Kawajiri, H., Takashima, T., Kashiwagi, S., Noda, S., Onoda, N., and Hirakawa, K. (2015). Pertuzumab in combination with trastuzumab and docetaxel for HER2-positive metastatic breast cancer. Expert Rev. Anticancer Ther. *15*, 17–26.

217. Kawasaki, Y., Sakimura, A., Park, C.M., Tomaru, R., Tanaka, T., Ozawa, T., Zhou, Y., Narita, K., Kishi, H., Muraguchi, A., et al. (2016). Feedback control of ErbB2 via ERK-mediated phosphorylation of a conserved threonine in the juxtamembrane domain. Sci. Rep. *6*, 31502.

218. Ke, Z., Caiping, S., Qing, Z., and Xiaojing, W. (2015). Sonic hedgehog-Gli1 signals promote epithelial-mesenchymal transition in ovarian cancer by mediating PI3K/AKT pathway. Med. Oncol. *32*, 368.

219. Kebenko, M., Drenckhan, A., Gros, S.J., Jucker, M., Grabinski, N., Ewald, F., Grottke, A., Schultze, A., Izbicki, J.R., Bokemeyer, C., et al. (2015). ErbB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma: identification of novel targets for concerted therapy concepts. Cell. Signal. *27*, 373–381.

220. Kern, D., Regl, G., Hofbauer, S.W., Altenhofer, P., Achatz, G., Dlugosz, A., Schnidar, H., Greil, R., Hartmann, T.N., and Aberger, F. (2015). Hedgehog/GLI and PI3K signaling in the initiation and maintenance of chronic lymphocytic leukemia. Oncogene *34*, 5341–5351.

221. Khalil, H.S., Langdon, S.P., Goltsov, A., Soininen, T., Harrison, D.J., Bown, J., and Deeni, Y.Y. (2016). A novel mechanism of action of HER2 targeted immunotherapy is explained by inhibition of NRF2 function in ovarian cancer cells. Oncotarget *7*, 75874–75901.

222. Khalil, S., Tan, G.A., Giri, D.D., Zhou, X.K., and Howe, L.R. (2012). Activation status of Wnt/ss-catenin signaling in normal and neoplastic breast tissues: relationship to HER2/neu expression in human and mouse. PLoS One *7*, e33421.

223. Khramtsov, A.I., Khramtsova, G.F., Tretiakova, M., Huo, D., Olopade, O.I., and Goss, K.H. (2010). Wnt/β-Catenin Pathway Activation Is Enriched in Basal-Like Breast Cancers and Predicts Poor Outcome. Am. J. Pathol. *176*, 2911–2920.

224. Kim, E.K., Kim, K.A., Lee, C.Y., and Shim, H.S. (2017). The frequency and clinical impact of HER2 alterations in lung adenocarcinoma. PLoS One *12*, e0171280.

225. Kim, H., Choi, J.-A., and Kim, J.-H. (2014). Ras promotes transforming growth factor-beta (TGF-beta)-induced epithelial-mesenchymal transition via a leukotriene B4 receptor-2-linked cascade in mammary epithelial cells. J. Biol. Chem. *289*, 22151–22160.

226. Kim, K., Lu, Z., and Hay, E.D. (2002). Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. Cell Biol. Int. *26*, 463–476.

227. Kim, R.-K., Kaushik, N., Suh, Y., Yoo, K.-C., Cui, Y.-H., Kim, M.-J., Lee, H.-J., Kim, I.-G., and Lee, S.-J. (2016). Radiation driven epithelial-mesenchymal transition is mediated by Notch signaling in breast cancer. Oncotarget.

228. Kim, T.-K., Hemberg, M., and Gray, J.M. (2015). Enhancer RNAs: a class of long noncoding RNAs synthesized at enhancers. Cold Spring Harb. Perspect. Biol. *7*, a018622.

229. King, C.R., Borrello, I., Porter, L., Comoglio, P., and Schlessinger, J. (1989). Ligand-independent tyrosine phosphorylation of EGF receptor and the erbB-2/neu proto-oncogene product is induced by hyperosmotic shock. Oncogene *4*, 13–18.

230. Kinzler, K.W., and Vogelstein, B. (1990). The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol. Cell. Biol. *10*, 634–642.
231. Kinzler, K.W., Ruppert, J.M., Bigner, S.H., and Vogelstein, B. (1988). The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature *332*, 371–374.
232. Klapper, L.N., Waterman, H., Sela, M., and Yarden, Y. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. Cancer Res. *60*, 3384–3388.
233. Köhrmann, A., Kammerer, U., Kapp, M., Dietl, J., and Anacker, J. (2009). Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer *9*, 188.
234. Koinuma, D., Tsutsumi, S., Kamimura, N., Taniguchi, H., Miyazawa, K., Sunamura, M., Imamura, T., Miyazono, K., and Aburatani, H. (2009). Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor beta signaling. Mol. Cell. Biol. *29*, 172–186.
235. Kondo, M., Cubillo, E., Tobiume, K., Shirakihara, T., Fukuda, N., Suzuki, H., Shimizu, K., Takehara, K., Cano, A., Saitoh, M., et al. (2004). A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal transdifferentiation. Cell Death Differ. *11*, 1092–1101.
236. Koninki, K., Barok, M., Tanner, M., Staff, S., Pitkanen, J., Hemmila, P., Ilvesaro, J., and Isola, J. (2010). Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. Cancer Lett. *294*, 211–219.
237. Korkaya, H., Paulson, A., Iovino, F., and Wicha, M.S. (2008). HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene *27*, 6120–6130.
238. Korpal, M., Lee, E.S., Hu, G., and Kang, Y. (2008). The miR-200 Family Inhibits Epithelial-Mesenchymal Transition and Cancer Cell Migration by Direct Targeting of E-cadherin Transcriptional Repressors ZEB1 and ZEB2. J. Biol. Chem. *283*, 14910–14914.
239. Kostyal, D., Welt, R.S., Danko, J., Shay, T., Lanning, C., Horton, K., and Welt, S. (2012). Trastuzumab and lapatinib modulation of HER2 tyrosine/threonine phosphorylation and cell signaling. Med. Oncol. *29*, 1486–1494.
240. Kovacs, E., Zorn, J.A., Huang, Y., Barros, T., and Kuriyan, J. (2015). A structural

perspective on the regulation of the epidermal growth factor receptor. Annu. Rev. Biochem. *84*, 739–764.

241. Kowanetz, M., Valcourt, U., Bergstrom, R., Heldin, C.-H., and Moustakas, A. (2004). Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. Mol. Cell. Biol. *24*, 4241–4254.
242. Kuleshov, M. V, Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., et al. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. *44*, W90-7.
243. Kulkarni, S., Saju, L., Farver, C., and Tubbs, R. (2012). Calpain4 is required for activation of HER2 in breast cancer cells exposed to trastuzumab and its suppression decreases survival and enhances response. Int. J. Cancer *131*, 2420–2432.
244. Kute, T., Stehle, J.R.J., Ornelles, D., Walker, N., Delbono, O., and Vaughn, J.P. (2012). Understanding key assay parameters that affect measurements of trastuzumab-mediated ADCC against Her2 positive breast cancer cells. Oncoimmunology *1*, 810–821.
245. Lamb, R., Ablett, M.P., Spence, K., Landberg, G., Sims, A.H., and Clarke, R.B. (2013). Wnt pathway activity in breast cancer sub-types and stem-like cells. PLoS One *8*, e67811.
246. Lamouille, S., and Derynck, R. (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J. Cell Biol. *178*, 437–451.
247. Lamouille, S., Connolly, E., Smyth, J.W., Akhurst, R.J., and Derynck, R. (2012). TGF-beta-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. J. Cell Sci. *125*, 1259–1273.
248. Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. Nat. Rev. Mol. Cell Biol. *15*, 178–196.
249. Lanske, B., Karaplis, A.C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L.H., Ho, C., Mulligan, R.C., et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science *273*, 663–666.
250. Le, X.-F., Bedrosian, I., Mao, W., Murray, M., Lu, Z., Keyomarsi, K., Lee, M.-H., Zhao, J., and Bast, R.C.J. (2006). Anti-HER2 antibody trastuzumab inhibits CDK2-mediated

NPAT and histone H4 expression via the PI3K pathway. Cell Cycle *5*, 1654–1661.

251. Lee, C.Y.-F., Lin, Y., Bratman, S. V, Feng, W., Kuo, A.H., Scheeren, F.A., Engreitz, J.M., Varma, S., West, R.B., and Diehn, M. (2014). Neuregulin autocrine signaling promotes self-renewal of breast tumor-initiating cells by triggering HER2/HER3 activation. Cancer Res. *74*, 341–352.
252. Lehmann, B.D., Bauer, J.A., Chen, X., Sanders, M.E., Chakravarthy, A.B., Shyr, Y., and Pietenpol, J.A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J. Clin. Invest. *121*, 2750–2767.
253. Lemmon, M.A. (2009). Ligand-induced ErbB receptor dimerization. Exp. Cell Res. *315*, 638–648.
254. Lemmon, M.A., and Schlessinger, J. (2010). Cell signaling by receptor-tyrosine kinases. Cell *141*, 1117–1134.
255. Lendeckel, U., Kohl, J., Arndt, M., Carl-McGrath, S., Donat, H., and Rocken, C. (2005). Increased expression of ADAM family members in human breast cancer and breast cancer cell lines. J. Cancer Res. Clin. Oncol. *131*, 41–48.
256. Leong, K.G., Niessen, K., Kulic, I., Raouf, A., Eaves, C., Pollet, I., and Karsan, A. (2007). Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. J. Exp. Med. *204*, 2935–2948.
257. Lesniak, D., Sabri, S., Xu, Y., Graham, K., Bhatnagar, P., Suresh, M., and Abdulkarim, B. (2013). Spontaneous Epithelial-Mesenchymal Transition and Resistance to HER-2-Targeted Therapies in HER-2-Positive Luminal Breast Cancer. PLoS One *8*.
258. Li, J.Y., Perry, S.R., Muniz-Medina, V., Wang, X., Wetzel, L.K., Rebelatto, M.C., Hinrichs, M.J.M., Bezabeh, B.Z., Fleming, R.L., Dimasi, N., et al. (2016). A Biparatopic HER2-Targeting Antibody-Drug Conjugate Induces Tumor Regression in Primary Models Refractory to or Ineligible for HER2-Targeted Therapy. Cancer Cell *29*, 117–129.
259. Li, L.-Y., Chen, H., Hsieh, Y.-H., Wang, Y.-N., Chu, H.-J., Chen, Y.-H., Chen, H.-Y., Chien, P.-J., Ma, H.-T., Tsai, H.-C., et al. (2011). Nuclear ErbB2 enhances translation and cell growth by activating transcription of ribosomal RNA genes. Cancer Res. *71*, 4269–4279.
260. Li, X., Lewis, M.T., Huang, J., Gutierrez, C., Osborne, C.K., Wu, M.-F., Hilsenbeck, S.G., Pavlick, A., Zhang, X., Chamness, G.C., et al. (2008). Intrinsic resistance of tumorigenic

breast cancer cells to chemotherapy. J. Natl. Cancer Inst. *100*, 672–679.

