Regulation of Epidermal Growth Factor Receptor Cell Signaling and Endocytosis during Mitosis

by

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Abstract

EGFR-mediated signaling has been studied extensively since the 1980s due to its importance in several types of cancers. The majority of the research however has focused on the actions of the EGFR during G0/G1 and has neglected to characterize EGFR outside of this cell cycle phase. In this thesis, I characterized EGFR signaling and endocytosis during the other phases of the cell cycle, mostly during mitosis. I characterized the differential regulation of various EGFR-mediated signaling pathways during mitosis, including the mechanism of ERK inhibition during mitosis. In addition, I showed that EGFR endocytosis during mitosis proceeds exclusively by non-clathrin mediated endocytosis, since EGFR endocytosis during mitosis is unaffected by clathrin knockdown and is much more dependent on the E3 ligase CBL than during interphase. Finally, I assayed for differences in the activation of EGFR and EGFR-mediated pathways during the various cell cycle phases, specifically during G1, S, G2, prometaphase, metaphase, and anaphase/telophase. My work has contributed to a fuller understanding of EGFR signaling and endocytosis, which will be important, for example, to better understand modulations to the EGFR for pharmacological purposes.

Preface

Parts of Chapter 1 of this thesis has been published as P. Wee, and Z. Wang, 2017, "Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways," *Cancers*, vol 9, issue 5, 52, doi:10.3390/cancers9050052. I wrote the review. Z. Wang was the supervisory author and was involved in manuscript composition.

Chapter 2 of this thesis has been published as P. Wee, H. Shi, J. Jiang, Y. Wang, Z. Wang, 2015, "EGF stimulates the activation of EGF receptors and the selective activation of major signaling pathways during mitosis," *Cellular Signalling*, vol 27, issue 3, 638-651. I was responsible for data collection and analysis as well as the manuscript composition. H. Shi, J. Jiang, Y. Wang assisted with the data collection. Z. Wang was the supervisory author and was involved with concept formation and manuscript composition.

Parts of Chapter 4 of this thesis has been published as P. Wee, and Z. Wang, 2017, "Cell Cycle Synchronization of HeLa Cells to Assay EGFR Pathway Activation," In: Wang Z. (eds) ErbB Receptor Signaling. Methods in Molecular Biology, vol 1652. Humana Press, New York, NY. I was responsible for manuscript composition. Z. Wang was the supervisory author and was involved in manuscript composition.

All other work in this thesis are my original work.

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List of Abbreviations

Akt or PKB	protein kinase B
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
CBL	casitas B-lineage lymphoma
CME	clathrin-mediated endocytosis
c-Src	cellular sarcoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB	erythroblastic leukemia viral oncogene homolog
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GRB2	growth factor receptor bound protein 2
GTP	guanine triphosphate
GTPases	guanine triphosphatases
IB	immunoblot
IF	immunofluorescence
IP	immunoprecipitation
kD	kilo Daltons
MAPK	mitogen-activated protein kinases
MEK	MAPK kinase
NCE	non-clathrin mediated endocytosis
PBS	phosphate buffered saline
PI	phosphoinositide
PI3K	phosphatidyl-inositol 3-kinase
PKC	protein kinase C
PLC-γ1	phopholipase C- γ1
PM	plasma membrane
pY or pTyr	phosphorylated tyrosine
Ras	rat sarcoma
RTKs	receptor tyrosine kinase
SF	serum free
SH2	Src homology 2
SH3	Src homology 3
SHC	Src homologous and collagen like protein
SOS	son-of-sevenless

TM	transmembrane

1 Chapter 1

Introduction

1.1 Introduction

Epidermal growth factor (EGF) receptor (EGFR), also known as ErbB1/HER,1 is the prototype of the EGFR family that also includes ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 [1]. Driven largely by its role in promoting cell proliferation and opposing apoptosis, the EGFR has been vilified as a proto-oncogene. New facts regarding the complex signaling network activated by the receptor tyrosine kinase and its endocytosis are continuously emerging, highlighting the fact that there is still much to discover about the EGFR before we can optimally fine-tune effective EGFR-targeting therapies.

EGFR activation often leads to cell progression through the cell cycle. In this thesis, I characterized the signaling pathways and the endocytic route mediated by the EGFR throughout the cell cycle, primarily during mitosis. Prior to my research, little was known about the mechanisms regulating these processes throughout cell cycle phases other than interphase, more specifically, the G0/G1 phases. It was clear however that both the signaling and endocytosis of the EGFR are differentially regulated throughout the cell cycle [2–4]. Therefore, the research presented in this thesis not only helps gain a fuller understanding of the role of the receptor tyrosine kinase, but the characterization of the differences between cell cycle phases may lead to new exploitable options for drug treatment.

1.1.1 Role of EGFR

The main role of the ErbB receptors is to convey information from the outside of the cell into a cellular response: signal transduction. Following activation, usually by one of its ligands, ErbB receptors can promote a multitude of biological processes, many of which being pro-oncogenic processes, including cell proliferation, angiogenesis, inhibition of apoptosis, cell motility, adhesion, and metastasis.

Almost all cell types possess ErbB family members, with the exception of hematopoietic cells [5]. EGFR family genes are critical to the normal embryogenesis of vertebrates [6]. Null mutations of any of the ErbB genes in mice cause embryonic or perinatal lethality [7,8]. The lethality of EGFR null mice have been shown to be due to abnormalities in organs including in the brain, skin, lung, and gastrointestinal tract, as well as to the renewal of stem cells [9,10]. EGFR also plays roles in rat embryonic skin maturation, hair follicle development, hair cycling [11], and corneal development [12]. In adolescence, EGFR family genes play key roles in mammary ductal development. Female mice with a T743G substitution that impairs tyrosine kinase activity fail to develop proper mammary glands, due to defective ductal growth, causing their pups to die from malnutrition [13–15]. EGFR activity remains high in most parts of mature CNS [16]. However, in mouse and rat astrocytes, EGFR is present in high levels in developing astrocytes, but becomes absent in mature astrocytes [17,18].

The EGFR's link to cancer was first recognized when the transforming v-ErbB oncogene of the avian erythroblatosis virus was found to be a mutant homolog of human EGFR [19,20]. The v-erbB oncogene was found to contain recombinations of the transmembrane and cytoplasmic domains of the EGFR [21], implicating EGFR aberrations to cancer. In addition to mutations, overexpression of EGFR was associated with cancer progression, first in carcinomas [22,23], and later on in sarcomas [24], non-small cell lung cancer (NSCLC) [25], and malignant gliomas [26]. The levels of EGFR would soon be found to predict tumor grade, patient prognosis, and relapse in cancer [27,28].

1.1.2 EGFR Structure and Mutations

The EGFR is synthesized as a 1210 residue precursor that is cleaved at the N-terminal to result in the mature 1186 residue transmembrane EGFR [20]. From N-terminal to C-terminal, the

EGFR consists of (1) an extracellular ligand binding and dimerization arm (exons 1–16), (2) a hydrophobic transmembrane domain (exon 17), and (3) the intracellular tyrosine kinase and C-terminal tail domains (exons 18–28) [7]. Here, we will describe the structure and function of each domain in the EGFR.

The extracellular region of the EGFR is composed of 621 amino acids and is subdivided into four domains, I (amino acids 1–133, exons 1–4), II (amino acids 134–312, exons 5–7), III (amino acids 313–445, exons 8–12), IV (amino acids 446–621, exons 13–16). Domains I and III are leucine-rich fragments that participate in ligand binding. Domain II forms homo- or hetero-dimers with the analogous domain of family members. Domain IV can form disulfide bonds to domain II, and links to the TM domain. Domains II and IV do not make contacts with the ligand, and are cysteine-rich regions.

The TM domain is a 23 amino acid long hydrophobic single pass membrane structure, that anchors the receptor to the membrane [29]. It is 23 amino acids long, from Ile622 to Met644 [30]. The EGFR TM domain has been suggested to play a role in dimerization, as the N-terminal region of the TM helices have been hypothesized to contact during dimerization [31].

The intracellular domain is 542 amino acids long, and includes the flexible juxtamembrane segment (~40 aa), the tyrosine kinase domain (amino acids 690–953, exons 18–24), and the C-terminal tail (amino acids 954–1136, exons 25–28) [32]. The tyrosine kinase domain can be divided into an N-lobe (a mainly β-sheet structure) and a C-lobe (a mainly α-helical structure), with an ATP-binding site located between the two lobes [33]. Transautophosphorylation relies on the interaction of the N-lobe of one receptor to the C-lobe of the other [34]. The kinase domain also contains lysine residues that are the primary sites of

receptor ubiquitination. The C-terminal tail includes various tyrosine residues, which when phosphorylated, allow the anchoring of a variety of intracellular proteins to the activated receptor. These proteins then participate in the signal transduction relay.

EGFR mutations occur at mutational "hotspots" in the extracellular region, the kinase domain, and the C-terminal tail [1]. EGFR ectodomain oncogenic alterations often cause the loss of the inhibitory regulatory domains for dimerization. The most famous EGFR ectodomain mutant is the viral EGFR homologue, v-ERBB, which completely lacks the ectodomain, and exists primarily in dimers [19]. In addition, the EGFR variant EGFRvIII arises from the genomic deletion of exons 2–7, and occurs in approximately 20% of glioblastomas [35,36]. Interestingly, EGFRvIII displays ligand-independent signaling, but has low constitutive activity. The low constitutive activity is enough to impart cancer cells with increased signaling, however its growth advantage is due to the fact that these receptors are not downregulated by endocytosis [36,37]. In the kinase domain, the most commonly seen EGFR point mutation is L858R, and makes up approximately 45% of mutations in the tyrosine kinase domain [38,39]. Termed a "classical" activating mutation, the L858R mutation of the activation loop confers 50-fold more kinase activity and higher K_M for ATP than wild type EGFR [40,41]. Another kinase domain mutant, T790M, is often referred as the "gatekeeper residue," and is notorious for conferring resistance to pharmacological EGFR tyrosine kinase inhibitors in addition to increasing EGFR phosphorylation levels [42,43].

1.2 EGFR Signal Transduction

The first studies on the EGF in animals showed that it stimulated epidermal proliferation and keratinization [44]. We now know that the EGF can stimulate cell proliferation, cell differentiation, cell growth, migration, and inhibit apoptosis. It has been shown that the addition

of EGF to HeLa cells activates the EGFR to cause the global phosphorylation of 2244 proteins at 6600 sites [45]. Furthermore, it was shown that EGF stimulation causes significant differences in expression of 3172 genes and 596 proteins in human mammary epithelial cells (HMEC) [46]. Even without factoring in other types of post-translational modifications, it is clear that the effects of the EGF on the cell are profound and wide-ranging. The signal transduction mediated by the EGFR is extremely complex. It begins with the EGFR being activated by one of its ligands, leading to receptor dimerization, the transphosphorylation of the C-terminal tail, and finally the propagation of the signal through various intricate signaling pathways to induce the expression of new genes. EGFR mutations and truncations can impart the EGFR with ligand-independent signaling, which lead to the upregulation of various pro-oncogenic processes, including chronic cell cycle proliferation. In this section, we will review EGFR signal transduction as it relates to the contents of this thesis.

1.2.1 Ligand – EGF

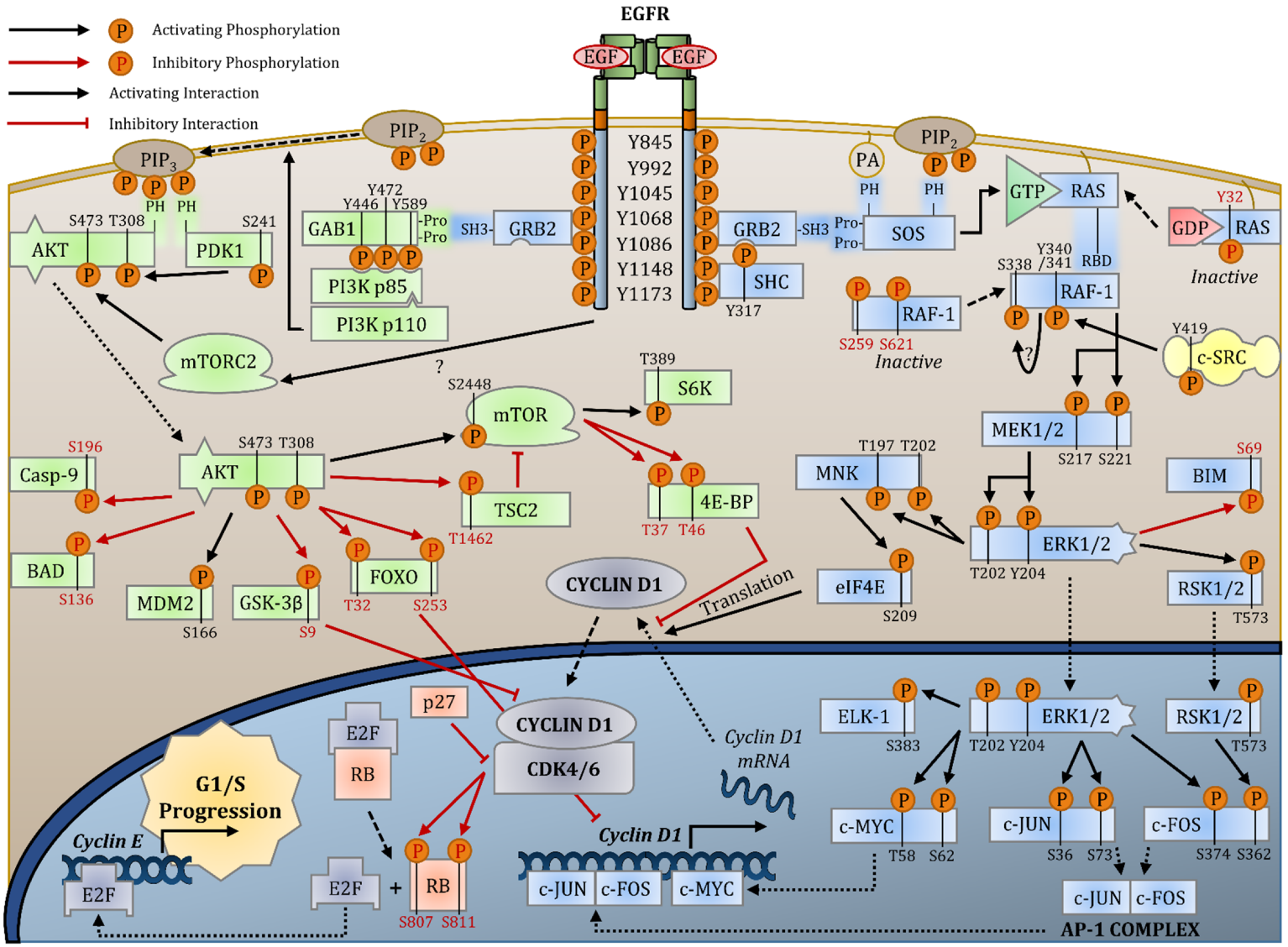
Human EGF is a 6 kDa protein made up of 53 amino acids. Physiologically in humans, various organs regulate their innate EGF concentrations [47]. For example, EGF is found at high concentrations (50–500 ng/mL) in bile, urine, milk, and prostate fluid, at medium concentrations (3–50 ng/mL) in tears, follicular fluid, sperm, and seminal plasma, and at low concentrations (1–2 ng/mL) in plasma, serum, and saliva (mice differ in that their saliva are high in EGF) [47,48]. The sources of EGF from the human body have been documented previously [49]. EGF is implicated in the morphogenesis of teeth, brain, reproductive tracts, skin, gastrointestinal tracts, in cardiovascular differentiation and function, epithelial regeneration, and corneal epithelia (reviewed in [50]). However, no disorders arising from EGF deficiency have been identified, likely due to the presence of other EGFR ligands [51].

1.2.2 EGFR Dimerization

For full EGFR activation, ligand binding and EGFR dimerization are crucial. EGF binding to the EGFR monomers at domains I and III promotes a domain rearrangement to expose the dimerization arm in domain II, leading to a stabilized “open” or “extended” conformation. [52,53]. Two receptors interact through domain II and form an asymmetric EGFR dimer pair, in that the C-terminus of the activating kinase inserts into the active site of the receiving kinase, so that this allosteric interaction can activate the receiving kinase, resulting in transautophosphorylation [34].

1.2.3 Signaling Pathways

The signal transduction pathways activated by the EGFR comprise the most important reasons the EGFR has been studied to such lengths. The powerful capacity of each of the multitude of pathways under the control of the EGFR to drive cell proliferation and resist apoptosis has formed a strong motivation for their cancer-related researches. Large strides have been accomplished in elucidating the pathways involved in mediating EGFR activity. Ligand binding and dimerization of EGFR leads to its transautophosphorylation, which initiates intracellular signaling (Figure 1.1).


Activating Phosphorylation
Inhibitory Phosphorylation
Activating Interaction
Inhibitory Interaction
EGFR
EGF
EGF
Y845
Y992
Y1045
Y1068
Y1086
Y1148
Y1173
PIP2
PIP3
PH
PH
AKT
S473 T308
PDK1
S241
GAB1
Y472
Y446 Y589
Pro
Pro
SH3-
GRB2
PI3K p85
PI3K p110
mTORC2
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GRB2
-SH3
SHC
Y317
PA
PH
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Pro-
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RAS
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Y32
Inactive
RAF-1
S259 S621
Inactive
Y340
/341
S338
RBD
RAF-1
?
c-SRC
Y419
S2448
mTOR
T389
S6K
MEK1/2
S217 S221
S196
Casp-9
AKT
S473 T308
TSC2
T1462
4E-BP
T37 T46
MNK
T197 T202
BIM
S69
BAD
S136
MDM2
S166
GSK-3β
S9
FOXO
T32
S253
CYCLIN D1
Translation
eIF4E
S209
ERK1/2
T202 Y204
RSK1/2
T573
p27
CYCLIN D1
CDK4/6
Cyclin D1
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ELK-1
S383
ERK1/2
T202 Y204
RSK1/2
T573
G1/S
Progression
Cyclin E
E2F
RB
E2F
+
RB
S807 S811
Cyclin D1
c-JUN
c-FOS
c-MYC
c-MYC
T58 S62
c-JUN
S36 S73
c-FOS
S374 S362
c-JUN
c-FOS
AP-1 COMPLEX

Figure 1.1. Epidermal growth factor receptor (EGFR) signaling pathways leading to G1/S cell cycle progression activated by EGF activation. Depicted are the RAS-RAF-MEK-ERK MAPK and PI3K-AKT-mTOR pathways. EGF activation of the EGFR induces receptor dimerization and transphosphorylation of the C-terminal domain. The phosphorylated C-terminal domain binds SHC and GRB2, along with PLC-γ1 at Y992 (not pictured). The GRB2 SH3 domain recruits the proline-rich domains of SOS or GAB1 to initiate ERK MAPK or AKT signaling respectively. SOS is also recruited to the plasma membrane (PM) by the interaction of its PH (pleckstrin homology) domains with PIP_2 (phosphatidylinositol-4,5-bisphosphate) and PA (phosphatidic acid). SOS catalyzes the conversion of GDP to GTP of RAS. Active RAS uses its RAS RAF-binding domain (RBD) to recruit RAF-1. RAF-1 is activated by dephosphorylation and phosphorylation events, and activates MEK1/2. Activated MEK1/2 activates ERK1/2. ERK1/2 has various cytoplasmic and nuclear targets, which aid in the transcription and translation of Cyclin D1. For example, ELK-1 transcribes the c-FOS gene (not pictured), and the protein product together with c-JUN make up the AP-1 complex. The AP-1 complex as well as c-MYC induce the transcription of CYCLIN D1. On the other hand, the receptor-bound GRB2 also binds GAB1. GAB1 recruits the p85 regulatory subunit of PI3K, which binds the p110 catalytic subunit. PI3K converts PIP_2 into PIP_3 (phosphatidylinositol-3,4,5-triphosphate). PIP_3 recruits AKT, and is phosphorylated and activated by PDK1 and mTORC2. AKT has many phosphorylation substrates, including various inhibitory phosphorylations to proteins that negatively regulate CYCLIN D1 activity such as GSK-3β (normally induces CYCLIN D1 degradation) and FOXO (normally represses CYCLIN D1 transcription). Furthermore, AKT inhibition of TSC2 allows the activation of mTOR, which inhibits the inhibitor of translation 4E-BP, thus allowing eIF4E-mediated translation of CYCLIN D1. Increased levels of CYCLIN D1 correlates with increased CDK4/6 activity, which phosphorylates RB. Phosphorylated RB releases the E2F transcription factor, which participates in the transcription of Cyclin E and leads to G1/S progression. The CDK inhibitor protein p27 inhibits CYCLIN D-CDK4/6 activity. However, activated ERK, c-MYC, AKT, all inhibit p27 activity. Importantly, the activated ERK MAPK and AKT pathways also inhibit various pro-apoptotic proteins, including BIM, Caspase-9, and BAD.

1.2.4 EGFR Transautophosphorylation

Binding of EGF to the EGFR leads to the transphosphorylation of various tyrosine residues on the intracellular C-terminal tail. The tyrosine residues phosphorylated by EGF addition to cells include Y703, Y920, Y992, Y1045, Y1068, Y1086, Y1148, and Y1173. In addition to these autophosphorylated sites, there are also residues that are phosphorylated by other kinases, which interestingly appear downstream in the EGFR-activation cascade. For example, Y845 is phosphorylated by c-SRC [54], and T654 is phosphorylated by PKC [55]. Regardless, the newly phosphorylated tyrosine residues serve as docking sites for proteins harboring phosphor-tyrosine-binding residues, such as those with Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains [56]. Other important domains in the EGFR's signal transduction include SH3 (binds proline-rich), 14-3-3 (binds phosphoserine), bromo (binds acetylated lysine), and PH domains (binds phosphorylated inositides).

Of the four family members, the EGFR signals to the largest number of unique signaling pathways, including the ERK MAPK, PI3K-AKT, SRC, PLC-γ1-PKC, JNK, and JAK-STAT pathways. As these pathways are inter-linked, the activation of the EGFR actually stimulates an entire signaling network associated with a wide number of outcomes, such as cell proliferation, growth, differentiation, migration, and inhibition of apoptosis. Many proteins within the EGFR's signal transduction network have been the subject of pharmaceutical targeting in malignancies, illustrating the potency of the receptor.

1.2.5 RAS-RAF-MEK-ERK MAPK Pathway

Following receptor transphosphorylation, the activated EGFR's Y1068 and Y1086 residues bind directly to GRB2 (growth factor receptor binding protein 2), by its SH2 domain from residues 60–158 [57–59]. Additionally, the activated EGFR Y1148 and Y1173 residues can

also recruit SHC (Src homology and collagen) [60–62] preferentially through it PTB domains, but also through its SH2 domain [63]. These two adaptors link the ligand-activated EGFR to complex intracellular biochemical pathways. Upon binding to the EGFR, SHC is phosphorylated at Y317, which becomes a binding site for GRB2 [61,64]. GRB2's two flanking SH3 domains bind to the proline rich carboxy-terminal tail in SOS1 (son of sevenless 1), which includes residues 1069 to 1138 [65–69]. SOS is a guanine nucleotide exchange factor (GEF) for RAS small guanosine triphosphatase (GTPase), and activates RAS by inducing it to exchange GDP to GTP [70]. RAS can then interact with the RAF-1 Ras-GTP-binding domain (RBD), which contains amino acids 55–131 [71]. RAF-1 is a complex protein, but phosphorylation of its Ser338 and Tyr341 residues have been shown to be important binding sites for MEK1/2 [72–75]. The phosphorylation of two other sites of RAF-1, S259 and S621 is inhibitory. Both pS259 and pS621 are bound by 14-3-3, which keeps RAF-1 in an inactivate conformation [76,77]. The phosphorylation of S259 is catalyzed by AKT [78]. RAF-1 directly activates MEK1/2 by phosphorylation at serine residues 217 and 221 [79]. MEK (mitogen-activated protein kinase kinase-MAPKK) 1/2 are a rare class of tyrosine and threonine/serine dual-specificity kinases that activate ERK1/2. MEK1/2 phosphorylates the Thr-Glu-Tyr motif in the ERK1/2 activation loop, at T202 and Y204 [80]. ERK1/2 then phosphorylates multiple substrates to induce various biological response. ERK1 and ERK2 are serine/threonine kinases that always appear to be activated together [81]. Unlike the narrow substrate specificity of RAF and MEK, ERK has over one hundred downstream cytoplasmic and nuclear substrates [82]. In the cytosol, it can activate RSK1 (p90 ribosomal S6 kinase 1) by phosphorylation at T573 [83]. Activated ERK itself also translocates to the nucleus to activate ternary complex factor (TCF) transcription factors, which play a major role in the induction of immediate early genes (IEGs) [84]. IEG products include c-

FOS and c-MYC, which induce late-response genes, to promote various phenotypes associated with ERK signaling [85]. Moreover, nuclear ERK can activate ELK-1, ETS, SP-1, and c-JUN [86–88].

Interestingly, in HeLa cells, the concentrations of RAS, RAF, and downstream proteins MEK and ERK have been estimated at 400, 13, 1400, and 960 nM respectively, and revealed that RAF inhibition could be mostly effective in inhibiting this signaling pathway [89].

1.2.6 PI3K-AKT-mTOR Pathway

The PI3K-AKT-mTOR signaling cascade controls metabolism, proliferation, cell size, survival and motility. In cancer, this pathway is often hyper-activated due to activating mutations to EGFR family members, PI3K, AKT, and downregulation of the famous tumour suppressor PTEN, which antagonizes PI3K activity. The PI3K-AKT-mTOR pathway is also dysregulated in diabetes, autism, and aging [90].

PI3K was discovered in the 1980s by the Cantley group [91]. It was shortly found to be activated by EGF stimulation [92]. There are three classes of PI3K, Class I, II, and III, which differ in structure, regulation, and function [93]. Here, we will only discuss Class I PI3K, the major downstream effector of EGFR. Class I PI3K are further subdivided by subclass: subclass IA (PI3Kα, β, and δ) is activated by receptor tyrosine kinases, and subclass IB (PI3Kγ) is activated by G protein coupled receptors [94]. PI3K is comprised of a regulatory p85 subunit that mediates binding to the receptor, and a catalytic p110 domain that phosphorylates the 3-OH group of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3) [91,95,96]. The newly formed PIP_3 is a potent secondary messenger and is the predominant mediator of PI3K activity [97]. The strong

signaling potential of PIP_3 is highlighted by the fact that the PI3K antagonist PTEN, which dephosphorylates and limits the activity PIP_3, is frequently inactivated in cancer [98]. PIP_3 links the lipid kinase activity of PI3K to the network of downstream signaling pathways, including the PH (pleckstrin homology) domain-containing serine/threonine kinase AKT/PKB. PI3K also indirectly stimulates the production of phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2), which can also recruit PH domain-containing proteins, including AKT. Other than AKT, cells contain 50–100 downstream effectors of PI3K [99]. Once localized at the PM, AKT is phosphorylated at T308 and S473. T308 phosphorylation is necessary and sufficient for AKT activation, however maximal activation is achieved by phosphorylation at S473 [100,101]. PDK1 phosphorylates AKT at T308, a residue located in the kinase domain [102]. Phosphorylation at the tail domain residue S473 is mediated by mTORC2, through a little understood process that involves PI3K activity [90,103].

There are three members of the AKT family: AKT1, AKT2, and AKT3. AKT2 and AKT3 have 81% and 83% amino acid homology to AKT1 respectively [99]. AKT isoform protein and mRNA levels have been characterized in various cell lines [104]. AKT1 and AKT2 are broadly expressed, whereas AKT3 is only expressed in the brain, heart, and kidney [105,106], which may explain why AKT3 is less well studied than the first two isoforms. There is no consensus on the distinct role of each isoform in cells, due to the controversial pool of data regarding their function. AKT1 has been found localized to the cytoplasm and nucleus, AKT2 to mitochondria, and AKT3 to the nucleus and nuclear membrane [104], although their localization may differ based on the cell type [107]. AKT2 appears to be the main AKT responsible for glucose metabolism and the induction of apoptosis, which supports its mitochondrial role. Regarding the regulation of AKT isoforms under the EGFR, all isoforms appear to be under the

control of PI3K upon EGF stimulation in various esophageal cancer cells [108]. However, depending on the cell line, the AKT isoforms are differentially activated in a little understood RAS-dependant manner. EGF stimulation does not appear to change AKT expression levels [104].