261. Lin, L., Hutzen, B., Lee, H.-F., Peng, Z., Wang, W., Zhao, C., Lin, H.-J., Sun, D., Li, P.-K., Li, C., et al. (2013). Evaluation of STAT3 signaling in ALDH+ and ALDH+/CD44+/CD24- subpopulations of breast cancer cells. PLoS One *8*, e82821.
262. Lipton, A., Goodman, L., Leitzel, K., Cook, J., Sperinde, J., Haddad, M., Kostler, W.J., Huang, W., Weidler, J.M., Ali, S., et al. (2013). HER3, p95HER2, and HER2 protein expression levels define multiple subtypes of HER2-positive metastatic breast cancer. Breast Cancer Res. Treat. *141*, 43–53.
263. Liu, H., Patel, M.R., Prescher, J.A., Patsialou, A., Qian, D., Lin, J., Wen, S., Chang, Y.-F., Bachmann, M.H., Shimono, Y., et al. (2010). Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. Proc. Natl. Acad. Sci. U. S. A. *107*, 18115–18120.
264. Liu, J., Chen, X., Mao, Y., Qu, Q., and Shen, K. (2014). Association of epithelial-mesenchymal transition with lapatinib resistance through multipe pathways activation in HER2-positive breast cancer. J Clin Oncol , 2014 *32*, (suppl; abstr e11579).
265. Liu, J.C., Voisin, V., Bader, G.D., Deng, T., Pusztai, L., Symmans, W.F., Esteva, F.J., Egan, S.E., and Zacksenhaus, E. (2012). Seventeen-gene signature from enriched Her2/Neu mammary tumor-initiating cells predicts clinical outcome for human HER2(+):ERα(−) breast cancer. Proc. Natl. Acad. Sci. U. S. A. *109*, 5832–5837.
266. Liu, L., Greger, J., Shi, H., Liu, Y., Greshock, J., Annan, R., Halsey, W., Sathe, G.M., Martin, A.-M., and Gilmer, T.M. (2009). Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res. *69*, 6871–6878.
267. Liu, P.C.C., Liu, X., Li, Y., Covington, M., Wynn, R., Huber, R., Hillman, M., Yang, G., Ellis, D., Marando, C., et al. (2006a). Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells. Cancer Biol. Ther. *5*, 657–664.
268. Liu, S., Dontu, G., Mantle, I.D., Patel, S., Ahn, N., Jackson, K.W., Suri, P., and Wicha, M.S. (2006b). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res. *66*, 6063–6071.
269. Loibl, S., and Gianni, L. (2017). HER2-positive breast cancer. Lancet (London, England) *389*, 2415–2429.

270. Longva, K.E., Pedersen, N.M., Haslekas, C., Stang, E., and Madshus, I.H. (2005). Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. Int. J. Cancer *116*, 359–367.

271. Loven, J., Hoke, H.A., Lin, C.Y., Lau, A., Orlando, D.A., Vakoc, C.R., Bradner, J.E., Lee, T.I., and Young, R.A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell *153*, 320–334.

272. Lu, Y., Ma, W., Mao, J., Yu, X., Hou, Z., Fan, S., Song, B., Wang, H., Li, J., Kang, L., et al. (2015). Salinomycin exerts anticancer effects on human breast carcinoma MCF-7 cancer stem cells via modulation of Hedgehog signaling. Chem. Biol. Interact. *228*, 100–107.

273. Lu, Y., Gao, Y., Tao, R., Liao, M., Huang, J., Wu, G., Han, F., and Li, X. (2016). Calpain-Dependent ErbB4 Cleavage Is Involved in Brain Ischemia-Induced Neuronal Death. Mol. Neurobiol. *53*, 2600–2609.

274. Lucs, A. V, Muller, W.J., and Muthuswamy, S.K. (2010). Shc is required for ErbB2-induced inhibition of apoptosis but is dispensable for cell proliferation and disruption of cell polarity. Oncogene *29*, 174–187.

275. Ma, I., and Allan, A.L. (2011). The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev. *7*, 292–306.

276. Maadi, H., Nami, B., and Wang, Z. (2017). Dimerization Assessment of Epithelial Growth Factor Family of Receptor Tyrosine Kinases by Using Cross-Linking Reagent. Methods Mol. Biol. *1652*, 101–108.

277. Maadi, H., Nami, B., Tong, J., Li, G., and Wang, Z. (2018). The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2. BMC Cancer *18*, 238.

278. Mackay, A., Jones, C., Dexter, T., Silva, R., Bulmer, K., Jones, A., Simpson, P., Harris, R., Jat, P., Neville, A., et al. (2003). DNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. Oncogene *22*, 2680–2688.

279. Magnifico, A., Albano, L., Campaner, S., Delia, D., Castiglioni, F., Gasparini, P., Sozzi, G., Fontanella, E., Menard, S., and Tagliabue, E. (2009). Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to

trastuzumab. Clin. Cancer Res. *15*, 2010–2021.

280. Majumder, M., Xin, X., Liu, L., Tutunea-Fatan, E., Rodriguez-Torres, M., Vincent, K., Postovit, L.-M., Hess, D., and Lala, P.K. (2016). COX-2 Induces Breast Cancer Stem Cells via EP4/PI3K/AKT/NOTCH/WNT Axis. Stem Cells *34*, 2290–2305.
281. Mamaeva, V., Niemi, R., Beck, M., Ozliseli, E., Desai, D., Landor, S., Gronroos, T., Kronqvist, P., Pettersen, I.K.N., McCormack, E., et al. (2016). Inhibiting Notch Activity in Breast Cancer Stem Cells by Glucose Functionalized Nanoparticles Carrying gamma-secretase Inhibitors. Mol. Ther. *24*, 926–936.
282. Mani, S.A., Guo, W., Liao, M.-J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133*, 704–715.
283. Mansour, M.R., Abraham, B.J., Anders, L., Berezovskaya, A., Gutierrez, A., Durbin, A.D., Etchin, J., Lawton, L., Sallan, S.E., Silverman, L.B., et al. (2014). Oncogene regulation. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. Science *346*, 1373–1377.
284. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H. 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., et al. (2001). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell *7*, 801–809.
285. Marchetti, A., Colletti, M., Cozzolino, A.M., Steindler, C., Lunadei, M., Mancone, C., and Tripodi, M. (2008). ERK5/MAPK is activated by TGFbeta in hepatocytes and required for the GSK-3beta-mediated Snail protein stabilization. Cell. Signal. *20*, 2113–2118.
286. Marone, R., Hess, D., Dankort, D., Muller, W.J., Hynes, N.E., and Badache, A. (2004). Memo mediates ErbB2-driven cell motility. Nat. Cell Biol. *6*, 515–522.
287. Marotta, L.L.C., Almendro, V., Marusyk, A., Shipitsin, M., Schemme, J., Walker, S.R., Bloushtain-Qimron, N., Kim, J.J., Choudhury, S.A., Maruyama, R., et al. (2011). The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(-) stem cell-like breast cancer cells in human tumors. J. Clin. Invest. *121*, 2723–2735.
288. Marty, M., Cognetti, F., Maraninchi, D., Snyder, R., Mauriac, L., Tubiana-Hulin, M., Chan, S., Grimes, D., Anton, A., Lluch, A., et al. (2005). Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-

line treatment: the M77001 study group. J. Clin. Oncol. *23*, 4265–4274.

289. Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G.P., Bartunik, H., Ellestad, G.A., Reddy, P., Wolfson, M.F., Rauch, C.T., Castner, B.J., et al. (1998). Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. Proc. Natl. Acad. Sci. U. S. A. *95*, 3408–3412.
290. Massague, J. (2012). TGFbeta signalling in context. Nat. Rev. Mol. Cell Biol. *13*, 616–630.
291. Masuda, H., Zhang, D., Bartholomeusz, C., Doihara, H., Hortobagyi, G.N., and Ueno, N.T. (2012). Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res. Treat. *136*, 331–345.
292. Matevossian, A., and Resh, M.D. (2015). Hedgehog Acyltransferase as a target in estrogen receptor positive, HER2 amplified, and tamoxifen resistant breast cancer cells. Mol. Cancer *14*.
293. De Mattos-Arruda, L., and Cortes, J. (2013). Use of pertuzumab for the treatment of HER2-positive metastatic breast cancer. Adv. Ther. *30*, 645–658.
294. McGowan, P.M., Ryan, B.M., Hill, A.D.K., McDermott, E., O'Higgins, N., and Duffy, M.J. (2007). ADAM-17 expression in breast cancer correlates with variables of tumor progression. Clin. Cancer Res. *13*, 2335–2343.
295. McGowan, P.M., Simedrea, C., Ribot, E.J., Foster, P.J., Palmieri, D., Steeg, P.S., Allan, A.L., and Chambers, A.F. (2011). Notch1 inhibition alters the CD44hi/CD24lo population and reduces the formation of brain metastases from breast cancer. Mol. Cancer Res. *9*, 834–844.
296. Mei, S., Qin, Q., Wu, Q., Sun, H., Zheng, R., Zang, C., Zhu, M., Wu, J., Shi, X., Taing, L., et al. (2017). Cistrome Data Browser: a data portal for ChIP-Seq and chromatin accessibility data in human and mouse. Nucleic Acids Res. *45*, D658-62.
297. Memmi, E.M., Sanarico, A.G., Giacobbe, A., Peschiaroli, A., Frezza, V., Cicalese, A., Pisati, F., Tosoni, D., Zhou, H., Tonon, G., et al. (2015). p63 Sustains self-renewal of mammary cancer stem cells through regulation of Sonic Hedgehog signaling. Proc. Natl. Acad. Sci. U. S. A. *112*, 3499–3504.
298. Mendrola, J.M., Berger, M.B., King, M.C., and Lemmon, M.A. (2002). The single transmembrane domains of ErbB receptors self-associate in cell membranes. J. Biol. Chem. *277*, 4704–4712.

299. Meran, S., Luo, D.D., Simpson, R., Martin, J., Wells, A., Steadman, R., and Phillips, A.O. (2011). Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. J. Biol. Chem. *286*, 17618–17630.

300. Mercogliano, M.F., De Martino, M., Venturutti, L., Rivas, M.A., Proietti, C.J., Inurrigarro, G., Frahm, I., Allemand, D.H., Deza, E.G., Ares, S., et al. (2017). TNFalpha-Induced Mucin 4 Expression Elicits Trastuzumab Resistance in HER2-Positive Breast Cancer. Clin. Cancer Res. *23*, 636–648.

301. Merdad, A., Karim, S., Schulten, H.-J., Dallol, A., Buhmeida, A., Al-Thubaity, F., Gari, M.A., Chaudhary, A.G., Abuzenadah, A.M., and Al-Qahtani, M.H. (2014). Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res. *34*, 1355–1366.

302. Merilahti, J.A.M., and Elenius, K. (2019). Gamma-secretase-dependent signaling of receptor tyrosine kinases. Oncogene *38*, 151–163.

303. Merilahti, J.A.M., Ojala, V.K., Knittle, A.M., Pulliainen, A.T., and Elenius, K. (2017). Genome-wide screen of gamma-secretase-mediated intramembrane cleavage of receptor tyrosine kinases. Mol. Biol. Cell *28*, 3123–3131.

304. Messier, T.L., Gordon, J.A.R., Boyd, J.R., Tye, C.E., Browne, G., Stein, J.L., Lian, J.B., and Stein, G.S. (2016). Histone H3 lysine 4 acetylation and methylation dynamics define breast cancer subtypes. Oncotarget *7*, 5094–5109.

305. Mielgo, A., Seguin, L., Huang, M., Camargo, M.F., Anand, S., Franovic, A., Weis, S.M., Advani, S.J., Murphy, E.A., and Cheresh, D.A. (2011). A MEK-independent role for CRAF in mitosis and tumor progression. Nat. Med. *17*, 1641–1645.

306. von Mikecz, A., Zhang, S., Montminy, M., Tan, E.M., and Hemmerich, P. (2000). CREB-binding protein (CBP)/p300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. J. Cell Biol. *150*, 265–273.

307. Mikels, A.J., and Nusse, R. (2006). Wnts as ligands: processing, secretion and reception. Oncogene *25*, 7461–7468.

308. von Minckwitz, G., Procter, M., de Azambuja, E., Zardavas, D., Benyunes, M., Viale, G., Suter, T., Arahmani, A., Rouchet, N., Clark, E., et al. (2017). Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer. N. Engl. J. Med. *377*, 122–131.

309. Mitsui, Y., Mochizuki, S., Kodama, T., Shimoda, M., Ohtsuka, T., Shiomi, T., Chijiiwa, M., Ikeda, T., Kitajima, M., and Okada, Y. (2006). ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res. *66*, 9913–9920.

310. Mittal, S., Subramanyam, D., Dey, D., Kumar, R. V, and Rangarajan, A. (2009). Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. Mol. Cancer *8*, 128.

311. Mittendorf, E.A., Wu, Y., Scaltriti, M., Meric-Bernstam, F., Hunt, K.K., Dawood, S., Esteva, F.J., Buzdar, A.U., Chen, H., Eksambi, S., et al. (2009). Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. Clin. Cancer Res. *15*, 7381–7388.

312. Modi, S., D'Andrea, G., Norton, L., Yao, T.J., Caravelli, J., Rosen, P.P., Hudis, C., and Seidman, A.D. (2006). A phase I study of cetuximab/paclitaxel in patients with advanced-stage breast cancer. Clin. Breast Cancer *7*, 270–277.

313. Mohsin, S.K., Weiss, H.L., Gutierrez, M.C., Chamness, G.C., Schiff, R., Digiovanna, M.P., Wang, C.-X., Hilsenbeck, S.G., Osborne, C.K., Allred, D.C., et al. (2005). Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. J. Clin. Oncol. *23*, 2460–2468.

314. Molina, M.A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., and Baselga, J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res. *61*, 4744–4749.

315. Molina, M.A., Saez, R., Ramsey, E.E., Garcia-Barchino, M.-J., Rojo, F., Evans, A.J., Albanell, J., Keenan, E.J., Lluch, A., Garcia-Conde, J., et al. (2002). NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. Clin. Cancer Res. *8*, 347–353.

316. Morel, A.-P., Lievre, M., Thomas, C., Hinkal, G., Ansieau, S., and Puisieux, A. (2008). Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One *3*, e2888.

317. Mori, S., Kodaira, M., Ito, A., Okazaki, M., Kawaguchi, N., Hamada, Y., Takada, Y., and Matsuura, N. (2015). Enhanced Expression of Integrin alphavbeta3 Induced by TGF-beta

Is Required for the Enhancing Effect of Fibroblast Growth Factor 1 (FGF1) in TGF-beta-Induced Epithelial-Mesenchymal Transition (EMT) in Mammary Epithelial Cells. PLoS One *10*, e0137486.

318. Moriki, T., Maruyama, H., and Maruyama, I.N. (2001). Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. J. Mol. Biol. *311*, 1011–1026.
319. Morita, T., Mayanagi, T., and Sobue, K. (2007). Dual roles of myocardin-related transcription factors in epithelial mesenchymal transition via slug induction and actin remodeling. J. Cell Biol. *179*, 1027–1042.
320. Mullen, P., Cameron, D.A., Hasmann, M., Smyth, J.F., and Langdon, S.P. (2007). Sensitivity to pertuzumab (2C4) in ovarian cancer models: cross-talk with estrogen receptor signaling. Mol. Cancer Ther. *6*, 93–100.
321. Mullooly, M., McGowan, P.M., Kennedy, S.A., Madden, S.F., Crown, J., O' Donovan, N., and Duffy, M.J. (2015). ADAM10: a new player in breast cancer progression? Br. J. Cancer *113*, 945–951.
322. Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., and Kopan, R. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol. Cell *5*, 197–206.
323. Munch, R.C., Muhlebach, M.D., Schaser, T., Kneissl, S., Jost, C., Pluckthun, A., Cichutek, K., and Buchholz, C.J. (2011). DARPins: an efficient targeting domain for lentiviral vectors. Mol. Ther. *19*, 686–693.
324. Muraoka, R.S., Koh, Y., Roebuck, L.R., Sanders, M.E., Brantley-Sieders, D., Gorska, A.E., Moses, H.L., and Arteaga, C.L. (2003). Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. Mol. Cell. Biol. *23*, 8691–8703.
325. Musolino, A., Naldi, N., Bortesi, B., Pezzuolo, D., Capelletti, M., Missale, G., Laccabue, D., Zerbini, A., Camisa, R., Bisagni, G., et al. (2008). Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J. Clin. Oncol. *26*, 1789–1796.
326. Nagata, Y., Lan, K.-H., Zhou, X., Tan, M., Esteva, F.J., Sahin, A.A., Klos, K.S., Li, P., Monia, B.P., Nguyen, N.T., et al. (2004). PTEN activation contributes to tumor inhibition

by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell *6*, 117–127.

327. Nagy, P., Jenei, A., Kirsch, A.K., Szollosi, J., Damjanovich, S., and Jovin, T.M. (1999). Activation-dependent clustering of the erbB2 receptor tyrosine kinase detected by scanning near-field optical microscopy. J. Cell Sci. *112 (Pt 1*, 1733–1741.
328. Nahta, R., and O'Regan, R.M. (2010). Evolving strategies for overcoming resistance to HER2-directed therapy: targeting the PI3K/Akt/mTOR pathway. Clin. Breast Cancer *10 Suppl 3*, S72-8.
329. Nahta, R., Takahashi, T., Ueno, N.T., Hung, M.-C., and Esteva, F.J. (2004a). P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res. *64*, 3981–3986.
330. Nahta, R., Hung, M.-C., and Esteva, F.J. (2004b). The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. *64*, 2343–2346.
331. Nami, B., and Wang, Z. (2017a). HER2 in Breast Cancer Stemness: A Negative Feedback Loop towards Trastuzumab Resistance. Cancers (Basel). *9*.
332. Nami, B., and Wang, Z. (2017b). Application of Immunofluorescence Staining to Study ErbB Family of Receptor Tyrosine Kinases. Methods Mol. Biol. *1652*, 109–116.
333. Nami, B., Donmez, H., and Kocak, N. (2016a). Tunicamycin-induced endoplasmic reticulum stress reduces in vitro subpopulation and invasion of CD44+/CD24- phenotype breast cancer stem cells. Exp. Toxicol. Pathol.
334. Nami, B., Ghasemi-Dizgah, A., and Vaseghi, A. (2016b). Overexpression of molecular chaperons GRP78 and GRP94 in CD44hi/CD24lo breast cancer stem cells. Bioimpacts *6*, 105–110.
335. Nami, B., Maadi, H., and Wang, Z. (2018). Mechanisms Underlying the Action and Synergism of Trastuzumab and Pertuzumab in Targeting HER2-Positive Breast Cancer. Cancers (Basel). *10*.
336. Nami, B., Maadi, H., and Wang, Z. (2019). The Effects of Pertuzumab and Its Combination with Trastuzumab on HER2 Homodimerization and Phosphorylation. Cancers (Basel). *11*.
337. Naor, D., Sionov, R. V, and Ish-Shalom, D. (1997). CD44: structure, function, and

association with the malignant process. Adv. Cancer Res. *71*, 241–319.