AKT (also known as Protein Kinase B, or PKB) is a serine/threonine kinase with a wide variety of substrates that impact cell survival, proliferation, metabolism, protein synthesis, growth, and migration. It is activated by a dual regulatory mechanism, requiring its translocation to the PM and phosphorylation at two conserved residues. Activated EGFR stimulates AKT translocation to the PM by activating the PI3K-induced formation of PIP_3. AKT binds to PIP_3 through its PH domain [109]. AKT mediates its wide range of physiological responses through the activation or deactivation of several downstream proteins.

In terms of cell survival, AKT functions in an anti-apoptotic manner by directly phosphorylating components of the cell death machinery. AKT phosphorylates the pro-apoptotic BAD (Bcl-2-associated death promoter) protein at S136, which inactivates BAD and prevents it from binding and inhibiting the anti-apoptotic BCL-X_L protein [110]. AKT also inhibits the catalytic activity of caspase-9 by phosphorylation at S196 [111], as well as the activity of FOXO1 by phosphorylation at T32 and S253 [112]. FOXO1 downstream gene targets include pro-apoptotic proteins BIM and the FAS ligand [113]. AKT also phosphorylates MDM2 at S166 and facilitates its translocation to the nucleus, where it ubiquitinates and downregulates p53, the well-known tumour suppressor [114–116].

One of the most important AKT pathways is to signal to mTOR (mammalian target of rapamycin). mTORC1 is well known to regulate cell growth and autophagy. The mTOR receives

stimulatory signals from growth factors through RAS and PI3K, as well as from nutrient inputs through amino acids, glucose, and oxygen availability [117].

1.2.7 PLC-γ1-PKC Pathway

Prior to the discovery of PI3K, interest on PIP_2 focused on the results of receptor-mediated PIP_2 hydrolysis by phospholipase C (PLC) [99]. The PLC isotype PLC-γ1 has been shown to upregulate cell migration and invasion in vitro and in vivo, including upregulating metastasis in cancer [118,119]. Of relation to this thesis, PLC-γ1 binds directly to phosphorylated EGFR at Y992 and Y1173 using its SH2 domain [120–123]. PLC-γ1 can also be recruited to the PM using its PH domain by binding PIP_3 formed by PI3K in response to EGF stimulation [124,125]. Phosphorylation of PLC-γ1 at Y472, Y771, Y778, Y783, and Y1254 have been shown to be important for its activity [126,127]. Once recruited to the vicinity of the PM and activated, PLC-γ1 hydrolyzes PIP_2 into free intracellular 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), two important secondary messengers. IP_3 binds to IP_3-receptors at the endoplasmic reticulum to induce intracellular calcium release. Calcium release converges with the DAG pathway, as both DAG and Ca^{2+} activate protein kinase C (PKC) family proteins.

PKC has a host of cellular substrates, including EGFR, RAF-1, H-RAS, p21, GSK-3β, RHOA, BAD, and BCL-2 [128]. Interestingly, PKC-dependent phosphorylation of EGFR T654 blocks EGF-induced EGFR activation [129]. Another substrate is phospholipase D (PLD). PKC phosphorylates PLD at S2, T147, S561, which mediates a large signaling network within the EGFR network [130–132]. Elevated PLD activity has been shown to contribute to fibroblast transformation by synergizing with EGFR and SRC [133,134]. Phospholipase D hydrolyzes phosphatidylcholine to form phosphatidic acid (PA) and choline [135]. PA can interact with proteins such as SOS, RAF, RAC, PIP5K, mTOR, and S6K (reviewed in [136]).

1.2.8 SRC

The first virus ever identified to cause cancer encoded a viral isoform of c-SRC (also known as pp60src). The isoform, discovered in 1911 by Francis Peyton Rous, was named v-SRC, as it induced sarcomas in chickens [137]. v-SRC lacks an important Y527 (Y530 in human c-SRC) inhibitory site. In inactive c-SRC, Y527 is phosphorylated and induces an inhibitory intramolecular loop between itself and the SRC SH2 domain [138–140]. Furthermore, auto-phosphorylation at Y416 (Y419 in human c-SRC) displaces the pY416-containing activation loop from the catalytic cleft, thereby allowing SRC to gain access to substrates [141].

c-SRC is widely implicated in various aspects of EGFR signaling. For example, it can directly phosphorylate EGFR Y845, RAF Y341, SHC1, clathrin, and CBL [142]. It is not clear how EGFR-mediates c-SRC activation, however EGF stimulation indeed leads to c-SRC activation [143]. EGFR and ERBB4 both possess binding sites for SRC [144]. EGFR and SRC have been reported to functionally synergize to form more aggressive cancers [143,145,146]. c-SRC may help activate STAT (signal transducer and activator of transcription) transcription factors in a JAK-independent manner [147]. c-SRC phosphorylates EGFR at Y845, which is not an autophosphorylation site [148]. However, Y845F mutation does not prevent c-SRC interaction to EGFR, nor does it affect ERK MAPK signaling [141,143]. Rather, Y845 has been suggested to regulate the autonomous lateral propagation of EGFR signals, which is the propagation of EGFR signals from one EGFR molecule to another without requiring binding of the ligand, potentiating EGFR kinase activity [149]. Phosphorylation of Y845 has been suggested as a diagnostic marker for various cancer treatments, including for NSCLCs [150,151]. Interestingly, pY845 is targeted by two phosphatases, PTP1B and TCPTP [152,153].

Since SRC and EGFR appear to cooperate to increase tumorigenicity, dual inhibition of SRC and EGFR has been proposed, such as in head and neck squamous cell carcinoma and colorectal cancer [154,155]. However, as mentioned in Section 2.3.2, SRC negatively regulates RAS by phosphorylation at Y32, therefore SRC inhibition leads to increased RAS activity. In fact, EGF stimulation of SRC/YES/FYN triple knockout MEFs does not induce the phosphorylation of RAS as Y32, and as such exhibit increased RAS-RAF-1 interactions [156].

1.3 EGFR Endocytosis

Endocytosis is the process by which extracellular cargo enters the cell through nascent plasma membrane invaginations that pinch off as intracellular vesicles. The endocytosis of the EGF-bound EGFR is of huge consequence to the cell. EGF-activated EGFR at the plasma membrane (PM) is a major site of origin for many signaling pathways. Endocytosis removes this signaling complex from the PM, ending the signaling from this location. This endocytosis however, begins a complex series of physiologically-relevant actions by the EGFR. Firstly, the endocytosed EGFR travels as an intracellular vesicle to fuse to an endosome. Endosomal EGFR retains the ability to signal, and due to its relocation, can access substrates that were once inaccessible at the PM [157]. This leads to distinct “endosomal EGFR” signaling pathways with different biological responses to “PM EGFR” signaling [158]. Moreover, at the endosome, the fate of the receptor is determined, where the EGFR is targeted for either degradation or recycling depending on the internalization pathway. The outcome of this decision thus regulates total EGFR levels, which plays a key role in subsequent EGFR activation and signaling.

Evidently, endocytosis adds another layer to the already complex signaling pathways, controlling signaling temporally and spatially. As EGFR signaling also controls its endocytosis, the interplay between the two thus critically regulates cellular activity. Here, I will discuss the molecular pathways of EGFR endocytosis, and their consequences to the cell. We will focus significant attention on differentiating between clathrin-dependent and –independent endocytosis, and discuss the role of ubiquitination and CBL to EGFR endocytosis.

1.3.1 Types of EGFR Endocytosis

In general, the endocytosis of membrane-bound receptors can occur by two mechanisms: constitutive endocytosis or ligand-induced endocytosis. The foremost example of constitutive endocytosis is of the transferrin receptor, which is continuously recycled between the PM and the cytoplasm. In constitutive endocytosis, the presence of the ligand does not affect the internalization and recycling cycle. In ligand-induced endocytosis, as for the EGFR, the membrane receptor can control its own internalization by activating downstream signals following ligand activation.

Ligand binding to the EGFR is necessary to strongly stimulate its endocytosis. Unliganded EGFR undergoes background levels of endocytosis at a 10-fold slower rate than EGF-stimulated EGFR ($t_{1/2}$ ~30 minutes), though they also recycle back to the PM at a fast speed [159–161]. Here, we will focus on ligand-induced EGFR endocytosis, particularly as a response to EGF.

Generally, ligand-induced EGFR endocytosis occurs through two molecular mechanisms. The first and most major route is clathrin-mediated endocytosis (CME), where the EGFR is internalized inside clathrin-coated pits. CME is by definition inhibited by molecular knockdown

of clathrin. The second type of endocytosis is non-clathrin mediated endocytosis (NCE) (or also called clathrin-independent endocytosis (CIE)). NCE is a broad term used to encompass all pathways of endocytosis insensitive to clathrin ablation.

The major difference between CME and NCE is that CME appears to mostly target the EGFR for recycling, whereas NCE functions to target the EGFR for degradation [48,162–164]. It was shown that low EGF concentrations appear to only activate CME, whereas high EGF concentrations are needed to activate NCE (along with CME). Importantly, ubiquitination appears to be required for NCE, but not for CME [48,165]. Therefore, it appears that these two endocytic pathways confer cells the ability to sense the amount of EGF ligand in the extracellular environment, so to accordingly downregulate the levels of plasma membrane EGFR to prevent its overactivation. The CME pathway is also easily saturated, which could explain why NCE was evolutionarily necessary to compensate in situations of excess ligand [166,167]. Lastly, NCE appears to progress slower than CME, although it is much faster than constitutive endocytosis [168].

1.3.2 Clathrin-Mediated Endocytosis

CME is the most researched and best-characterized endocytic pathway [47], as it is used not only for the internalization of receptors, but also for pathogen entry and synaptic transmission. Here, clathrin, adaptor proteins, and accessory proteins are recruited to the site at the plasma membrane that is to be endocytosed. EGFR CME, as with other types of receptors, occurs in four major steps: nucleation, budding, scission, and uncoating. Nucleation occurs when EGFR activation causes clathrin to accumulate at the intracellular side of the PM. Budding is the process by which the clathrin-coated patches invaginate continuously to form deep clathrin-coated pits (CCPs). Here, invagination is mediated by the connection of clathrin triskelions to

form a rounded lattice that resembles the panels on a soccer ball, with each connection progressively pulling and molding the CCP into a clathrin-coated bud. Scission then pinches off the invaginated bud from the cell membrane, releasing what is called the clathrin-coated vesicle (CCV) into the cytosol. The scission process is mediated by the large GTPase dynamin. The CCV is then shuttled to the endosome, a heterogeneous collection of PM-derived vesicles whose function is to sort cargo to the lysosome or for recycling back to the PM. Prior to fusion with the endosome, the coat is dissociated from the vesicle, releasing clathrin and adaptor proteins that can engage in a new round of endocytosis.

It should be stated that the important modules on the EGFR itself that initiate the internalization of CME has been full of controversy. It has been difficult to obtain a clear consensus on the molecular determinants and important components of the EGFR endocytic machinery because many early works attempted to isolate single factors that could completely influence CME, although it is becoming evident that many factors work inter-connectedly to bring about CME. Furthermore, the contributions of ligand concentration and of EGFR expression levels were not well understood, leading to conclusions on CME that may have included processes based in NCE, largely based on the use of high EGF concentrations.

Recent works from the Sorkin lab have attempted to clarify which motifs and proteins are actually important to EGFR CME. By performing progressive mutations of various EGFR amino acids, the Sorkin group had proposed that four redundant and cooperative mechanisms could mediate EGFR CME [169]: 1) through the clathrin adaptor AP-2; 2) by the acetylation of three different lysine residues (K1155, K1158, and K1164) at the EGFR C-terminal; 3) through GRB2, and; 4) by the ubiquitination of the receptor kinase domain. These conclusions were based on an EGFR mutant, 21KRΔAP2, which lacked ubiquitination and acetylation sites, through the

mutation of 21 lysine sites, and also lacked AP-2 binding sites (mutations to the Y^{974}RAL and ^{1010}LL^{1011} sites). However, as the 21KRΔAP2 mutant was found to still internalize at relatively high rates of 25-30% that of WT EGFR when treated with 2 ng/mL, this model was more recently expanded to account for newly identified ubiquitination sites identified by mass spectrometry, including Lys851. The residual internalization could be strongly inhibited by depletion of GRB2 and components of the ubiquitination process, including CBL, UbcH5b/c (an E2 ligase), and ubiquitin-binding adaptors [170]. The authors concluded that EGFR CME is in fact mediated by only two redundant mechanisms, ubiquitination and interaction with AP-2, and that they mediate CME stochastically. It must be noted that this model is based strongly on a heavily mutated EGFR, for which a difference they showed was its weaker ability to maintain AKT activity.

The actin machinery has been shown to be important for CME of EGFR. Actin accumulation at EGFR-containing clathrin coated pits sites is likely important to generate the force necessary for the budding pit to overcome membrane tension from the cell [171]. N-WASP and the ARP2/3 have been shown to be recruited to these sites following EGF stimulation [172].

1.3.3 Non-Clathrin Mediated Endocytosis

NCE (also CIE for clathrin-independent endocytosis) is a broad term used to encompass all types of endocytosis insensitive to clathrin ablation. The types of mechanisms of EGFR NCE are still being elucidated, as the heterogeneity of the pathway complicates the research. Additionally, new forms of EGFR NCE continue to be discovered. researchers have generally classified the pathways based on the following features [173]. First, whether the process appears to be of small-scale, or of large scale. Large scale NCE resembles pinocytosis, typically involving actin cytoskeleton rearrangement and membrane ruffling that does not appear to

specifically target receptors into endocytic vesicles. Small scale NCE includes processes that are smaller than 200 nm, and are further subdivided into three classes. 1. their dependence on dynamin; 2. the type or presence of a coat-like protein, and; 3. their dependence on small GTPases. To date, types of NCE identified include caveolin-mediated endocytosis, flotillin-mediated endocytosis, CLIC/GEEK, and FEME pathway [47].