338. Ni, C.Y., Murphy, M.P., Golde, T.E., and Carpenter, G. (2001). gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science *294*, 2179–2181.
339. Ni, M., Chen, Y., Fei, T., Li, D., Lim, E., Liu, X.S., and Brown, M. (2013). Amplitude modulation of androgen signaling by c-MYC. Genes Dev. *27*, 734–748.
340. Niessen, K., Fu, Y., Chang, L., Hoodless, P.A., McFadden, D., and Karsan, A. (2008). Slug is a direct Notch target required for initiation of cardiac cushion cellularization. J. Cell Biol. *182*, 315–325.
341. Niikura, N., Liu, J., Hayashi, N., Mittendorf, E.A., Gong, Y., Palla, S.L., Tokuda, Y., Gonzalez-Angulo, A.M., Hortobagyi, G.N., and Ueno, N.T. (2012). Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. J. Clin. Oncol. *30*, 593–599.
342. Nilsson, G.M.A., Akhtar, N., Kannius-Janson, M., and Baeckstrom, D. (2014). Loss of E-cadherin expression is not a prerequisite for c-erbB2-induced epithelial-mesenchymal transition. Int. J. Oncol. *45*, 82–94.
343. Nord, A.S., Blow, M.J., Attanasio, C., Akiyama, J.A., Holt, A., Hosseini, R., Phouanenavong, S., Plajzer-Frick, I., Shoukry, M., Afzal, V., et al. (2013). Rapid and pervasive changes in genome-wide enhancer usage during mammalian development. Cell *155*, 1521–1531.
344. Noseda, M., McLean, G., Niessen, K., Chang, L., Pollet, I., Montpetit, R., Shahidi, R., Dorovini-Zis, K., Li, L., Beckstead, B., et al. (2004). Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ. Res. *94*, 910–917.
345. O'Brien, N.A., McDonald, K., Tong, L., von Euw, E., Kalous, O., Conklin, D., Hurvitz, S.A., di Tomaso, E., Schnell, C., Linnartz, R., et al. (2014). Targeting PI3K/mTOR overcomes resistance to HER2-targeted therapy independent of feedback activation of AKT. Clin. Cancer Res. *20*, 3507–3520.
346. O'Shea, C., McKie, N., Buggy, Y., Duggan, C., Hill, A.D.K., McDermott, E., O'Higgins, N., and Duffy, M.J. (2003). Expression of ADAM-9 mRNA and protein in human breast cancer. Int. J. Cancer *105*, 754–761.

347. Ohnishi, Y., Yasui, H., Kakudo, K., and Nozaki, M. (2016). Lapatinib-resistant cancer cells possessing epithelial cancer stem cell properties develop sensitivity during sphere formation by activation of the ErbB/AKT/cyclin D2 pathway. Oncol. Rep. *36*, 3058–3064.

348. Okano, J., Gaslightwala, I., Birnbaum, M.J., Rustgi, A.K., and Nakagawa, H. (2000). Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. J. Biol. Chem. *275*, 30934–30942.

349. Olayioye, M.A., Beuvink, I., Horsch, K., Daly, J.M., and Hynes, N.E. (1999). ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J. Biol. Chem. *274*, 17209–17218.

350. Oliveras-Ferraros, C., Vazquez-Martin, A., Martin-Castillo, B., Cufi, S., Del Barco, S., Lopez-Bonet, E., Brunet, J., and Menendez, J.A. (2010). Dynamic emergence of the mesenchymal CD44(pos)CD24(neg/low) phenotype in HER2-gene amplified breast cancer cells with de novo resistance to trastuzumab (Herceptin). Biochem. Biophys. Res. Commun. *397*, 27–33.

351. Oliveras-Ferraros, C., Corominas-Faja, B., Cufi, S., Vazquez-Martin, A., Martin-Castillo, B., Iglesias, J.M., Lopez-Bonet, E., Martin, A.G., and Menendez, J.A. (2012). Epithelial-to-mesenchymal transition (EMT) confers primary resistance to trastuzumab (Herceptin). Cell Cycle *11*, 4020–4032.

352. Opyrchal, M., Salisbury, J.L., Iankov, I., Goetz, M.P., McCubrey, J., Gambino, M.W., Malatino, L., Puccia, G., Ingle, J.N., Galanis, E., et al. (2014). Inhibition of Cdk2 kinase activity selectively targets the CD44(+)/CD24(-)/Low stem-like subpopulation and restores chemosensitivity of SUM149PT triple-negative breast cancer cells. Int. J. Oncol. *45*, 1193–1199.

353. Osipo, C., Patel, P., Rizzo, P., Clementz, A.G., Hao, L., Golde, T.E., and Miele, L. (2008). ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor. Oncogene *27*, 5019–5032.

354. Oskarsson, T., Acharyya, S., Zhang, X.H.-F., Vanharanta, S., Tavazoie, S.F., Morris, P.G., Downey, R.J., Manova-Todorova, K., Brogi, E., and Massagué, J. (2011). Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. Nat. Med. *17*, 867–874.

355. Ounzain, S., and Pedrazzini, T. (2016). Super-enhancer lncs to cardiovascular development

and disease. Biochim. Biophys. Acta *1863*, 1953–1960.

356. Pandya, K., Meeke, K., Clementz, A.G., Rogowski, A., Roberts, J., Miele, L., Albain, K.S., and Osipo, C. (2011). Targeting both Notch and ErbB-2 signalling pathways is required for prevention of ErbB-2-positive breast tumour recurrence. Br. J. Cancer *105*, 796–806.
357. Pandya, K., Wyatt, D., Gallagher, B., Shah, D., Baker, A., Bloodworth, J., Zlobin, A., Pannuti, A., Green, A., Ellis, I.O., et al. (2016). PKCα Attenuates Jagged-1-mediated Notch Signaling in ErbB-2 positive Breast Cancer to Reverse Trastuzumab Resistance. Clin. Cancer Res. *22*, 175–186.
358. Pang, M.-F., Georgoudaki, A.-M., Lambut, L., Johansson, J., Tabor, V., Hagikura, K., Jin, Y., Jansson, M., Alexander, J.S., Nelson, C.M., et al. (2016). TGF-beta1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis. Oncogene *35*, 748–760.
359. Papageorgis, P. (2015). TGFβ Signaling in Tumor Initiation, Epithelial-to-Mesenchymal Transition, and Metastasis. J. Oncol. *2015*.
360. Papageorgis, P., Lambert, A.W., Ozturk, S., Gao, F., Pan, H., Manne, U., Alekseyev, Y.O., Thiagalingam, A., Abdolmaleky, H.M., Lenburg, M., et al. (2010). Smad signaling is required to maintain epigenetic silencing during breast cancer progression. Cancer Res. *70*, 968–978.
361. Papewalis, J., AYu, N., and Rajewsky, M.F. (1991). G to A polymorphism at amino acid codon 655 of the human erbB-2/HER2 gene. Nucleic Acids Res. *19*, 5452.
362. Park, J., and Schwarzbauer, J.E. (2014). Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition. Oncogene *33*, 1649–1657.
363. Park, S.-M., Gaur, A.B., Lengyel, E., and Peter, M.E. (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. *22*, 894–907.
364. Parker, S.C.J., Stitzel, M.L., Taylor, D.L., Orozco, J.M., Erdos, M.R., Akiyama, J.A., van Bueren, K.L., Chines, P.S., Narisu, N., Black, B.L., et al. (2013). Chromatin stretch enhancer states drive cell-specific gene regulation and harbor human disease risk variants. Proc. Natl. Acad. Sci. U. S. A. *110*, 17921–17926.
365. Parmantier, E., Lynn, B., Lawson, D., Turmaine, M., Namini, S.S., Chakrabarti, L., McMahon, A.P., Jessen, K.R., and Mirsky, R. (1999). Schwann cell-derived Desert

hedgehog controls the development of peripheral nerve sheaths. Neuron *23*, 713–724.

366. Pedersen, K., Angelini, P.-D., Laos, S., Bach-Faig, A., Cunningham, M.P., Ferrer-Ramon, C., Luque-Garcia, A., Garcia-Castillo, J., Parra-Palau, J.L., Scaltriti, M., et al. (2009). A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. Mol. Cell. Biol. *29*, 3319–3331.
367. Pegram, M.D., Konecny, G., and Slamon, D.J. (2000). The molecular and cellular biology of HER2/neu gene amplification/overexpression and the clinical development of herceptin (trastuzumab) therapy for breast cancer. Cancer Treat. Res. *103*, 57–75.
368. Pereira, B., Chin, S.-F., Rueda, O.M., Vollan, H.-K.M., Provenzano, E., Bardwell, H.A., Pugh, M., Jones, L., Russell, R., Sammut, S.-J., et al. (2016). The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. Nat. Commun. *7*, 11479.
369. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., et al. (2000). Molecular portraits of human breast tumours. Nature *406*, 747–752.
370. Petricevic, B., Laengle, J., Singer, J., Sachet, M., Fazekas, J., Steger, G., Bartsch, R., Jensen-Jarolim, E., and Bergmann, M. (2013). Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent in both adjuvant and metastatic HER2/neu breast cancer patients. J. Transl. Med. *11*, 307.
371. Pham, D.-H., Kim, J.-S., Kim, S.-K., Shin, D.-J., Uong, N.-T.-T., Hyun, H., Yoon, M.S., Kang, S.J., Ryu, Y.J., Cho, J.S., et al. (2017). Effects of ADAM10 and ADAM17 Inhibitors on Natural Killer Cell Expansion and Antibody-dependent Cellular Cytotoxicity Against Breast Cancer Cells In Vitro. Anticancer Res. *37*, 5507–5513.
372. Pham, P. V, Phan, N.L.C., Nguyen, N.T., Truong, N.H., Duong, T.T., Le, D. V, Truong, K.D., and Phan, N.K. (2011). Differentiation of breast cancer stem cells by knockdown of CD44: promising differentiation therapy. J. Transl. Med. *9*, 209.
373. Phillips, T.M., McBride, W.H., and Pajonk, F. (2006). The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J. Natl. Cancer Inst. *98*, 1777–1785.
374. Piccart-Gebhart, M.J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., Jackisch, C., et al. (2005). Trastuzumab after adjuvant

chemotherapy in HER2-positive breast cancer. N. Engl. J. Med. *353*, 1659–1672.

375. Pierce, B.G., Wiehe, K., Hwang, H., Kim, B.-H., Vreven, T., and Weng, Z. (2014). ZDOCK server: interactive docking prediction of protein-protein complexes and symmetric multimers. Bioinformatics *30*, 1771–1773.
376. Pietras, R.J., Pegram, M.D., Finn, R.S., Maneval, D.A., and Slamon, D.J. (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. Oncogene *17*, 2235–2249.
377. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B.J., Sela, M., et al. (1996). Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. EMBO J. *15*, 2452–2467.
378. Pivot, X., Manikhas, A., Zurawski, B., Chmielowska, E., Karaszewska, B., Allerton, R., Chan, S., Fabi, A., Bidoli, P., Gori, S., et al. (2015). CEREBEL (EGF111438): A Phase III, Randomized, Open-Label Study of Lapatinib Plus Capecitabine Versus Trastuzumab Plus Capecitabine in Patients With Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer. J. Clin. Oncol. *33*, 1564–1573.
379. Plaks, V., Brenot, A., Lawson, D.A., Linneman, J., Van Kappel, E.C., Wong, K., de Sauvage, F., Klein, O.D., and Werb, Z. (2013). Lgr5 expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell Rep. *3*, 70–78.
380. Ponta, H., Sherman, L., and Herrlich, P.A. (2003). CD44: from adhesion molecules to signalling regulators. Nat. Rev. Mol. Cell Biol. *4*, 33–45.
381. Porter, J.R., Fisher, B.E., Baranello, L., Liu, J.C., Kambach, D.M., Nie, Z., Koh, W.S., Luo, J., Stommel, J.M., Levens, D., et al. (2017). Global Inhibition with Specific Activation: How p53 and MYC Redistribute the Transcriptome in the DNA Double-Strand Break Response. Mol. Cell *67*, 1013-1025.e9.
382. Pulyaeva, H., Bueno, J., Polette, M., Birembaut, P., Sato, H., Seiki, M., and Thompson, E.W. (1997). MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. Clin. Exp. Metastasis *15*, 111–120.
383. Raab, S., Steinbacher, J., Schmiedel, B.J., Kousis, P.C., Steinle, A., Jung, G., Grosse-Hovest, L., and Salih, H.R. (2014). Fc-optimized NKG2D-Fc constructs induce NK cell

antibody-dependent cellular cytotoxicity against breast cancer cells independently of HER2/neu expression status. J. Immunol. *193*, 4261–4272.

384. Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., and Wysocka, J. (2011). A unique chromatin signature uncovers early developmental enhancers in humans. Nature *470*, 279–283.
385. Radisky, E.S., and Radisky, D.C. (2015). Matrix metalloproteinases as breast cancer drivers and therapeutic targets. Front. Biosci. (Landmark Ed. *20*, 1144–1163.
386. Ramaswamy, B., Lu, Y., Teng, K., Nuova, G., Li, X., Shapiro, C.L., and Majumder, S. (2012). Hedgehog Signaling Is a Novel Therapeutic Target in Tamoxifen-Resistant Breast Cancer Aberrantly Activated by PI3K/AKT Pathway. *72*.
387. Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., and Nathans, J. (1997). A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc. Natl. Acad. Sci. U. S. A. *94*, 2859–2863.
388. Razis, E., Bobos, M., Kotoula, V., Eleftheraki, A.G., Kalofonos, H.P., Pavlakis, K., Papakostas, P., Aravantinos, G., Rigakos, G., Efstratiou, I., et al. (2011). Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. Breast Cancer Res. Treat. *128*, 447–456.
389. Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell *67*, 687–699.
390. Rebay, I., Fehon, R.G., and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell *74*, 319–329.
391. Reibenwein, J., and Krainer, M. (2008). Targeting signaling pathways in ovarian cancer. Expert Opin. Ther. Targets *12*, 353–365.
392. Reim, F., Dombrowski, Y., Ritter, C., Buttmann, M., Hausler, S., Ossadnik, M., Krockenberger, M., Beier, D., Beier, C.P., Dietl, J., et al. (2009). Immunoselection of breast and ovarian cancer cells with trastuzumab and natural killer cells: selective escape of CD44high/CD24low/HER2low breast cancer stem cells. Cancer Res. *69*, 8058–8066.
393. Ren, Z., and Schaefer, T.S. (2002). ErbB-2 activates Stat3 alpha in a Src- and JAK2-

dependent manner. J. Biol. Chem. *277*, 38486–38493.

394. Rennstam, K., Jonsson, G., Tanner, M., Bendahl, P.-O., Staaf, J., Kapanen, A.I., Karhu, R., Baldetorp, B., Borg, A., and Isola, J. (2007). Cytogenetic characterization and gene expression profiling of the trastuzumab-resistant breast cancer cell line JIMT-1. Cancer Genet. Cytogenet. *172*, 95–106.
395. Rennstam, K., McMichael, N., Berglund, P., Honeth, G., Hegardt, C., Ryden, L., Luts, L., Bendahl, P.-O., and Hedenfalk, I. (2010). Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer. Breast Cancer Res. Treat. *122*, 315–324.
396. Rexer, B.N., Chanthaphaychith, S., Dahlman, K., and Arteaga, C.L. (2014). Direct inhibition of PI3K in combination with dual HER2 inhibitors is required for optimal antitumor activity in HER2+ breast cancer cells. Breast Cancer Res. *16*, R9.
397. Rimawi, M.F., Schiff, R., and Osborne, C.K. (2015). Targeting HER2 for the treatment of breast cancer. Annu. Rev. Med. *66*, 111–128.
398. Roskoski, R.J. (2014). The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol. Res. *79*, 34–74.
399. Rotin, D., Honegger, A.M., Margolis, B.L., Ullrich, A., and Schlessinger, J. (1992). Presence of SH2 domains of phospholipase C gamma 1 enhances substrate phosphorylation by increasing the affinity toward the epidermal growth factor receptor. J. Biol. Chem. *267*, 9678–9683.
400. Roy, R., Wewer, U.M., Zurakowski, D., Pories, S.E., and Moses, M.A. (2004). ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J. Biol. Chem. *279*, 51323–51330.
401. Ruff, M., Leyme, A., Le Cann, F., Bonnier, D., Le Seyec, J., Chesnel, F., Fattet, L., Rimokh, R., Baffet, G., and Theret, N. (2015). The Disintegrin and Metalloprotease ADAM12 Is Associated with TGF-beta-Induced Epithelial to Mesenchymal Transition. PLoS One *10*, e0139179.
402. Ruiz-Saenz, A., Dreyer, C., Campbell, M.R., Steri, V., Gulizia, N., and Moasser, M.M. (2018). HER2 Amplification in Tumors Activates PI3K/Akt Signaling Independent of HER3. Cancer Res. *78*, 3645–3658.
403. Saez, R., Molina, M.A., Ramsey, E.E., Rojo, F., Keenan, E.J., Albanell, J., Lluch, A.,

Garcia-Conde, J., Baselga, J., and Clinton, G.M. (2006). p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin. Cancer Res. *12*, 424–431.

404. Sahlgren, C., Gustafsson, M. V, Jin, S., Poellinger, L., and Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. Proc. Natl. Acad. Sci. U. S. A. *105*, 6392–6397.
405. Sajot, N., and Genest, M. (2000). Structure prediction of the dimeric neu/ErbB-2 transmembrane domain from multi-nanosecond molecular dynamics simulations. Eur. Biophys. J. *28*, 648–662.
406. Sajot, N., and Genest, M. (2001). Dimer interface of transmembrane domains for neu/erbB-2 receptor dimerization and transforming activation: a model revealed by molecular dynamics simulations. J. Biomol. Struct. Dyn. *19*, 15–31.
407. Sak, M.M., Szymanska, M., Bertelsen, V., Hasmann, M., Madshus, I.H., and Stang, E. (2013). Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. Carcinogenesis *34*, 2031–2038.
408. Sakai, K., Yokote, H., Murakami-Murofushi, K., Tamura, T., Saijo, N., and Nishio, K. (2007). Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway. Cancer Sci. *98*, 1498–1503.
409. Saleem, A., Searle, G.E., Kenny, L.M., Huiban, M., Kozlowski, K., Waldman, A.D., Woodley, L., Palmieri, C., Lowdell, C., Kaneko, T., et al. (2015). Lapatinib access into normal brain and brain metastases in patients with Her-2 overexpressing breast cancer. EJNMMI Res. *5*.
410. Sanderson, M.P., Dempsey, P.J., and Dunbar, A.J. (2006). Control of ErbB signaling through metalloprotease mediated ectodomain shedding of EGF-like factors. Growth Factors *24*, 121–136.
411. Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., Ceccarelli, C., Santini, D., Paterini, P., Marcu, K.B., et al. (2007a). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J. Clin. Invest. *117*, 3988–4002.
412. Sansone, P., Storci, G., Giovannini, C., Pandolfi, S., Pianetti, S., Taffurelli, M., Santini, D., Ceccarelli, C., Chieco, P., and Bonafe, M. (2007b). p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland

expanded in vitro as mammospheres. Stem Cells *25*, 807–815.