For the EGFR, high ligand concentrations and/or conditions of high receptor expression activate NCE, whereas low or high levels of each condition can activate CME [174–177]. For example, the ablation of clathrin by siRNA only inhibits endocytosis when low EGF concentrations are used [48]. Also, downregulation of clathrin or the α-adaptin subunit of AP-2 by siRNA does not abolish internalization or sorting in HeLa cells treated with high EGF dosages [162,178]. NCE frequently depends on the formation of lipid rafts, which are cholesterol-rich PM microdomains that are sensitive to cholesterol ablation, such as by the agent filipin [48,179,180]. Also, NCE appears to occur more slowly than CME [166]. Functionally, NCE appears to target EGFR for degradation rather than recycling to the PM [163,165]. Ubiquitination appears to be dispensable for CME, but required for NCE [48]

1.3.4 Ubiquitination

Ubiquitin is ubiquitously expressed in mammals, and is a post-translational modification that is well known to regulate proteasomal degradation. However, ubiquitination can also regulate receptor trafficking, sorting, and downregulation. Ubiquitination of a substrate involves the sequential action of three classes of enzymes, E1, E2, and E3 proteins. CBL is the main E3 ligase for EGFR ubiquitination [181,182]. Here, we will focus on the role of ubiquitination for EGFR endocytosis, and we will discuss CBL in the following section.

Treatment of high EGF concentrations induce a rapid increase in EGFR ubiquitination. This ubiquitination is dependent on EGFR phosphorylation [163,183]. EGFR ubiquitination is minimal in cells treated with 1 ng/mL EGF, and nearly maximal a 10 ng/mL [47].

The correlation between NCE and EGFR ubiquitination was first reported in the seminal paper by Sigismund and colleagues [48]. Low EGF concentrations caused EGFR to localize predominantly to clathrin-coated pits, whereas high EGF led to near equal distributions of EGFR to clathrin- and non-clathrin-coated pits. Furthermore, an EGFR mutant fused to ubiquitin (EGFR/Ubmut) was constitutively internalized, and co-localized almost exclusively in non-clathrin-coated pits.

Ubiquitination of the EGFR is mainly through both mono- and poly-ubiquitination, and mainly through Lys63 chains, although Lys48 and Lys11 polyubiquitin chains have also been observed. Lys63 polyubiquitination, rather than multi-ubiquitination, has been suggested to be important for sorting to degradation [184]. Monoubiquitination is thought to signal for receptor internalization and sorting, whereas polyubiquitination targets proteins for degradation.

In one study, mass spectrometry has identified lysine residues 692, 713, 730, 843, 905, and 946 as EGF-dependent ubiquitination sites. A further study from the group recently identified lysines 851 and 855 as ubiquitination sites [170]

Ubiquitination appears to be unnecessary for EGFR CME. Mutation of 15 major lysine residues, including 6 major ubiquitin-acceptor sites that abolished receptor ubiquitination did not affect EGFR internalization rates [185]. However, mutation of 16 major lysine residues resulted in slower internalization, attributed to lower EGFR tyrosine phosphorylation. Interestingly, add-back of two major ubiquitin-acceptor sites to this mutant could promote EGFR CME (although

EGFR tyrosine phosphorylation of this add-back mutant also increased, but below that of WT EGFR), which was taken to mean ubiquitination could promote CME.

Ubiquitination of the EGFR appears to target it for lysosomal degradation [47], although the molecular mechanism is not known. In other words, how ubiquitination mediates EGFR trafficking from PM, to vesicles, to endosomes, to multivesicular bodies, and then to lysosomes is unclear. Proteins with ubiquitin interacting motifs (UIMs), including EPS15 and EPSIN have been shown to be important [48]. The elucidation of this pathway would be of wide-ranging impact.

1.3.5 CBL

The role of CBL in EGFR endocytosis has been controversial, due to the controversial role of ubiquitination itself. Furthermore, the CBL interactome reveals its key role in many of the EGFR's pathways, highlighting its complexity in the pathway [186].

In the CBL family, the isoform c-CBL is the best studied, followed by Cbl-b and then Cbl-3 (or Cbl-c). CBL-b has been shown to compensate for c-CBL knockdown and could ubiquitinate EGFR at a later time compared to c-CBL [187]. CBL-c is reported to be primarily expressed in epithelial tissues [188]. Here, I will mostly discuss c-CBL (henceforth CBL), as both CBL-b and CBL-c are lowly expressed in HeLa cells [163], the cell line in which most of my experiments were conducted.

CBL can be recruited to activated EGFR directly by interaction at pY1045 [182]. A phospho-deficient pY1045F EGFR mutant is ubiquitination impaired, but ubiquitination can still be observed, as CBL can also bind to the EGFR indirectly through GBR2, which binds to pY1068 and pY1086 [59,189]. The recruitment of CBL to the EGFR is therefore kinase-

dependent. Complex mathematical modelling and experimental validation have shown an EGF dose-response threshold-controlled EGFR-CBL interaction [163] (Figure 1.2). This model takes into account the synergism by which pY1045, pY1068, pY1086, and GRB2 can all contribute to CBL binding, and predicts that ubiquitination of the EGFR rises more quickly compared to EGFR phosphorylation in response to EGF doses between 2-10 ng/mL, effectively setting a threshold effect. As such, EGFR ubiquitination is minimal in cells treated with 1 ng/mL EGF, and nearly maximal a 10 ng/mL, whereas EGFR phosphorylation is maximal at 100 ng/mL.

CBL contains a tyrosine kinase binding (TKB) domain, a proline-rich domain (PRD), and a RING finger domain. The TKB binds to pY sites of the activated EGFR. The PRD can be used to be recruited by SH3-containing proteins such as GRB2, SRC, and CIN85 [186,190]. The RING finger domain importantly catalyzes the transfer of ubiquitin from an E2 ligase to ubiquitinate the EGFR. CBL mutations in the RING finger domain or in the linker between the RING finger and tyrosine kinase-binding domain significantly reduce EGFR ubiquitination and down-regulation [191]. The oncogenic variant 70z-CBL (used in this thesis), originally isolated from a 70Z/3 mouse pre-B-lymphoma cell line, contains a 17 amino acid deletion that disrupts the RING finger motif [192]. Furthermore, mutations in RING and linker domains, deletions and insertion that inhibit receptor ubiquitination can result in malignancies [193,194]. Oncogenic CBL has been observed in AML, and lung cancers [195]. Furthermore, c-CBL overexpression has been shown to inhibit migration, reduce cell proliferation, and inhibit tumour growth in vitro in NSCLC cell lines, and in vivo [196]. The role of various CBL mutations and its effect on cellular transformation, EGFR ubiquitination, internalization, and recycling has been studied in extensively [197], which has unfortunately also led to controversial data. Various publications argue for a role for CBL in endocytosis, whereas others argue that it is dispensable.

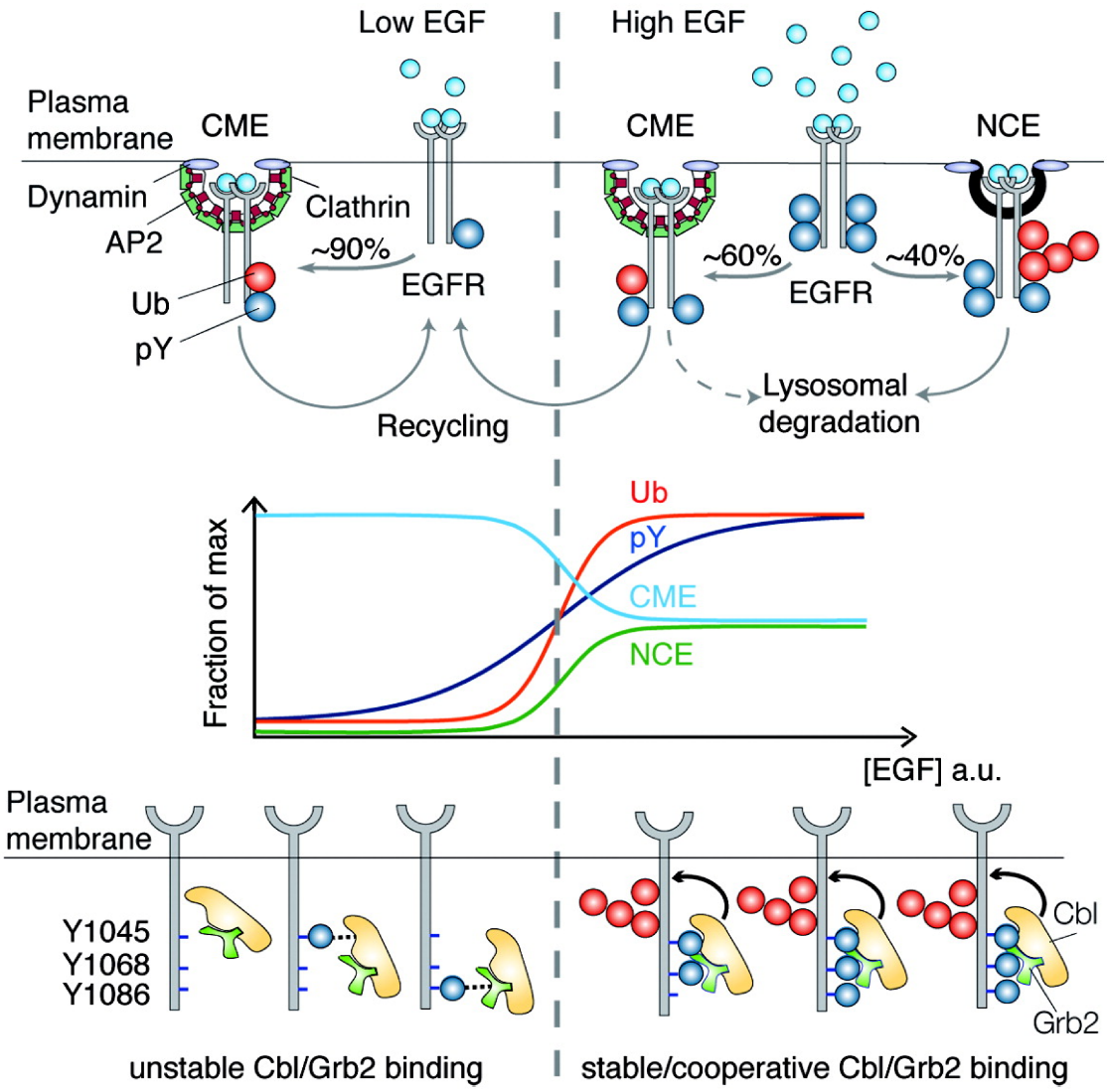

Figure 1.2. Model of EGFR-Ub threshold. EGFR ubiquitination is dependent on CBL-binding to EGFR. CBL-binding to EGFR however is dependent on various factors: pY1045, pY1068, pY1086, and GRB2-binding to EGFR. At low doses, increasing EGF concentrations slowly increases EGFR tyrosine phosphorylation, but is not yet sufficient to induce CBL-binding to EGFR. However, with increasing EGF doses toward X_T (half-maximal dose EGF dose for EGFR ubiquitination), the chance of EGFR phosphorylation and GRB2-binding to EGFR continue increasing, however the chance of CBL-binding increases dramatically.

Figure adapted from Polo, S., Di Fiore, P. P., & Sigismund, S. (2014). Keeping EGFR signaling in check: Ubiquitin is the guardian. *Cell cycle*, *13*(5), 681-682.

In CME, GRB2 recruitment of CBL has been shown to be important for endocytosis [198]. GRB2 knockdown by siRNA inhibits endocytosis. This was rescued by GRB2-YFP transfection, as well as with transfection with a chimera of the SH2 domain of GRB2 fused to c-CBL, even if it was fused with only the RING domain of c-CBL. Although EGFR CME appears to require GRB2 and c-CBL, the EGFR ubiquitination sites do not appear to play a significant role. To mediate this discrepancy, two hypotheses have been proposed, that: there exists an unidentified receptor-associated protein that is (1) ubiquitinated by c-CBL or (2) that mediates the internalization of the receptor [199].

The role of CBL in NCE is much clearer. An EGFR/ubiquitin chimera that signals exclusively through its ubiquitin moiety is internalized exclusively by NCE [48]. The ubiquitination of the EGFR is dependent on CBL, and cells downregulated for both CBL and clathrin cannot undergo significant NCE (although low background levels of internalization were still observed) [163].

1.3.6 Endosomal Sorting

Following endocytosis, endocytic vesicles fuse to early endosomes (EEs), a combination of small vesicles and tubules. EGF and EGFR can be detected in early endosomes after 1-5 min of EGF stimulation at 37°C. It is here that the fate of the receptors are determined. To first deactivate the EGFR, endosomes use their mild acidity (pH 6.0-6.8) to dissociate the ligand from the EGFR [200], The free ligand is transferred to the late endosome for degradation, however the free receptor has two choices. Firstly, the EGFR may be recycled to the plasma membrane. This can happen in two ways: by being retrieved from early endosomes and being rapidly recycled back to the PM (~1-3 mins), or by forming recycling endosomes at the perinuclear region before returning to the PM (~10-15 mins) [201]. Secondly, the EGFR may be targeted for degradation,

by sequestering the receptor away into intraluminal vesicles. This gives the endosomes the appearance of a multi-vesicular body (MVB), and become referred to as late endosomes. Late endosomes pour their contents into lysosomes to initiate the degradation of the cargo. This entire process occurs rapidly in the cell: for cells stimulated with EGF at 37°C, EGF and EGFR can be detected in early endosomes after 1-5 min, in late endosomes after 10-20 min, and in lysosomes after 40-60 min [168,202].

EGFR sorting decisions rely on the recruitment of RAB GTPases [203]. This has led to the use of RAB proteins as markers of endocytic trafficking. For example, RAB5 is found in early endosomes, RAB7 destines EGFR late endosomal maturation, and RAB11 targets EGFR for recycling [201].

Unlike the extremely complicated internalization of the EGFR, the lysosomal degradation sorting signal has been attributed to single ubiquitin-based mechanism of EGFR [181,204,205]. The endosomal sorting of the EGFR to the lysosome requires CBL-mediated ubiquitination [191,206–208]. However, the precise molecular mechanism linking ubiquitination to EGFR internalization and degradation is still lacking.

1.4 Cell Cycle

1.4.1 Cell Cycle

Almost all cells of the human body go through the cell cycle. Excluding post-mitotic cells such as neurons and muscle cells, the cell cycle grants cells a way to replenish its numbers so to grow, or balance out the cells lost by cell death. The division of a single parent cell to two perfectly-copied daughter cells is fundamental to life itself.

The typical cell cycle is composed of the following cycling phases: G1, S-phase, G2, mitosis, and cytokinesis. Mitosis is also made up of prophase, prometaphase, metaphase, anaphase, and telophase.

1.4.2 CDKs and Cyclins: Molecular Drivers of the Cell Cycle

Progression through the cell cycle is mediated by cyclin-dependent kinases (CDKs) and their regulatory cyclin subunits. CDKs are major cell cycle serine/threonine kinases with a wide variety of substrates, and include CDK4/6, CDK2, and CDK1 (also known as CDC2). CDKs are activated in large part by the binding to their cyclin partners, whose expression rise and fall throughout the cell cycle to mediate the temporal activation of each CDKs [209–211]. There are four different classes of cyclins, A-, B-, D-, and E-type, and various subtypes of each class, bringing the human cyclin count to ten [212].

Quiescent cells do not express any detectable levels of cyclins, and thus CDKs are not activated. The presence of mitogenic factors, such as EGF, induces the expression of CYCLIN D, which then accumulates throughout G1 and bind to their catalytic partners CDK4 and CDK6. Full activation of CDK4/6 requires dephosphorylation by CDC25A phosphatase. A key phosphorylation target of CYCLIN D-associated CDKs is the retinoblastoma (RB) protein, which normally binds and inhibits E2F family members. Phosphorylation of RB, including at Ser807 and Ser811, releases E2F, and free E2F can help transcribe key genes for the transition of G1/S, including CYCLIN E and CYCLIN A [213,214]. CYCLIN E binds CDK2 and further phosphorylates RB to complete its inactivation [215]. This triggers S-phase by de-repressing the transcription of genes encoding proteins required for DNA synthesis, such as those that drive the assembly of the pre-replication complex [216]. Upon the assembly of the pre-replication complex, CYCLIN A levels rise sufficiently to begin replacing Cyclin E on CDK2, and initiates

DNA replication [217]. During G2, CDK2-CYCLIN A also catalyzes phosphorylations to FoxM1, whose target genes drive mitotic entry, such as regulators of mitosis and components of the spindle assembly checkpoint [218,219]. At the same time, signals activate the CDC25C phosphatase and inhibit the WEE1 and MYT1 kinases, which together activate CDK1-CYCLIN B by dephosphorylating CDK1 at Y14 and Y15 [220]. The CDK1-cyclin B complex, also called the maturation promoting factor (MPF), drive cells through mitosis by inducing nuclear envelope breakdown, a process that coincides with the degradation of CYCLIN A, facilitating the formation of CDK1-CYCLIN B complexes. Further activity of the MPF induces chromosome condensation, and activation of mitotic kinases. Chromosomes line up at the metaphase plate, and once the SAC is satisfied, the chromosomes are pulled apart and CYCLIN B1 is degraded. Cells then complete mitosis, including cellular fission by cytokinesis. The progression of cells through each of these phases is tightly guarded by cell cycle checkpoints.

1.4.3 Cell Cycle Checkpoints

Various cell cycle checkpoints exist to ensure that critical parts are in place prior to cell cycle progression into the next phase. The cell cycle checkpoints are the G1 (restriction) checkpoint, the G2/M DNA damage checkpoint, and the metaphase or spindle assembly checkpoint (SAC). In cancer, components of these checkpoints are often inactivated, so that cell cycle arrest does not occur, and cell cycle progression can proceed unchecked. The family of cyclin dependent kinase inhibitors (CDKi) form important mediators of cell cycle arrest, and includes the INK4A family ($p16^{Ink4A}$) and the Cip1/Kip1 family ($p21^{Cip1/Waf1}$ and $p27^{Kip1}$). $p16^{Ink4A}$ can prevent the activation of CDK4 and CDK6, $p21^{Cip1/Waf1}$ can prevent the activation of CDK1, and $p27^{Kip1}$ can prevent the activation of CDK2 and CDK4. Furthermore, DNA damage

including single stranded or double stranded breaks can induce cell cycle arrest, often through a p53-dependent mechanism.

G1 arrest occurs when mostly double-strand DNA breaks are sensed by ATM (ataxia telangiectasia mutated), which phosphorylate CHK2 [221,222]. CHK2 then phosphorylate and induce the degradation of CDC25A, a protein which normally helps activate CDK2-CYCLIN E. The degradation of CDC25A following DNA damage thus inhibits the ability of CDK2-CYCLIN E to cause progression to S-phase [223,224]. Furthermore, ATM can phosphorylate p53, which reduces p53 affinity to its negative regulator MDM2, thus stabilizing p53. Stabilized p53 can activate $p21^{Waf1/Cip1}$, which inhibits both CDK2/CYCLIN A and CDK2/CYCLIN E, further ensuring cell cycle arrest until the DNA damage can be fixed, or if the damage is irreparable, lead to cell apoptosis [225–227].

S-phase arrest occurs in the event of DNA damage arising from stalled replication forks or nucleotide repair processes. ATR (ataxia telangiectasia and Rad3 related) senses these mostly at tracts of single-stranded DNA and phosphorylates CHK1, which also induces CDC25A degradation to prevent progression through S phase [223,224,228].

CHK1 is also involved in G2 arrest at the G2/M checkpoint, since it can also inhibit CDC25C and activate WEE1, and prevent entry into mitosis [229–231]. CHK1 phosphorylation of CDC25C forms an inhibitory binding site for 14-3-3 to sequester it [232]. During G2, CHK1 is also able to activate $p21^{Waf1/Cip1}$ [233].

The spindle assembly checkpoint (SAC, also known as the mitotic or metaphase checkpoint) at metaphase ensures that mitotic cells can faithfully divide its chromosomal contents prior to anaphase allocation to each daughter cell. If all chromosomes are not properly

attached to the mitotic spindle, the SAC is not satisfied and mitotic exit is not allowed. In this way, the SAC importantly guards against whole-chromosome gains or losses that may result from uneven distribution of chromosomes. The SAC is an important component to the function of a class of cancer drugs known as anti-mitotics. As cancer cells often undergo cell proliferation more frequently, anti-mitotic drugs slow their growth more specifically than most normal cells. Anti-mitotics prevent cells from proceeding past the SAC through various mechanisms, and induce the mitotic catastrophe of these cells. Mitotic catastrophe is a term more recently defined as a mechanism that senses mitotic failure and results in an irreversible fate, such as apoptosis, necrosis, or senescence [234]. Causes of mitotic catastrophe may range from premature or inappropriate entry into mitosis, to the failure of cell cycle checkpoints to fix cellular damage [235]. Mitotic catastrophe is a potent oncosuppressive mechanism, as it can catch cancer cells that have evaded the previous checkpoints. These cells are usually less potent, since their improperly-managed DNA contents make it more difficult to form viable daughter cells, leading to genomic instability or aneuploidy. However, evasion of mitotic catastrophe is considered an advantage to cancer development [235]. Cancer cells that find ways around the SAC often replicate despite having non-normal numbers of chromosomes. This further increases the chances for *de novo* mutations that may be beneficial to cancer cells.

1.4.4 EGFR and Cell Cycle

The Restriction Point (R point) is the instant at G1 at which the cell commits to the completion of the cell cycle and at which the cell no longer requires growth factors to complete the cell cycle [236]. For cell cycle progression, based on the definition of the R point, it would appear that EGFR activation is only necessary from quiescence to the R point. Little is known about the functional significance of EGFR activation in phases other than G1. It is clear

that EGF stimulation can activate the EGFR of cells in all cell cycle phases (our unpublished observations). What then is the role of activated EGFR in S, G2, or mitosis? The EGFR controls a vast array of other physiological functions, such as migration, cell growth, and cell survival. For example, EGF stimulation during G1 also controls cell spreading, and this morphological change has been shown to be important for DNA replication [237]. Does the EGFR play roles in impacting those functions during S, G2, or mitosis?

1.4.4.1 EGFR During G0 to G1

The best described role for the EGFR is to drive cells through G1, largely by inducing the expression of cyclin D. In cancers, activating mutations to the EGFR are correlated with higher expression levels of cyclin D [238–243]. These types of cancers also have poor prognoses. The activation of proteins in the MAPK ERK pathway are crucial to cyclin D induction, as sustained RAS or ERK1/2 activation are sufficient for the accumulation of Cyclin D1 and the inactivation of RB during G1 [244–249]. Furthermore, PI3K-AKT signaling also upregulates Cyclin D1. Active GSK-3β normally causes Cyclin D proteolysis through phosphorylation at T286 [250]. However, activated AKT phosphorylates and deactivates GSK-3β, which inhibits the repression of Cyclin D, thus upregulating Cyclin D levels. Since Cyclin D can activate CDK4, selective CDK4/6 inhibitors have been of intense research, including for cancers driven by HER2 or PI3K [251].

It has been shown that a pulse of EGF stimulation during early G1 and another at late G1 are sufficient to drive cells past the R point [252,253]. Inhibition of EGFR in various cancers with EGFR inhibitors, including monoclonal antibodies or tyrosine kinase inhibitors, lead to G1/S arrest [254]. Interestingly, many researches point that the cause of the G1/S arrest is due to the upregulation of the $p27^{Kip1}$ [255–261]. Upregulation of $p27^{Kip1}$ leads the downregulation of

Cyclin D1 activity and hypophosphorylation of RB. ERK activity is thought to contribute to $p27^{Kip1}$ down-regulation [262–264]. Also, $p27^{Kip1}$ has been found to downregulate Egfr transcription [265,266]. Interestingly, c-MYC, a downstream effector of ERK, activates Cyclin D1 transcription and inhibits $p21^{Cip1}$ and $p27^{Kip1}$ activity, demonstrating the central role of c-MYC in G1/S progression [267,268].

1.4.4.2 EGFR During S phase

Both low and high concentrations of EGF stimulation (1 or 100 ng/mL) of EGFR during S-phase has been shown to promote premature centrosome separation in HeLa cells [269]. This premature centrosome separation further leads to a 44% decrease in the duration of mitosis, and an increase in the accuracy of mitosis [270]. Interestingly, this premature centrosome separation occurred in an AKT-dependant manner through the Mst2-hSav1-Nek2A module, thus putting less dependence on the kinesin-5 motor protein Eg5 for centrosome separation. As such, Eg5 inhibitors did not arrest cells in mitotic if they also had high levels of basal EGFR signaling. Therefore, S-phase EGF response can play a large role in drug resistance.

Interestingly, radiation therapy has been shown to activate EGFR and ERK and induce progression to S phase and cryoprotection [271,272]. A report showed that in EGFR-transfected CHO cells, cell death only occurred to quiescent cells that progressed through the cell cycle (due to EGFR activation), and not to cells that were already proliferating [273]. This difference in radiosensitivity between cell cycle phases is a poignant example of how cell cycle phase can affect therapeutic results.

1.4.4.3 EGFR During G2

There has been more research performed regarding the role of the EGFR during G2, mainly because a requirement for basal EGFR activity has been shown for the G2/M transition. However, the mechanism by which this requirement is mediated is still unknown.

The G2/M DNA damage checkpoint may represent a beneficial point for tumour cells to repair their DNA prior to entering mitosis. Some studies have shown that EGFR inhibition may allow cells to bypass the G2/M checkpoint. If these cells bypass the DNA repair mechanism of the G2/M checkpoint, these cells are more likely to possess DNA unsuitable for proper mitosis, which can lead to mitotic catastrophe. In addition, if these cells are also treated with an anti-mitotic drug, mitotic catastrophe may be more likely.

For example, a study showed that EGFR-targeted hybrid plasmonic magnetic nanoparticles (named 225-NP) could override the G2/M checkpoint by inhibiting CHK1 signaling [274]. In endometrial cancer cells lacking p53, the combination of paclitaxel (arrests cells in mitosis) and gefitinib (EGFR kinase inhibitor) appeared to increased cell death from paclitaxel alone [275]. The authors showed that the combination of paclitaxel and gefitinib for 24h induced a more efficient arrest of cells in G2/M compared to paclitaxel alone. They also showed that this combination somehow increased CDC25C activity and decreased WEE1 activity, which likely helped activate the Cyclin B-CDK1 complex and allowed cells to commence mitosis, although the cells would be arrested before reaching anaphase and undergo mitotic catastrophe. Another study showed that nimotuzumab (a humanised monoclonal antibody against EGFR) sensitized human lung adenocarcinoma A549 to radiotherapy [276]. This combination arrested more cells in G2/M compared to radiotherapy alone, which arrested more cells in G0/G1. A study has shown that nimotuzumab may inhibit radiation-induced activation of DNA-PKcs (DNA-dependent protein kinase, catalytic subunit) by blocking the AKT-PI3K

pathway, which are normally required for NHEJ DNA repair [277]. Furthermore, a newer small molecule EGFR inhibitor, WB-308, has been shown to cause G2/M arrest and apoptosis [278].

1.4.4.4 EGFR During Mitosis

The ability of EGFR to be activated and to be endocytosed during mitosis has been somewhat controversial. There are no changes to EGFR expression at the plasma membrane in mitotic cells [4,279,280]. EGF-induced EGFR signaling had been thought to be suppressed during mitosis, and this was explained as due to decreased ligand binding affinity and an inability to induce receptor dimerization, although cells with higher EGFR expression levels could overcome the suppression [2]. However, EGFR dimerization was shown to occur in nocodazole-arrested mitotic HeLa cells following EGF stimulation for 5 min [281]. Another report showed that EGFR, ERK2, and PLC-γ are less phosphorylated in mitosis-arrested cells [282]. Another study showed CDK1 interacted with and inhibited several proteins in the EGFR pathway, including EGFR, GRB2, SOS1, and RAF-1 [3]. EGFR kinase activity was also shown to be necessary for its endocytosis during mitosis [4]. EGFR signaling can also critically affect surrounding mitotic cells. In *D. melanogaster* tracheal placode, EGFR-induced myosin-II contractility was found to help epithelial invagination driven by surrounding cells undergoing mitotic cell rounding [283].