413. Santibanez, J.F. (2006). JNK mediates TGF-beta1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes. FEBS Lett. *580*, 5385–5391.
414. Sartor, M.A., Mahavisno, V., Keshamouni, V.G., Cavalcoli, J., Wright, Z., Karnovsky, A., Kuick, R., Jagadish, H. V, Mirel, B., Weymouth, T., et al. (2010). ConceptGen: a gene set enrichment and gene set relation mapping tool. Bioinformatics *26*, 456–463.
415. Sato, N., Sano, M., Tabei, T., Asaga, T., Ando, J., Fujii, H., Yamamoto, N., Kurosumi, M., Inoue, K., and Kimura, M. (2006). Combination docetaxel and trastuzumab treatment for patients with HER-2-overexpressing metastatic breast cancer: a multicenter, phase-II study. Breast Cancer *13*, 166–171.
416. Savagner, P., Yamada, K.M., and Thiery, J.P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J. Cell Biol. *137*, 1403–1419.
417. Scaltriti, M., Rojo, F., Ocana, A., Anido, J., Guzman, M., Cortes, J., Di Cosimo, S., Matias-Guiu, X., Ramon y Cajal, S., Arribas, J., et al. (2007). Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J. Natl. Cancer Inst. *99*, 628–638.
418. Scaltriti, M., Verma, C., Guzman, M., Jimenez, J., Parra, J.L., Pedersen, K., Smith, D.J., Landolfi, S., Ramon y Cajal, S., Arribas, J., et al. (2009). Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene *28*, 803–814.
419. Scaltriti, M., Eichhorn, P.J., Cortes, J., Prudkin, L., Aura, C., Jimenez, J., Chandarlapaty, S., Serra, V., Prat, A., Ibrahim, Y.H., et al. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc. Natl. Acad. Sci. U. S. A. *108*, 3761–3766.
420. Schabath, H., Runz, S., Joumaa, S., and Altevogt, P. (2006). CD24 affects CXCR4 function in pre-B lymphocytes and breast carcinoma cells. J. Cell Sci. *119*, 314–325.
421. Schechter, A.L., Stern, D.F., Vaidyanathan, L., Decker, S.J., Drebin, J.A., Greene, M.I., and Weinberg, R.A. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature *312*, 513–516.
422. Scheel, C., Eaton, E.N., Li, S.H.-J., Chaffer, C.L., Reinhardt, F., Kah, K.-J., Bell, G., Guo,

W., Rubin, J., Richardson, A.L., et al. (2011). Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell *145*, 926–940.

423. Scheuer, W., Friess, T., Burtscher, H., Bossenmaier, B., Endl, J., and Hasmann, M. (2009). Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. Cancer Res. *69*, 9330–9336.
424. Schnetz, M.P., Bartels, C.F., Shastri, K., Balasubramanian, D., Zentner, G.E., Balaji, R., Zhang, X., Song, L., Wang, Z., Laframboise, T., et al. (2009). Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. Genome Res. *19*, 590–601.
425. Schroeder, J.A., Adriance, M.C., McConnell, E.J., Thompson, M.C., Pockaj, B., and Gendler, S.J. (2002). ErbB-beta-catenin complexes are associated with human infiltrating ductal breast and murine mammary tumor virus (MMTV)-Wnt-1 and MMTV-c-Neu transgenic carcinomas. J. Biol. Chem. *277*, 22692–22698.
426. Schroeter, E.H., Kisslinger, J.A., and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature *393*, 382–386.
427. Schulze, W.X., Deng, L., and Mann, M. (2005). Phosphotyrosine interactome of the ErbB-receptor kinase family. Mol. Syst. Biol. *1*, 2005.0008.
428. Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R., and Virshup, D.M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089–2091.
429. Seo, S., Richardson, G.A., and Kroll, K.L. (2005). The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. Development *132*, 105–115.
430. Serrano-Olvera, A., Duenas-Gonzalez, A., Gallardo-Rincon, D., Candelaria, M., and De la Garza-Salazar, J. (2006). Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer. Cancer Treat. Rev. *32*, 180–190.
431. Seton-Rogers, S.E., Lu, Y., Hines, L.M., Koundinya, M., LaBaer, J., Muthuswamy, S.K., and Brugge, J.S. (2004). Cooperation of the ErbB2 receptor and transforming growth factor beta in induction of migration and invasion in mammary epithelial cells. Proc. Natl. Acad. Sci. U. S. A. *101*, 1257–1262.
432. Shao, S., Zhao, X., Zhang, X., Luo, M., Zuo, X., Huang, S., Wang, Y., Gu, S., and Zhao, X. (2015). Notch1 signaling regulates the epithelial-mesenchymal transition and invasion

of breast cancer in a Slug-dependent manner. Mol. Cancer *14*, 28.

433. Sharma, N., Nanta, R., Sharma, J., Gunewardena, S., Singh, K.P., Shankar, S., and Srivastava, R.K. (2015). PI3K/AKT/mTOR and sonic hedgehog pathways cooperate together to inhibit human pancreatic cancer stem cell characteristics and tumor growth. Oncotarget *6*, 32039–32060.
434. Sharpe, S., Barber, K.R., and Grant, C.W.M. (2002). Evidence of a tendency to self-association of the transmembrane domain of ErbB-2 in fluid phospholipid bilayers. Biochemistry *41*, 2341–2352.
435. Shi, Y., Fan, X., Deng, H., Brezski, R.J., Rycyzyn, M., Jordan, R.E., Strohl, W.R., Zou, Q., Zhang, N., and An, Z. (2015). Trastuzumab triggers phagocytic killing of high HER2 cancer cells in vitro and in vivo by interaction with Fcgamma receptors on macrophages. J. Immunol. *194*, 4379–4386.
436. Shipitsin, M., Campbell, L.L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M., et al. (2007). Molecular definition of breast tumor heterogeneity. Cancer Cell *11*, 259–273.
437. Shirakihara, T., Saitoh, M., and Miyazono, K. (2007). Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. Mol. Biol. Cell *18*, 3533–3544.
438. Shlyueva, D., Stampfel, G., and Stark, A. (2014). Transcriptional enhancers: from properties to genome-wide predictions. Nat. Rev. Genet. *15*, 272–286.
439. Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., and Darnell, J.E. (1993). Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature *366*, 580–583.
440. Siegel, P.M., Shu, W., Cardiff, R.D., Muller, W.J., and Massague, J. (2003). Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. Proc. Natl. Acad. Sci. U. S. A. *100*, 8430–8435.
441. Sims, A.H., Zweemer, A.J.M., Nagumo, Y., Faratian, D., Muir, M., Dodds, M., Um, I., Kay, C., Hasmann, M., Harrison, D.J., et al. (2012). Defining the molecular response to trastuzumab, pertuzumab and combination therapy in ovarian cancer. Br. J. Cancer *106*, 1779–1789.
442. Skeen, J.E., Bhaskar, P.T., Chen, C.-C., Chen, W.S., Peng, X., Nogueira, V., Hahn-

Windgassen, A., Kiyokawa, H., and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell *10*, 269–280.

443. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177–182.
444. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., and Ullrich, A. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science *244*, 707–712.
445. Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. *344*, 783–792.
446. So, J.Y., Smolarek, A.K., Salerno, D.M., Maehr, H., Uskokovic, M., Liu, F., and Suh, N. (2013). Targeting CD44-STAT3 signaling by Gemini vitamin D analog leads to inhibition of invasion in basal-like breast cancer. PLoS One *8*, e54020.
447. Soady, K.J., Kendrick, H., Gao, Q., Tutt, A., Zvelebil, M., Ordonez, L.D., Quist, J., Tan, D.W.-M., Isacke, C.M., Grigoriadis, A., et al. (2015). Mouse mammary stem cells express prognostic markers for triple-negative breast cancer. Breast Cancer Res. *17*, 31.
448. Son, M.-Y., Kim, J., Han, H.-W., Woo, S.-M., Cho, Y.S., Kang, Y.-K., and Han, Y.-M. (2008). Expression profiles of protein tyrosine kinase genes in human embryonic stem cells. Reproduction *136*, 423–432.
449. Spector, N.L., and Blackwell, K.L. (2009). Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. J. Clin. Oncol. *27*, 5838–5847.
450. Sperinde, J., Jin, X., Banerjee, J., Penuel, E., Saha, A., Diedrich, G., Huang, W., Leitzel, K., Weidler, J., Ali, S.M., et al. (2010). Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin. Cancer Res. *16*, 4226–4235.
451. Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., and Pellegrini, S. (1994). Association and activation of Jak-

Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science *263*, 92–95.

452. Stankic, M., Pavlovic, S., Chin, Y., Brogi, E., Padua, D., Norton, L., Massague, J., and Benezra, R. (2013). TGF-beta-Id1 signaling opposes Twist1 and promotes metastatic colonization via a mesenchymal-to-epithelial transition. Cell Rep. *5*, 1228–1242.
453. Subik, K., Lee, J.-F., Baxter, L., Strzepek, T., Costello, D., Crowley, P., Xing, L., Hung, M.-C., Bonfiglio, T., Hicks, D.G., et al. (2010). The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. Breast Cancer (Auckl). *4*, 35–41.
454. Sullivan, N.J., Sasser, A.K., Axel, A.E., Vesuna, F., Raman, V., Ramirez, N., Oberyszyn, T.M., and Hall, B.M. (2009). Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene *28*, 2940–2947.
455. Sun, M., Zhang, N., Wang, X., Li, Y., Qi, W., Zhang, H., Li, Z., and Yang, Q. (2016). Hedgehog pathway is involved in nitidine chloride induced inhibition of epithelial-mesenchymal transition and cancer stem cells-like properties in breast cancer cells. Cell Biosci. *6*, 44.
456. Swain, S.M., Kim, S.-B., Cortes, J., Ro, J., Semiglazov, V., Campone, M., Ciruelos, E., Ferrero, J.-M., Schneeweiss, A., Knott, A., et al. (2013). Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. Lancet. Oncol. *14*, 461–471.
457. Swain, S.M., Baselga, J., Kim, S.-B., Ro, J., Semiglazov, V., Campone, M., Ciruelos, E., Ferrero, J.-M., Schneeweiss, A., Heeson, S., et al. (2015). Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. N. Engl. J. Med. *372*, 724–734.
458. Takai, N., Jain, A., Kawamata, N., Popoviciu, L.M., Said, J.W., Whittaker, S., Miyakawa, I., Agus, D.B., and Koeffler, H.P. (2005). 2C4, a monoclonal antibody against HER2, disrupts the HER kinase signaling pathway and inhibits ovarian carcinoma cell growth. Cancer *104*, 2701–2708.
459. Takaku, M., Grimm, S.A., Shimbo, T., Perera, L., Menafra, R., Stunnenberg, H.G., Archer, T.K., Machida, S., Kurumizaka, H., and Wade, P.A. (2016). GATA3-dependent cellular reprogramming requires activation-domain dependent recruitment of a chromatin remodeler. Genome Biol. *17*, 36.

460. Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z., and He, X. (2004). A mechanism for Wnt coreceptor activation. Mol. Cell *13*, 149–156.

461. Tamura, K., Shimizu, C., Hojo, T., Akashi-Tanaka, S., Kinoshita, T., Yonemori, K., Kouno, T., Katsumata, N., Ando, M., Aogi, K., et al. (2011). FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *22*, 1302–1307.

462. Tan, X., Thapa, N., Sun, Y., and Anderson, R.A. (2015). A kinase-independent role for EGF receptor in autophagy initiation. Cell *160*, 145–160.

463. Tanaka, H., Nakamura, M., Kameda, C., Kubo, M., Sato, N., Kuroki, S., Tanaka, M., and Katano, M. (2009). The Hedgehog signaling pathway plays an essential role in maintaining the CD44+CD24-/low subpopulation and the side population of breast cancer cells. Anticancer Res. *29*, 2147–2157.

464. Tandon, A.K., Clark, G.M., Chamness, G.C., Ullrich, A., and McGuire, W.L. (1989). HER-2/neu oncogene protein and prognosis in breast cancer. J. Clin. Oncol. *7*, 1120–1128.

465. Tavares, A.L.P., Mercado-Pimentel, M.E., Runyan, R.B., and Kitten, G.T. (2006). TGF beta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. Dev. Dyn. *235*, 1589–1598.

466. Teng, L., He, B., Wang, J., and Tan, K. (2015). 4DGenome: a comprehensive database of chromatin interactions. Bioinformatics *31*, 2560–2564.

467. Tester, A.M., Ruangpanit, N., Anderson, R.L., and Thompson, E.W. (2000). MMP-9 secretion and MMP-2 activation distinguish invasive and metastatic sublines of a mouse mammary carcinoma system showing epithelial-mesenchymal transition traits. Clin. Exp. Metastasis *18*, 553–560.

468. Theodoropoulos, P.A., Polioudaki, H., Agelaki, S., Kallergi, G., Saridaki, Z., Mavroudis, D., and Georgoulias, V. (2010). Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer. Cancer Lett. *288*, 99–106.

469. Thomas, M., Halsall, S., and Peters, T.J. (1982). Role of hepatic acetaldehyde dehydrogenase in alcoholism: demonstration of persistent reduction of cytosolic activity in abstaining patients. Lancet (London, England) *2*, 1057–1058.

470. Thuault, S., Valcourt, U., Petersen, M., Manfioletti, G., Heldin, C.-H., and Moustakas, A.

(2006). Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. J. Cell Biol. *174*, 175–183.

471. Thurman, R.E., Rynes, E., Humbert, R., Vierstra, J., Maurano, M.T., Haugen, E., Sheffield, N.C., Stergachis, A.B., Wang, H., Vernot, B., et al. (2012). The accessible chromatin landscape of the human genome. Nature *489*, 75–82.
472. Tiede, B., and Kang, Y. (2011). From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. Cell Res. *21*, 245–257.
473. Timmerman, L.A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J.M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J.C., et al. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev. *18*, 99–115.
474. Tomita, H., Tanaka, K., Tanaka, T., and Hara, A. (2016). Aldehyde dehydrogenase 1A1 in stem cells and cancer. Oncotarget *7*, 11018–11032.
475. Tural, D., Akar, E., Mutlu, H., and Kilickap, S. (2014). P95 HER2 fragments and breast cancer outcome. Expert Rev. Anticancer Ther. *14*, 1089–1096.
476. Ueda, Y., Wang, S., Dumont, N., Yi, J.Y., Koh, Y., and Arteaga, C.L. (2004). Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. J. Biol. Chem. *279*, 24505–24513.
477. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., and Schlessinger, J. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature *309*, 418–425.
478. Valabrega, G., Montemurro, F., Sarotto, I., Petrelli, A., Rubini, P., Tacchetti, C., Aglietta, M., Comoglio, P.M., and Giordano, S. (2005). TGFalpha expression impairs Trastuzumab-induced HER2 downregulation. Oncogene *24*, 3002–3010.
479. Varchetta, S., Gibelli, N., Oliviero, B., Nardini, E., Gennari, R., Gatti, G., Silva, L.S., Villani, L., Tagliabue, E., Menard, S., et al. (2007). Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res. *67*, 11991–11999.
480. Vasudevan, D., Hickok, J.R., Bovee, R.C., Pham, V., Mantell, L.L., Bahroos, N., Kanabar, P., Cao, X.-J., Maienschein-Cline, M., Garcia, B.A., et al. (2015). Nitric Oxide Regulates

Gene Expression in Cancers by Controlling Histone Posttranslational Modifications. Cancer Res. *75*, 5299–5308.

481. Veltmaat, J.M., Van Veelen, W., Thiery, J.P., and Bellusci, S. (2004). Identification of the mammary line in mouse by Wnt10b expression. Dev. Dyn. *229*, 349–356.
482. Venturutti, L., Romero, L. V, Urtreger, A.J., Chervo, M.F., Cordo Russo, R.I., Mercogliano, M.F., Inurrigarro, G., Pereyra, M.G., Proietti, C.J., Izzo, F., et al. (2016). Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis. Oncogene *35*, 2208–2222.
483. Vinkemeier, U., Cohen, S.L., Moarefi, I., Chait, B.T., Kuriyan, J., and Darnell, J.E.J. (1996). DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. EMBO J. *15*, 5616–5626.
484. Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., et al. (2009). ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature *457*, 854–858.
485. Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., et al. (2002). Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J. Clin. Oncol. *20*, 719–726.
486. Vu, T., and Claret, F.X. (2012). Trastuzumab: Updated Mechanisms of Action and Resistance in Breast Cancer. Front. Oncol. *2*.
487. Wang, S.E. (2011). The Functional Crosstalk between HER2 Tyrosine Kinase and TGF-β Signaling in Breast Cancer Malignancy. J. Signal Transduct. *2011*.
488. Wang, Z. (2017). ErbB Receptors and Cancer. Methods Mol. Biol. *1652*, 3–35.
489. Wang, K., Ma, Q., Ren, Y., He, J., Zhang, Y., Zhang, Y., and Chen, W. (2007a). Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/beta-catenin signaling in HER2 overexpressing human breast cancer cells. Oncol. Rep. *17*, 89–96.
490. Wang, L., Schulz, T.C., Sherrer, E.S., Dauphin, D.S., Shin, S., Nelson, A.M., Ware, C.B., Zhan, M., Song, C.-Z., Chen, X., et al. (2007b). Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood

110, 4111–4119.

491. Wang, Q., Zhu, F., and Wang, Z. (2007c). Identification of EGF receptor C-terminal sequences 1005-1017 and di-leucine motif 1010LL1011 as essential in EGF receptor endocytosis. Exp. Cell Res. *313*, 3349–3363.
492. Wang, S.-C., Lien, H.-C., Xia, W., Chen, I.-F., Lo, H.-W., Wang, Z., Ali-Seyed, M., Lee, D.-F., Bartholomeusz, G., Ou-Yang, F., et al. (2004). Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. Cancer Cell *6*, 251–261.
493. Wang, X., Wang, G., Zhao, Y., Liu, X., Ding, Q., Shi, J., Ding, Y., and Wang, S. (2012). STAT3 mediates resistance of CD44(+)CD24(-/low) breast cancer stem cells to tamoxifen in vitro. J. Biomed. Res. *26*, 325–335.
494. Ward, C.W., Hoyne, P.A., and Flegg, R.H. (1995). Insulin and epidermal growth factor receptors contain the cysteine repeat motif found in the tumor necrosis factor receptor. Proteins *22*, 141–153.
495. Watabe, T., and Miyazono, K. (2009). Roles of TGF-beta family signaling in stem cell renewal and differentiation. Cell Res. *19*, 103–115.
496. Weber, F., de Villiers, J., and Schaffner, W. (1984). An SV40 “enhancer trap” incorporates exogenous enhancers or generates enhancers from its own sequences. Cell *36*, 983–992.
497. Wei, W., Tweardy, D.J., Zhang, M., Zhang, X., Landua, J., Petrovic, I., Bu, W., Roarty, K., Hilsenbeck, S.G., Rosen, J.M., et al. (2014). STAT3 signaling is activated preferentially in tumor-initiating cells in claudin-low models of human breast cancer. Stem Cells *32*, 2571–2582.
498. Wei, Y., Zhang, S., Shang, S., Zhang, B., Li, S., Wang, X., Wang, F., Su, J., Wu, Q., Liu, H., et al. (2016). SEA: a super-enhancer archive. Nucleic Acids Res. *44*, D172-9.
499. Weigelt, B., Horlings, H.M., Kreike, B., Hayes, M.M., Hauptmann, M., Wessels, L.F.A., de Jong, D., Van de Vijver, M.J., Van’t Veer, L.J., and Peterse, J.L. (2008). Refinement of breast cancer classification by molecular characterization of histological special types. J. Pathol. *216*, 141–150.
500. Weihua, Z., Tsan, R., Huang, W.-C., Wu, Q., Chiu, C.-H., Fidler, I.J., and Hung, M.-C. (2008). Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell *13*, 385–393.
501. Wen, X.-F., Yang, G., Mao, W., Thornton, A., Liu, J., Bast, R.C.J., and Le, X.-F. (2006).

HER2 signaling modulates the equilibrium between pro- and antiangiogenic factors via distinct pathways: implications for HER2-targeted antibody therapy. Oncogene *25*, 6986–6996.