1.5 Hypothesis and Aims

The EGFR's role in stimulating cell cycle progression is well known. The role of the EGFR has been intensely studied mostly during the G0 and G1 phase of the cell cycle. However, the signaling and endocytosis of the EGFR have not been extensively studied during mitosis. This is because the majority of the interest in the EGFR is regarding its ability to stimulate cell

cycle progression past the G1. Moreover, interphase cells make up the majority of cells in cell cultures and animal models, meaning mitotic cells are often disregarded.

Since it was clear that the EGFR signals and is endocytosed differently between cell cycle phases, here, I studied the function and role of the EGFR throughout the cell cycle, primarily during mitosis. I first characterized EGFR signaling during mitosis to find the molecular mechanisms behind the differential signaling outputs of the EGFR between mitosis and interphase (Chapter 2) [279]. Second, I studied the endocytosis of the EGFR during mitosis, to uncover the molecular mechanism behind the slower endocytosis previously observed as compared to interphase cells [4] (Chapter 3). Lastly, I characterized EGFR signaling throughout the other phases of the cell cycle (Chapter 4). Therefore, my research has contributed to the most in-depth characterization of the EGFR during mitosis. This knowledge may be used to fine-tune the pharmacological targeting of the EGFR.

2 Chapter 2

Regulation of epidermal growth factor receptor cell signaling during mitosis

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Note: All experiments were carried out by Ping Wee, except for the work on CHO and Cos7 cell lines conducted by H. Shi. J. Jiang and Y. Wang contributed to data collection.

2.1 Chapter Abstract

Mitosis and epidermal growth factor (EGF) receptor (EGFR) are both targets for cancer therapy. The role of EGFR signaling in mitosis has been rarely studied and poorly understood. The limited studies indicate that the activation of EGFR and downstream signaling pathways are mostly inhibited during mitosis. However, we recently showed that EGFR is phosphorylated in response to EGF stimulation in mitosis. Here, we studied EGF-induced EGFR activation and the activation of major signaling pathways downstream of EGFR during mitosis. We showed that EGFR was strongly activated by EGF during mitosis as all the five major tyrosine residues including Y992, Y1045, Y1068, Y1086, and Y1173 were phosphorylated to a level similar to that in interphase. We further showed that the activated EGFR is able to selectively activate some downstream signaling pathways while avoiding others. Activated EGFR is able to activate PI3K, AKT2, but not AKT1, which may be responsible for the observed effects of EGF against nocodazole-induced cell death. Activated EGFR is also able to activate c-SRC, c-CBL and PLC-γ1 during mitosis. However, activated EGFR is unable to activate ERK1/2 and their downstream substrates RSK and ELK-1. While it activated RAS, EGFR failed to fully activate RAF-1 in mitosis due to the lack of phosphorylation at Y341 and the lack of dephosphorylation at pS259. We conclude that contrary to the dogma, EGFR is activated by EGF during mitosis. Moreover, EGFR-mediated cell signaling is regulated differently from interphase to specifically serve the needs of the cell in mitosis.

2.2 Introduction

EGFR plays important roles in initiating cell signaling to produce specific effects on cell growth and development [284]. The activated EGFR forms signaling complexes with many signaling proteins including GRB2, SHC, phospholipase C-γ1 (PLC-γ1), the p85α subunit of phosphoinositide 3-kinase (PI3K), SRC, and CBL [285,286]. The formation of the receptor-signaling protein complexes then initiates the activation of various signaling pathways. For example, the interaction between EGFR and SHC/GRB2 results in the recruitment of SOS to the plasma membrane to activate RAS. Activated RAS mediates RAF activation, which then phosphorylates and activates mitogen-activated protein kinase kinase (MEK), leading to the activation of extracellular signal-regulated kinases (ERK). Activated ERK phosphorylates RSK that in turn translocates into the nucleus to activate transcription factors such as c-FOS and SRF. Activated ERK may also translocate into the nucleus to activate transcription factors such as ELK1 and c-FOS[158,285–287]. The activation of PLC-γ1 by EGFR regulates cell mitogenesis and migration [158,288,289]. The activation of PI3K by EGFR stimulates AKT/PKB activity, which protects the cell from undergoing apoptosis [108,290,291]. The activation of CBL results in the ubiquitination and downregulation of EGFR [181,182,187].

However, all of the above knowledge regarding EGFR may only reflect its activation and function during interphase, especially G1 phase as these studies were conducted with the cells mostly in G1 phase. The cell cycle is a series of events leading to cell replication. When plated at low cell densities in serum-containing medium, cultured cells start to proliferate, moving through the four phases of the cell cycle: G1, S, G2, and M. Progression through each phase and transition from one phase to the next is regulated by the coordinated action of kinases and proteases. When deprived of serum, cells continue to cycle until they complete mitosis,

whereupon they exit from the cell cycle into the G0 state. These cells can re-enter the G1 phase of the cell cycle by the addition of serum or growth factors [292,293]. Growth factors, including EGF, regulate cell cycle progression, especially the G0 to G1 transition and G1-S progression. Growth factors must be present until the restriction point (R point) in G1 phase to stimulate entry into the cell cycle and proliferation. After the R point, growth factors are not required to complete the other stages of the cell cycle [292]. We and others also showed that two pulses (30 min) of growth factors including platelet-derived growth factor and EGF, separated by 8 hours, are also able to drive the cell cycle [252,253]. While it has been proposed that the G0 to S interval is the only portion of the cell cycle that is regulated by growth factors [292], there have been sporadic publications showing a minor role for growth factor-mediated cell signaling in S and G2 phase [270,294–298].

The role of growth factor-induced cell signaling in mitosis has been rarely and poorly studied. Mitosis (M phase) is the most dynamic period of the cell cycle, involving a major reorganization of many cell components. A hallmark of cancer involves the cancer cell's ability to sustain chronic proliferation [299]. A major difference between cancer cells and normal cells is that the cancer cells are much more mitogenic and show a higher frequency of mitosis. Therefore, most cancer drugs are designed to specifically target mitotic cells [300]. It has been reported that while there is no change in the number of cell surface EGFR between interphase and M phase of the cell cycle, EGF-induced activation of EGFR and downstream signaling is tightly suppressed in M phase due to a decrease in ligand binding affinity and the inability of EGF to induce receptor dimerization [2]. It has also been shown that EGFR, ERK2, GTPase-activating protein (GAP) and PLC-γ1 are less phosphorylated in M-arrested cells than they are in interphase[282]. A further study showed that CDC2 (also known as CDK1) inhibits EGFR-

stimulated ERK activation during mitosis by primarily targeting signaling proteins that are upstream of MEK1 including EGFR [3]. These studies suggest that inhibition of EGFR activity and its downstream signaling pathways underlie the importance of keeping the cell sheltered from extracellular signals when it undergoes division, and is beneficial for preventing gene expression so to preserve the energy needs that are required for mitotic structural changes [2,3,282].

However, in a recent study of EGF-induced EGFR endocytosis during M phase, we have shown that at M phase, EGFR is expressed at the same level as in interphase and is also activated by EGF to the same level as in interphase as shown by pY992[4]. Here we further studied EGF-induced activation of EGFR and the major downstream signaling pathways during cell mitosis. We showed that EGFR was strongly activated by EGF during mitosis as all five major tyrosine residues including Y992, Y1045, Y1068, Y1086, and Y1173 were phosphorylated to a level similar to that in interphase. We further showed that the activated EGFR selectively activated some downstream signaling pathways while avoiding others. Activation of EGFR resulted in the activation of AKT2, but not AKT1, which may be responsible for the observed effects of EGF against nocodazole-induced cell death. Activated EGFR also activated c-CBL and PLC-γ1, two signaling protein with multiple cellular functions, during M phase. However, activated EGFR is unable to activate ERK1/2 and their downstream substrates RSK and Elk-1. While it activated RAS, EGFR failed to fully activate RAF-1 in mitosis due to the lack of phosphorylation at Y341 and the lack of dephosphorylation at pS259. These studies suggest a differential activation of EGFR-pathway genes during mitosis, for which targeted drug therapy should consider.

2.3 Materials and Methods

2.3.1 Antibodies and Chemicals

Mouse monoclonal anti-PLC-γ1 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). All of rest primary antibodies were from Santa Cruz Biotech (Santa Cruz, CA). The horseradish peroxidase (HRP) - conjugated secondary antibodies were from Bio-Rad (Hercules, CA) and the fluorescence-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). The cell culture reagents were from Invitrogen (Carlsbad, CA). Mammalian Protein Extraction Reagent (M-Per) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL USA). Vybrant MTT Cell Proliferation Assay Kit were from Invitrogen (Grand Island, NY). Unless otherwise specified, all other chemicals were from Sigma (St. Louis, MO)

2.3.2 Cell Culture and Treatment

The cell lines that were used include HeLa cell line stably expressing H2B-GFP (HeLa H2B-GFP), Cos7 fibroblasts and CHO cell lines stably expressing a YFP-tagged, wild-type EGFR (CHO-EGFR) [301]. The HeLa H2B stable cell line is a generous gift from Dr. Wahl (The Salk Institute for Biological Studies). It has been shown that the H2B-GFP fusion protein is incorporated into nucleosomes without affecting cell cycle [302].

All cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 10% FBS and were maintained in a 5% CO2 atmosphere. For CHO cell lines, G418 was added to a final concentration of 500 μg/ml. For the HeLa H2B-GFP cell line, blasticidine was added as a supplement to a concentration of 2 μg/ml to maintain the trans-gene expression. EGF was used at a final concentration of 1 to 50 ng/ml. To inhibit EGFR kinase activity, cells were incubated with AG1478 at 1 μM for 30 min.

To collect lysates for mitotic cells, cells were arrested in prometaphase by treating with nocodazole (200 ng/mL) for 16 h. The nocodazole-arrested cells were treated with EGF (50 ng/ml, except indicated otherwise) for 5, 15, 30 min, 1 or 2 h. Mitotic cells in serum-free media were

dislodged by gently tapping the plates on ice. The cells were washed with PBS and centrifuged at 1000 rpm. The obtained mitotic cells were then lysed with M-Per in the presence of phosphatase and protease inhibitors including 100 mm NaF, 5 mM MgCl2, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A. To collect lysates for interphase cells, cells were serum starved for 16 h and then treated with EGF for 5, 15, 30 min, 1 or 2 h. The cells were collected by scraping on ice in cold M-per buffer in the presence of phosphatase and protease inhibitors.

2.3.3 Immunoblotting

Immunoblotting was performed as previously described [158]. Briefly, protein samples were separated by SDS-PAGE and then were transferred onto nitrocellulose and probed with primary antibodies. The primary antibodies were detected with a HRP conjugated secondary antibody followed by enhanced chemiluminescence development (Pierce Chemical, Rockford, IL) and light detection on Fuji Super RX Film (Tokyo, Japan). For graphical analysis, sub-saturated band exposures were scanned using a GS-800 calibrated densitometer. Quantification of band intensity was finished by using ImageJ software. For the quantification of the phosphorylation of various proteins, the band intensity of phosphorylated proteins is normalized against the band intensity of non-phosphorylated proteins. Two-tailed student t-tests were completed using MedCalc software. ** indicates $p<0.01$ and * indicates $p<0.05$.

2.3.4 RAS Activation Assay

Glutathione S-transferase (GST) fused to the RAF RAS binding domain (GST-RBD), precoupled to glutathione-agarose beads in BOS buffer (50 nM Tris-HCl, pH 7.4, 200 mM NaCl, 1%, Nonidet P-40, 10% glycerol, 10 mM NaF, 2.5 mM $MgCl_2$, 1 mM. EDTA), was added, and the lysates were incubated at 4°C for 1 h. Beads were collected by centrifugation and washed three

times with BOS buffer, and then loading buffer was added. RAS was detected with the monoclonal anti-RAS antibody (Santa Cruz, cat: sc-166691), followed by a horseradish peroxidase (HRP)-coupled anti-mouse antibody.

2.3.5 Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously [4]. Briefly, cells were grown on glass coverslips and serum starved for 16 h. After treatment, the cells were fixed by methanol and permeabilized with 0.2% Triton X-100. Next, the cells were incubated with primary antibody at room temperature for 1 h followed by rhodamine-labeled secondary antibody for 1 h.

2.3.6 Immunoprecipitation

Immunoprecipitation experiments were carried out as described previously [4]. Cells were lysed with immunoprecipitation buffer (20 mM Tris, pH7.5, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml aprotinin, 1 µM pepstatin A) overnight at 4°C. Cell lysates were then centrifuged at 21 000 x g for 30 min to remove debris. The supernatants, containing 1 mg of total protein, were used to incubate with 1 µg of mouse anti-EGFR antibody Ab1 and sheep anti-EGFR antibody to immunoprecipitate EGFR from Cos7 cells. For control experiments, primary antibodies were substituted with normal mouse or sheep IgG (Sigma), and no EGFR was precipitated by normal IgG.

2.3.7 Cell survival assay

Hela H2B-GFP Cells were plated onto 96-well plates, 10,000 cells/well. Forty-eight hours later, the culture medium was replaced by fresh medium containing 10% FBS, nocodazole, EGF (50 ng/ml), or EGF/nocodazole as indicated for 36 h. The percentages of viable cells were then determined by the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2yl)-2,5-

diphenyltetrazolium bromide) to an insoluble formazan, relative to drug-free controls, using the Vybrant MTT Cell Proliferation Assay Kit. After incubation for 4 h, the formazan crystals were dissolved in dimethyl sulfoxide and the absorbance intensity was measured by a microplate reader (Bio-RAD 680, USA) at 540 nm. All cell survival data shown are the means of at least three independent experiments.