502. Whyte, W.A., Bilodeau, S., Orlando, D.A., Hoke, H.A., Frampton, G.M., Foster, C.T., Cowley, S.M., and Young, R.A. (2012). Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature *482*, 221–225.
503. Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes. Cell *153*, 307–319.
504. Wieduwilt, M.J., and Moasser, M.M. (2008). The epidermal growth factor receptor family: Biology driving targeted therapeutics. Cell. Mol. Life Sci. *65*, 1566–1584.
505. Wilken, J.A., and Maihle, N.J. (2010). Primary trastuzumab resistance: new tricks for an old drug. Ann. N. Y. Acad. Sci. *1210*, 53–65.
506. Wilson, K.J., Gilmore, J.L., Foley, J., Lemmon, M.A., and Riese, D.J. 2nd (2009). Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol. Ther. *122*, 1–8.
507. Wilson, K.S., Roberts, H., Leek, R., Harris, A.L., and Geradts, J. (2002). Differential gene expression patterns in HER2/neu-positive and -negative breast cancer cell lines and tissues. Am. J. Pathol. *161*, 1171–1185.
508. Wong, H., Leung, R., Kwong, A., Chiu, J., Liang, R., Swanton, C., and Yau, T. (2011). Integrating Molecular Mechanisms and Clinical Evidence in the Management of Trastuzumab Resistant or Refractory HER-2(+) Metastatic Breast Cancer. Oncologist *16*, 1535–1546.
509. Woodward, W.A., Chen, M.S., Behbod, F., Alfaro, M.P., Buchholz, T.A., and Rosen, J.M. (2007). WNT/β-catenin mediates radiation resistance of mouse mammary progenitor cells. Proc. Natl. Acad. Sci. U. S. A. *104*, 618–623.
510. Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F., and Massague, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003–1014.
511. Wu, Y., Ginther, C., Kim, J., Mosher, N., Chung, S., Slamon, D., and Vadgama, J. V (2012). Expression of Wnt3 activates Wnt/beta-catenin pathway and promotes EMT-like

phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells. Mol. Cancer Res. *10*, 1597–1606.

512. Xia, W., Mullin, R.J., Keith, B.R., Liu, L.-H., Ma, H., Rusnak, D.W., Owens, G., Alligood, K.J., and Spector, N.L. (2002). Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene *21*, 6255–6263.
513. Xia, W., Bisi, J., Strum, J., Liu, L., Carrick, K., Graham, K.M., Treece, A.L., Hardwicke, M.A., Dush, M., Liao, Q., et al. (2006). Regulation of survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. Cancer Res. *66*, 1640–1647.
514. Xiao, Y., Nimmer, P., Sheppard, G.S., Bruncko, M., Hessler, P., Lu, X., Roberts-Rapp, L., Pappano, W.N., Elmore, S.W., Souers, A.J., et al. (2015). MCL-1 Is a Key Determinant of Breast Cancer Cell Survival: Validation of MCL-1 Dependency Utilizing a Highly Selective Small Molecule Inhibitor. Mol. Cancer Ther. *14*, 1837–1847.
515. XIE, G., YAO, Q., LIU, Y., DU, S., LIU, A., GUO, Z., SUN, A., RUAN, J., CHEN, L., YE, C., et al. (2012). IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. Int. J. Oncol. *40*, 1171–1179.
516. Xu, W., Yuan, X., Beebe, K., Xiang, Z., and Neckers, L. (2007). Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. Mol. Cell. Biol. *27*, 220–228.
517. Yakes, F.M., Chinratanalab, W., Ritter, C.A., King, W., Seelig, S., and Arteaga, C.L. (2002). Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res. *62*, 4132–4141.
518. Yamaguchi, H., Chang, S.-S., Hsu, J.L., and Hung, M.-C. (2014). Signaling cross-talk in the resistance to HER family receptor targeted therapy. Oncogene *33*, 1073–1081.
519. Yamashita-Kashima, Y., Iijima, S., Yorozu, K., Furugaki, K., Kurasawa, M., Ohta, M., and Fujimoto-Ouchi, K. (2011). Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models. Clin. Cancer Res. *17*, 5060–5070.
520. Yamashita-Kashima, Y., Shu, S., Yorozu, K., Moriya, Y., and Harada, N. (2017). Mode of

action of pertuzumab in combination with trastuzumab plus docetaxel therapy in a HER2-positive breast cancer xenograft model. Oncol. Lett. *14*, 4197–4205.

521. Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C.H. (1994). Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J. Biol. Chem. *269*, 20172–20178.
522. Yan, M., Schwaederle, M., Arguello, D., Millis, S.Z., Gatalica, Z., and Kurzrock, R. (2015). HER2 expression status in diverse cancers: review of results from 37,992 patients. Cancer Metastasis Rev. *34*, 157–164.
523. Yang, D., Jang, I., Choi, J., Kim, M.-S., Lee, A.J., Kim, H., Eom, J., Kim, D., Jung, I., and Lee, B. (2018). 3DIV: A 3D-genome Interaction Viewer and database. Nucleic Acids Res. *46*, D52–D57.
524. Yang, L., Tang, H., Kong, Y., Xie, X., Chen, J., Song, C., Liu, X., Ye, F., Li, N., Wang, N., et al. (2015). LGR5 Promotes Breast Cancer Progression and Maintains Stem-Like Cells Through Activation of Wnt/beta-Catenin Signaling. Stem Cells *33*, 2913–2924.
525. Yang, N., Zhou, T.-C., Lei, X., Wang, C., Yan, M., Wang, Z.-F., Liu, W., Wang, J., Ming, K.-H., Wang, B.-C., et al. (2016). Inhibition of Sonic Hedgehog Signaling Pathway by Thiazole Antibiotic Thiostrepton Attenuates the CD44+/CD24-Stem-Like Population and Sphere-Forming Capacity in Triple-Negative Breast Cancer. Cell. Physiol. Biochem. *38*, 1157–1170.
526. Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. Nat. Rev. Mol. Cell Biol. *2*, 127–137.
527. Yardley, D.A. (2013). Drug resistance and the role of combination chemotherapy in improving patient outcomes. Int. J. Breast Cancer *2013*, 137414.
528. Yonemori, K., Tsuta, K., Shimizu, C., Hatanaka, Y., Hirakawa, A., Ono, M., Kouno, T., Katsumata, N., Ando, M., Tamura, K., et al. (2010). Immunohistochemical expression of HER1, HER3, and HER4 in HER2-positive breast cancer patients treated with trastuzumab-containing neoadjuvant chemotherapy. J. Surg. Oncol. *101*, 222–227.
529. Yu, L., Hebert, M.C., and Zhang, Y.E. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. *21*, 3749–3759.
530. Yu, Y., Wang, Y., Ren, X., Tsuyada, A., Li, A., Liu, L.J., and Wang, S.E. (2010). Context-dependent bidirectional regulation of the MutS homolog 2 by transforming growth factor

beta contributes to chemoresistance in breast cancer cells. Mol. Cancer Res. *8*, 1633–1642.

531. Yuan, C.-X., Lasut, A.L., Wynn, R., Neff, N.T., Hollis, G.F., Ramaker, M.L., Rupar, M.J., Liu, P., and Meade, R. (2003). Purification of Her-2 extracellular domain and identification of its cleavage site. Protein Expr. Purif. *29*, 217–222.
532. Yuste, L., Montero, J.C., Esparis-Ogando, A., and Pandiella, A. (2005). Activation of ErbB2 by overexpression or by transmembrane neuregulin results in differential signaling and sensitivity to herceptin. Cancer Res. *65*, 6801–6810.
533. Zhang, Y.E. (2009). Non-Smad pathways in TGF-beta signaling. Cell Res. *19*, 128–139.
534. Zhang, F., Li, C., Halfter, H., and Liu, J. (2003). Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene *22*, 894–905.
535. Zhang, H., Berezov, A., Wang, Q., Zhang, G., Drebin, J., Murali, R., and Greene, M.I. (2007). ErbB receptors: from oncogenes to targeted cancer therapies. J. Clin. Invest. *117*, 2051–2058.
536. Zhang, S., Huang, W.-C., Li, P., Guo, H., Poh, S.-B., Brady, S.W., Xiong, Y., Tseng, L.-M., Li, S.-H., Ding, Z., et al. (2011). Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat. Med. *17*, 461–469.
537. Zhao, D., Mo, Y., Li, M.-T., Zou, S.-W., Cheng, Z.-L., Sun, Y.-P., Xiong, Y., Guan, K.-L., and Lei, Q.-Y. (2014). NOTCH-induced aldehyde dehydrogenase 1A1 deacetylation promotes breast cancer stem cells. J. Clin. Invest. *124*, 5453–5465.
538. Zhao, H., Tang, H., Xiao, Q., He, M., Zhao, L., Fu, Y., Wu, H., Yu, Z., Jiang, Q., Yan, Y., et al. (2016). The Hedgehog signaling pathway is associated with poor prognosis in breast cancer patients with the CD44+/CD24 phenotype. Mol. Med. Rep. *14*, 5261–5270.
539. Zheng, L., Ren, J.Q., Li, H., Kong, Z.L., and Zhu, H.G. (2004). Downregulation of wild-type p53 protein by HER-2/neu mediated PI3K pathway activation in human breast cancer cells: its effect on cell proliferation and implication for therapy. Cell Res. *14*, 497–506.
540. Zhou, B.P., Deng, J., Xia, W., Xu, J., Li, Y.M., Gunduz, M., and Hung, M.-C. (2004). Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat. Cell Biol. *6*, 931–940.
541. Zhou, X., Lowdon, R.F., Li, D., Lawson, H.A., Madden, P.A.F., Costello, J.F., and Wang, T. (2013). Exploring long-range genome interactions using the WashU Epigenome

Browser. Nat. Methods *10*, 375–376.

542. Zhou, Y., Li, S., Hu, Y.P., Wang, J., Hauser, J., Conway, A.N., Vinci, M.A., Humphrey, L., Zborowska, E., Willson, J.K. V, et al. (2006). Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma GEO cells to apoptosis. Cancer Res. *66*, 404–411.
543. Zhu, J., Sammons, M.A., Donahue, G., Dou, Z., Vedadi, M., Getlik, M., Barsyte-Lovejoy, D., Al-awar, R., Katona, B.W., Shilatifard, A., et al. (2015). Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. Nature *525*, 206–211.
544. Zhuang, G., Brantley-Sieders, D.M., Vaught, D., Yu, J., Xie, L., Wells, S., Jackson, D., Muraoka-Cook, R., Arteaga, C., and Chen, J. (2010). Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res. *70*, 299–308.
545. Zuin, J., Dixon, J.R., van der Reijden, M.I.J.A., Ye, Z., Kolovos, P., Brouwer, R.W.W., van de Corput, M.P.C., van de Werken, H.J.G., Knoch, T.A., van IJcken, W.F.J., et al. (2014). Cohesin and CTCF differentially affect chromatin architecture and gene expression in human cells. Proc. Natl. Acad. Sci. U. S. A. *111*, 996–1001.