2.4 Results

2.4.1 Collection of mitotic cells

To study EGF-induced EGFR signaling during mitosis, we used nocodazole to arrest the Hela H2B-GFP cell at prometaphase and collected these mitotic cell by shaking as we previously described [4]. Cells without nocodazole treatment were used as interphase cells. By checking under the microscope, the non-treated cells only contained less than 5% mitotic cells. After shaking to remove most of these mitotic cells, the rest of the cells were used as interphase cells. To determine whether we had well separated mitotic cells from interphase cells, we examined the cell lysates by using cyclin B1 and cyclin E as markers for mitosis and interphase, respectively. It is well established that cyclin E is only expressed in interphase and cyclin B is only expressed in M phase [303]. We showed that our lysates of M phase cells express high levels of cyclin B1, but not cyclin E, and our lysates of interphase cells express high levels of cyclin E, but not cyclin B1 (Figure 2.1).

2.4.2 EGF-induced EGFR activation during mitosis

We previously showed that EGFR is activated by EGF in M phase [4]. Given the dogma that EGFR and downstream signaling is tightly suppressed in mitosis [2,3,282], we examined whether EGFR is fully activated by EGF addition during mitosis. We examined the phosphorylation of five

tyrosine residues within EGFR C-terminus including Y992, Y1045, Y1068, Y1086, and Y1173 that are known to be strongly phosphorylated by EGF during interphase by using specific antibodies in Hela H2B-GFP cells (Figure 2.2A-F). We showed that all these five major tyrosine residues were strongly phosphorylated by addition of EGF in M phase. The phosphorylation levels at the peak were similar in M phase as in interphase cells (Figure 2.2A-F). However, in M phase, EGF-induced EGFR phosphorylation reached its peak more slowly (at 15 min) than in interphase (at 5 min). Moreover, while the phosphorylation of EGFR in interphase is quickly diminished after reaching its peak, the EGFR phosphorylation level remained high for much longer time in M phase. For Y1068 and Y1086, the phosphorylation reduced sharply 30 min after addition of EGF in interphase, but remained high 1 h after EGF addition in M phase. For Y992, Y1148 and Y1173, the phosphorylation reduced sharply 1 h after addition of EGF in interphase, but remained high 1 h after EGF addition in M phase (Figure 2.2A-F).

To confirm these results and to examine EGFR activation during mitosis in the absence of nocodazole, EGFR phosphorylation at all five pY sites was examined by indirect immunofluorescence. As show in Figure 2.2G, following EGF stimulation for 5 min and 30 min, pY992, pY1068, pY1086 and pY1173 were all as strongly phosphorylated in mitotic cells as in interphase cells. The localization of the phosphorylated EGFR were also in consistence with its pattern of endocytosis as previously described [4]. The antibody to pY1045 did not work for indirect immunofluorescence.

We also examined EGF-induced EGFR activation in other cells including Cos7 and CHO-EGFR cells. CHO-EGFR cells are a selected cell line that stably expresses YFP-tagged EGFR [301]. As shown in Figure 2.3A&B, during mitosis, EGF stimulated EGFR activation to a level very similar to interphase in both Cos7 and CHO-EGFR cells. When we examined the individual

pY sites, we also found that all of the major pY sites on EGFR C-terminus including pY992, pY1045, pY1068, pY1086 and pY1173 were phosphorylated during mitosis in response to EGF stimulation (Figure 2.3C&D)

Finally, we examined the dose-response of EGFR to EGF treatments. We treated Hela H2B-GFP, Cos7 and CHO-EGFR cells with EGF of various concentrations as indicated and examined the overall phosphorylation of EGFR during interphase and mitosis. As shown in Figure 2.3E, although the higher dosage of EGF (10 and 50 ng/ml) stimulated stronger EGFR phosphorylation, the low dose of EGF (5 and 1 ng/ml) also stimulated strong EGFR phosphorylation for the cells in either interphase or mitosis in all these three cell lines. These results indicate that during mitosis, EGFR is activated by addition of EGF to a similar level as in interphase.

2.4.3 The effects of activated EGFR on PI3K/AKT pathway in M phase

We next examined whether the activated EGFR stimulates the major signaling pathways during mitosis. We first examined the PI3K/AKT pathway that is known to be activated by EGF during interphase. Although no specific pY site at EGFR C-terminus has been shown to bind to the p85α subunit of PI3K, PI3K has been shown to co-immunoprecipitated with activated EGFR and was phosphorylated by EGF addition [158]. Here we showed that EGF-stimulated the phosphorylation of p85α subunit of PI3K during mitosis (Figure 2.4A&B). However, the pattern of p85α phosphorylation during mitosis was different from interphase. EGF-induced p85α phosphorylation in mitosis was delayed and the intensity was lower when compared with interphase (Figure 2.4A&B). We next examined EGF-induced phosphorylation of AKT during mitosis. We showed that two AKT isoforms including AKT1 (60 kDa) and AKT2 (56 kDa) were detected in both interphase and mitosis (Figure 2.4C). While EGF stimulated the phosphorylation of both AKT1 and AKT2 during interphase, EGF only stimulated the phosphorylation of AKT2 in

mitosis (Figure 2.4C-E). We further showed that treatment of the Hela H2B-GFP cells with AG1478 to inhibit EGFR activation blocked EGF-induced AKT phosphorylation in both interphase and mitosis (Figure 2.4F). Similarly, inhibition of PI3K activity by treatment of Hela H2B-GFP cells with wortmannin also blocked EGF-induced activation of AKT in both interphase and mitosis (Figure 2.4G).

The functional differences among AKT isoforms are not well defined, however, activation of AKT in general is critical to activate the cellular anti-apoptotic pathways to support cell survival. To examine whether the activation of AKT2 helps cell survival during mitosis, we examined whether addition of EGF during mitosis protects cells from nocodazole-induced apoptosis. It has been shown that treatment of the cells with nocodazole arrests cell at mitosis and the prolonged arrest at mitosis leads to cell apoptosis. Indeed, we showed by using a MTT assay that treatment with nocodazole for 24 h strongly reduced the number of living cells, possibly through apoptosis (Figure 2.4H). However, addition of EGF increased the number of surviving cells (Figure 2.4H), which suggests that EGF protects cell from apoptosis during mitosis.

2.4.4 The effects of activated EGFR on PLC-γ1, Cbl and c-Src in M phase

We also examined whether EGF was able to stimulate the activation of other important signaling proteins during mitosis. The signaling proteins examined include PLC-γ1, CBL and c-SRC. As shown in Fig. 8A&B, PLC-γ1 was expressed in mitosis at a level similar to interphase in Hela H2B-GFP cells. Moreover, EGF stimulated strong PLC-γ1 phosphorylation during mitosis. When compared with interphase, PLC-γ1 phosphorylation in mitosis is at similar intensity but lasts much longer. For the cells in interphase, PLC-γ1 phosphorylation peaked at 5 min of EGF addition and dropped to a very low level at 30 min; however, during mitosis, PLC-γ1 phosphorylation maintained at high level even 1 hour after EGF addition (Figure 2.5A&B). We further showed that

inhibition of EGFR activation by treating Hela H2B-GFP cells with AG1478 blocked EGF-induced PLC-γ1 phosphorylation in both interphase and mitosis (Figure 2.5C), which indicates that the observed PLC-γ1 phosphorylation was due to the activation of EGFR. When we examined EGF-induced PLC-γ1 phosphorylation in M phase in Cos7 cells, we got very similar results (Suppl Figure 2.1A-C). We showed that EGF stimulated PLC-γ1 phosphorylation in mitosis (Suppl Figure 2.1A). The activation of PLC-γ1 occurred at various EGF dosages ranging from 5 ng/ml to 50 ng/ml and EGF concentration of 1ng/ml stimulated very little PLC-γ1 phosphorylation (Suppl Figure 2.1B). Moreover, inhibition of EGFR kinase activity by AG1478 blocked EGF-induced PLC-γ1 phosphorylation in both interphase and mitosis (Suppl Figure 2.1C). We also examined whether EGF stimulates PLC-γ1 phosphorylation in mitotic CHO-EGFR cells. We showed that at the concentration ranging from 1 ng/ml to 50 ng/ml EGF stimulated PLC-γ1 phosphorylation (Suppl Figure 2.1D).

We next examined whether the activated EGFR is able to phosphorylate c-CBL in mitotic Hela H2B-GFP cells. We showed that c-CBL was well expressed in M phase, although it was slightly lower than interphase (Figure 2.5D). EGF strongly stimulated c-CBL phosphorylation in the cells of both interphase and mitosis. Again, c-CBL phosphorylation in mitotic cells lasted longer than the cells in the interphase (Figure 2.5D&E). Similar results were obtained in Cos7 cells (Suppl Figure 2.1E). In Cos7 cells, EGF stimulated strong c-CBL phosphorylation in the cells of both interphase and M phase (Suppl Figure 2.1E) and the c-CBL phosphorylation was inhibited when cells were treated with AG1478 to block EGFR activation (Suppl Figure 2.1F).

We also examined c-SRC activation in Hela H2B-GFP cells during mitosis. We showed that c-SRC was well expressed in M phase (Figure 2.5F). EGF strongly stimulated c-SRC phosphorylation at its Y418 site in the cells of both interphase and mitosis. c-SRC phosphorylation

in mitosis was weaker, but lasted longer than the cells in the interphase (Figure 2.5F&G). It is interesting to notice that the c-SRC band is upshifted in mitotic cells, which suggests additional phosphorylation on the other sites.

2.4.5 The effects of activated EGFR on ERK activation in M phase

We next examined the effects of activated EGFR on ERK pathway in M phase. It has been reported that the ERK1/2 is not activated by EGFR activation during mitosis [2,3,282]. We showed here that indeed ERK1/2 was little phosphorylated by addition of EGF in M phase, although ERK1/2 was expressed in a level similar to interphase (Figure 2.6A-C). As a positive control, we showed that ERK1/2 were strongly activated by EGF in interphase (Figure 2.6A-C). We further showed that in interphase ERK1/2 were strongly phosphorylated even at the EGF dosage of 1 ng/ml (Figure 2.6D), which is consistent with our previous results [158]. However, ERK1/2 were little activated at the EGF dosage from 1ng/ml to 50 ng/ml during mitosis (Figure 2.6D). We then verified our results in other cell lines including Cos7 and CHO-EGFR cells. As shown in Fig. 3E, ERK1/2 were expressed at the same level during mitosis as in the interphase in both Cos7 and CHO-EGFR cells. While ERK1/2 were strongly phosphorylated by EGF during interphase, ERK1/2 were barely phosphorylated in response to EGF during mitosis in both Cos7 and CHO-EGFR cells (Figure 2.6E).

We also examined the phosphorylation of two ERK substrates, RSK and ELK1. We showed that RSK was also little activated in response to EGF in M phase as compared to interphase, although RSK expression levels were similar in mitotic cells as compared with interphase cells (Figure 2.6F). Similar results were obtained for ELK1. As shown in Figure 2.6G&H, ELK was well expressed in M phase as in interphase in Hela H2B-GFP cells. EGF stimulated strong phosphorylation of ELK1 during mitosis (Figure 2.6G&H). Although EGF

stimulated some phosphorylation of ELK1 during mitosis, the level of ELK1 phosphorylation was much lower in M phase than in interphase (Figure 2.6G&H). Together our results indicate that during M phase EGF was unable to stimulate activation of ERK1/2 and the ERK substrates RSK and ELK1.

2.4.6 The mechanisms underlying the inactivation of ERK by EGF during mitosis

Previous work suggested that the decoupling of EGFR activation from ERK activation was due to the mitotic phosphorylation of multiple ERK activators including SOS-1 and RAF-1 by CDC2. Inhibition of CDC2 activity by the CDK inhibitor roscovitine in mitotic cells restored ERK activation by EGF [40]. To test this, we treated Hela H2B-GFP cells with roscovitine to inhibit CDC2 activity and examined the effects on ERK activation. Interestingly, treatment with roscovitine did not restore the activation of ERK1/2 during M phase (Figure 2.7A). Moreover, roscovitine treatment did not change the phosphorylation of RAF-1 in Hela H2B-GFP cells during mitosis (Figure 2.7B). These results suggest that CdCD2 activity and its phosphorylation of RAF-1 are not responsible for the lack of activation of ERK in mitosis.

To understand why activated EGFR does not activate ERK during mitosis, we examined the activation of signaling proteins downstream of EGFR but upstream of ERK. First we examined the expression level of GRB2 and SHC in Hela H2B-GFP, Cos7 and CHO-EGFR cells during mitosis. We showed that in all three cell lines the expression levels of GRB2 and SHC were very similar between interphase and mitosis (Figure 2.7C). We next examined the interaction between EGFR and SHC in response to EGF stimulation in both Cos7 cells. The interaction between EGFR and SHC were determined by the co-immunoprecipitation (IP). As shown in Figure 2.7D, following EGF stimulation, SHC was co-IPed with EGFR in response to EGF in both mitotic cells and the cells in interphase.

We next showed that the SOS was also well expressed in mitosis at a level similar to that in interphase (Figure 2.8A). To examine RAS activation by EGF during mitosis, we first examined the expression and localization of RAS by indirect immunofluorescence. As shown in Figure 2.8B, RAS was properly localized to the plasma membrane at similar intensity with or without EGF stimulation in both interphase and M phase. We also showed by immunoblotting that in M phase RAS expressed at a level similar to that in interphase (Figure 2.8C). The RAS activity was determined by its interaction with RBD. With GST-tagged RBD pull-down experiment followed by immunoblotting, we showed that RAS was well activated in mitotic Hela H2B-GFP cells in response to EGF stimulation (Figure 2.8D).

We also checked MEK, the direct activator of ERK. We showed that while MEK was well expressed in mitosis at a level similar to that in interphase, MEK was not phosphorylated in response to EGF in mitotic Hela H2B-GFP cells (Figure 2.8E&F), which was consistent with the early results regarding EGF-induced ERK activation during mitosis (Figure 2.6). We further showed that in interphase ERK1/2 were strongly phosphorylated even at the EGF dosage of 1 ng/ml (Figure 2.8G), which is consistent with our previous results [158]. However, ERK1/2 were little activated at the EGF dosage from 1 to 50 ng/ml (Figure 2.8G). Moreover, we showed that inhibition of EGFR activation by AG1478 blocked EGF-induced MEK activation during interphase (Figure 2.8H), which suggests that the MEK activation observed during interphase is indeed due to the activation of EGFR. However, there was very little MEK activation observed for mitotic cells (Figure 2.8H). The effects of EGF on MEK activation during mitosis were also examined in Cos7 cells (Suppl Figure 2A-C). We showed that in Cos7 cells, EGF stimulated strong MEK activation during interphase, but did not activate MEK during mitosis (Suppl Figure 2A). Furthermore, the activation of MEK during interphase occurred through a wide range of EGF

concentration from 1 ng/ml to 50 ng/ml, and the activation of MEK during interphase was inhibited by AG1478 (Suppl Figure 2B&C). However, under all these conditions we did not observe MEK activation during mitosis (Suppl Figure 2B&C).

Finally, we examined the activation of RAF-1, which sits between RAS and MEK in the EGFR-ERK signaling cascade. It has been suggested that RAF-1 activation requires several steps: recruitment to the plasma membrane by activated RAS, de-phosphorylation of pS259 by PP2A, and phosphorylation of S338 and Y341 by various kinases at the plasma membrane [304]. Both S338 and Y341 must be phosphorylated to synergistically activate RAF-1 [305]. Thus, to assess EGF-induced RAF-1 activation during mitosis we examined the phosphorylation of RAF-1 at Y341, S338 and S259 with site-specific antibodies. We first examined RAF-1 S338 phosphorylation. We showed that RAF-1 was well expressed during mitosis in Hela H2B-GFP cells (Figure 2.9A). Interestingly, RAF-1 S338 was phosphorylated following EGF stimulation during mitosis at a similar level to interphase (Figure 2.9A&B). Moreover, RAF-1 S338 phosphorylation was abolished if we treated cells with AG1478 to inhibit EGFR activation (Figure 2.9C). We also examined EGF-induced RAF-1 S338 phosphorylation in Cos7 and CHO-EGFR cells. We showed that RAF-1 was well expressed in mitosis and EGF stimulated RAF-1 S338 phosphorylation in M phase to a level similar to interphase in both Cos7 and CHO-EGFR cells (Suppl Figure 2D). Moreover, the RAF-1 S338 phosphorylation occurred through a wide range of EGF concentrations from 1 ng/ml to 50 ng/ml, although the phosphorylation level was much lower at the low concentration of EGF (1ng/ml) for mitotic cells in both Cos7 and CHO-EGFR cells (Suppl Figure 2E). We also showed that EGF-induced RAF-1 S338 phosphorylation was inhibited in both interphase and mitosis when Cos7 cells were treated with AG1478 to inhibit EGFR activation (Suppl Figure 2F).

We next examined whether RAF-1 Y341 was phosphorylated by EGF during mitosis. We showed that in Hela H2B-GFP cells, EGF only stimulated RAF-1 Y340/341 phosphorylation of the cells in interphase, however, RAF-1 Y341 was only slightly phosphorylated by EGF during mitosis (Figure 2.9D&E). Finally, we determined the phosphorylation status of RAF-1 S259 during mitosis. Interestingly, RAF-1 S259 was very little phosphorylated during interphase and very strongly phosphorylated during mitosis, independent of EGF treatment (Figure 2.9F&G). Together, our data suggest that RAF-1 was not activated by EGF during mitosis as RAF-1 Y341 was not phosphorylated and S259 was highly phosphorylated. The inactivity of RAF-1 may explain the lack of activation of MEK and ERK1/2.

2.5 Discussion

The role of EGFR signaling in M phase has been rarely studied and poorly understood. The limited studies indicate that EGFR signaling is mostly inhibited during M phase. These studies suggest that inhibition of EGFR activity and its activation of downstream signaling pathways underlie the importance of keeping the cell sheltered from extracellular signals when it undergoes division, and is beneficial for preventing gene expression so to preserve the energy needs that are required for mitotic structural changes [2,3,282]. However, we recently showed that contrary to the dogma, EGF stimulates strong EGFR phosphorylation at Y992 during mitosis [4]. Here we further studied EGF-induced EGFR activation and the activation of various downstream signaling pathways. A model of mitotic EGFR signaling is presented in Figure 2.10.

To obtain large number of mitotic cells, we used nocodazole to arrest the cells at prometaphase. Nocodazole is the chemical most commonly used to arrest cells at prometaphase [4,305]. We showed that treatment with nocodazole for 16 hours arrested more than 80% of the cells at prometaphase. Treatment with nocodazole for 16 hours did not result in detectable cell

death, although longer exposure to nocodazole (>24 h) did increase cell death, probably by apoptosis (Figure 2.4h). By using cyclin B1 and cyclin E as markers for M phase and interphase, respectively [303] (Figure 2.1), and by examining the cells under microscope (data not shown), we showed that our fraction of M phase cells and interphase cells are very specific. Although nocodazole may have some other effects on cell, it is still the choice for studying mitotic cell in almost all of the research [2,3,282,305]. When it was possible, we also used indirect immunofluorescence to confirm our findings in the absence of nocodazole (Figure 2.2G & Figure 2.8B)

Our first major finding is that EGF could fully activated EGFR in M phase. We showed that all the five major tyrosine residues including Y992, Y1035, Y1068, Y1086, and Y1173 were as strongly phosphorylated in response to EGF in M phase as in interphase (Figure 2.2 and Figure 2.3). This finding is confirmed by both immunoblotting in the presence of nocodazole and indirect immunofluorescence in the absence of nocodazole. The peak intensity of the phosphorylation in M phase is similar to that in interphase. An interesting difference is that in M phase, EGFR phosphorylation lasts longer. This longer phosphorylation may be related to its delayed endocytosis and degradation as we reported previously [4]. We further showed that EGF-induced EGFR phosphorylation occurred at a wide range of EGF concentrations from 1 ng/ml to 50 ng/ml. Our observation is consistent among three different cell lines including Hela H2B-GFP, Cos7 and CHO-EGFR cells. Together, our results indicate that in M phase, EGFR is strongly activated by EGF. These results challenged the existing dogma that EGFR activation is strongly inhibited in M phase [2,3,282] and highlight the importance to study EGFR-mediated cell signaling in mitosis.

The second finding of this research is that activated EGFR actively regulates its downstream signaling pathways. We showed that during mitosis, EGFR is able to activate most of the major

signaling pathways, including PI3K/AKT, PLC-γ1, c-CBL, and c-SRC as in interphase. We showed that EGF stimulated the activation of PI3K and AKT-2 in mitosis and this activation is dependent on the activation of EGFR (Figure 2.4). It is interesting to notice that EGF is able to activate both AKT-1 and AKT-2 in interphase. The functional differences among AKT isoforms are not well defined, however, activation of AKT in general is critical to activate the cellular anti-apoptotic pathways to support cell survival. We showed that in mitosis PLC-γ1 phosphorylation has the similar peak intensity (at 5 min) and lasts much longer. The EGF-induced PLC-γ1 phosphorylation in M phase is also dependent on the activation of EGFR. PLC-γ1 has been implicated in various growth factor (GF)-induced cell signaling including cell proliferation, differentiation, receptor endocytosis, cell motility, membrane ruffle formation and branching tubulogenesis [306–312]. Although need to be elucidated, the strong phosphorylation of PLC-γ1 in response to EGF in M phase suggests that EGFR- PLC-γ1 signaling cascade is important in regulating cell functions in mitosis. EGF also stimulated strong c-CBL phosphorylation during mitosis. CBL plays important roles in regulating EGFR ubiquitination and endocytosis [181,182,187]. We also showed that EGF stimulated c-SRC phosphorylation at Y418. SRC plays diverse functions in cell signaling such as cell proliferation, differentiation, apoptosis, migration, and metabolism [151].

The third finding of this research is that the activation of EGFR during mitosis plays important roles in regulating cell functions. We showed that addition of EGF during mitosis protected cells from apoptosis induced by prolonged exposure to nocodazole (Figure 2.4H). It is well established that in interphase, EGF promotes cell survival by activating PI3K/AKT pathway and other pathways [108,290,291]. We also showed that EGF activated PI3K and AKT-2 through the activation of EGFR in this study (Figure 2.4A-G). Thus, it is possible that during mitosis, EGF

protects cells from apoptosis by activating PI3K/AKT pathways, although this needs to be confirmed by further experiments. Further research is also need to determine why activated EGFR is able to activate AKT-2, but not AKT-1, in mitosis and what is the functional significance of this selective activation. Moreover, we have shown previously that EGFR activation is required for EGF-induced EGFR endocytosis during mitosis [4]. It was reported that stimulation of cells with high concentration of EGF results in the activation of CBL and the ubiquitination of EGFR, which stimulate EGFR endocytosis through the caveolar pathway in interphase [48,162]. Interestingly, we showed here that c-CBL was strongly phosphorylated by EGF during mitosis (Figure 2.5). Thus, we hypothesized that the activation of c-CBL functions to regulate EGFR endocytosis in mitosis through non-clathrin mediated endocytosis. Its role is explored in depth in Chapter 3

Another important finding of this research is that activated EGFR is able to selectively activate some downstream signaling pathways without activating others. Beside the above mentioned selective activation of AKT-2, but not AKT-1, we also showed here that activated EGFR did not activate MEK/ERK pathway (Figure 2.6-2.9). We showed that EGF did not stimulate ERK1/2 activation during M phase in all three cell lines examined (Figure 2.6). We also showed that the two major ERK substrates, RSK and ELK1 were not activated by EGF stimulation during mitosis (Figure 2.6), which indicates that the ERK signaling pathways were not responsive to EGF stimulation during mitosis. While our finding that ERK was not activated by EGF during M phase is consistent with early reports, the previous reports suggest that the ERK inactivation is due to the inactivation of EGFR during M phase [2,3,282]. It was further reported that during M phase, high CDC2 activity inhibits EGFR-mediated ERK signaling by interacting with and phosphorylating multiple proteins including EGFR, SOS1, GRB2 and RAF-1 [3]. However, in our research, we find that EGFR is strongly activated by EGF during M phase, but the activated EGFR

was unable to activate downstream ERK signaling pathways. We also showed that inhibition of CDC2 activity with roscovitine did not restore EGF-induced ERK activation in Hela H2B-GFP cells (Figure 2.7).

We showed that inactivation of MEK/ERK pathway may be due to the lack of full activation of RAF-1 during mitosis. The major signaling proteins functioning between EGFR and ERK along the EGFR-ERK signaling cascade are well expressed during M phase (Figure 2.6-2.9). These proteins include SHC, GRB2, SOS1, RAS, RAF-1 and MEK. SHC is able to associate with EGFR following EGF stimulation (Figure 2.7). RAS is also strongly activated during M phase in response to EGF (Figure 2.8D). However, MEK, the direct activator of ERK, is not activated by EGF stimulation during M phase (Figure 2.8E-H and Suppl Figure 2A-C). We then focused on RAF-1, the protein between Ras and MEK in EGFR-ERK signaling cascades.

It has been suggested that RAF-1 activation requires several steps, recruitment to the plasma membrane by activated RAS, de-phosphorylation of pS259 by PP2A, and phosphorylation of S338 and Y341 by various kinases at the plasma membrane [304]. Both S338 and Y341 must be phosphorylated to synergistically activate RAF-1 [304]. Thus, to assess EGF-induced RAF-1 activation during mitosis we examined the phosphorylation of RAF-1 at Y341, S338 and S259 with site-specific antibodies (Figure 2.6 and SI Suppl Figure 2D-F). We showed that RAF-1 S338 is strongly phosphorylated in response to EGF during M phase. However, RAF-1 Y341 was not phosphorylated in response to EGF during M phase. Moreover, during M phase RAF-1 S259 was highly phosphorylated when compared with interphase. The strong phosphorylation of RAF-1 S259 during M phase is not dependent on EGF stimulation. These results indicate that RAF-1 was not activated in response to EGF during M phase. Further research is needed to understand what blocks the activation of RAF-1 by activated EGFR. RAF-1 is regulated by complex mechanisms.

Despite intensive studies, the RAF-1 regulation is not completely understood. In the inactive state, RAF-1 is in a closed conformation in which the N-terminal regulatory region folds over and occludes the catalytic region. This conformation is stabilized by a 14-3-3 dimer binding to pS259 in N-terminal domain, and pS621 in C-terminal domain [304,313]. Following stimulation, inactive RAF-1 may be recruited to the plasma membrane by activated RAS and/or by phosphatidic acid (PA). At the cell membrane RAF-1 pS259 will be dephosphorylated by phosphatase PP2A, leading to the release of 14-3-3 and the conformational change that enables RAS binding and the phosphorylation of S338 and Y341. Y341 can be phosphorylated by JAK family kinases. S338 can be phosphorylated by PAK, PKC, and CK2 [304,314,315]. Based on this activation model, our data suggest that there are two pools of RAF-1 during mitosis. The major pool of RAF-1 remains phosphorylated at S259 with or without EGF stimulation and thus is locked in inactivate form. The other pool of RAF-1 is dephosphorylated at S259 and is able to be activated by phosphorylation of S338 and Y341. However, while S338 is phosphorylated in response to EGF, Y341 phosphorylation was blocked by an unknown mechanism. As a result, both pools of RAF-1 are not phosphorylated by EGF stimulation during M phase. We do not know whether the failure to dephosphorylate pS259 is due to failed membrane translocation or due to the lack of PP2A activity during M phase. We also do not know whether the failure to phosphorylate Y341 is due to the low activity of JAK family kinase activities in response to EGF during M phase. Further research is needed.

2.6 Conclusions

Mitosis and EGFR are both the targets for cancer therapy. Mitosis is the most dynamic period of the cell cycle, involving a major reorganization of virtually all cell components. A hallmark of cancer involves the cancer cells' ability to sustain chronic proliferation. Therefore, many cancer

drugs are designed to specifically target mitotic cells. On the other hand, EGFR is overexpressed or over-activated in many epithelial tumors and plays important roles in cancer development and progression. EGFR and other EGFR family members such as ErbB2 and ErbB3 have also been targets for cancer therapy. Thus, the understanding of EGFR signaling during mitosis will certainly improve our means to combat cancer.

2.7 Figures

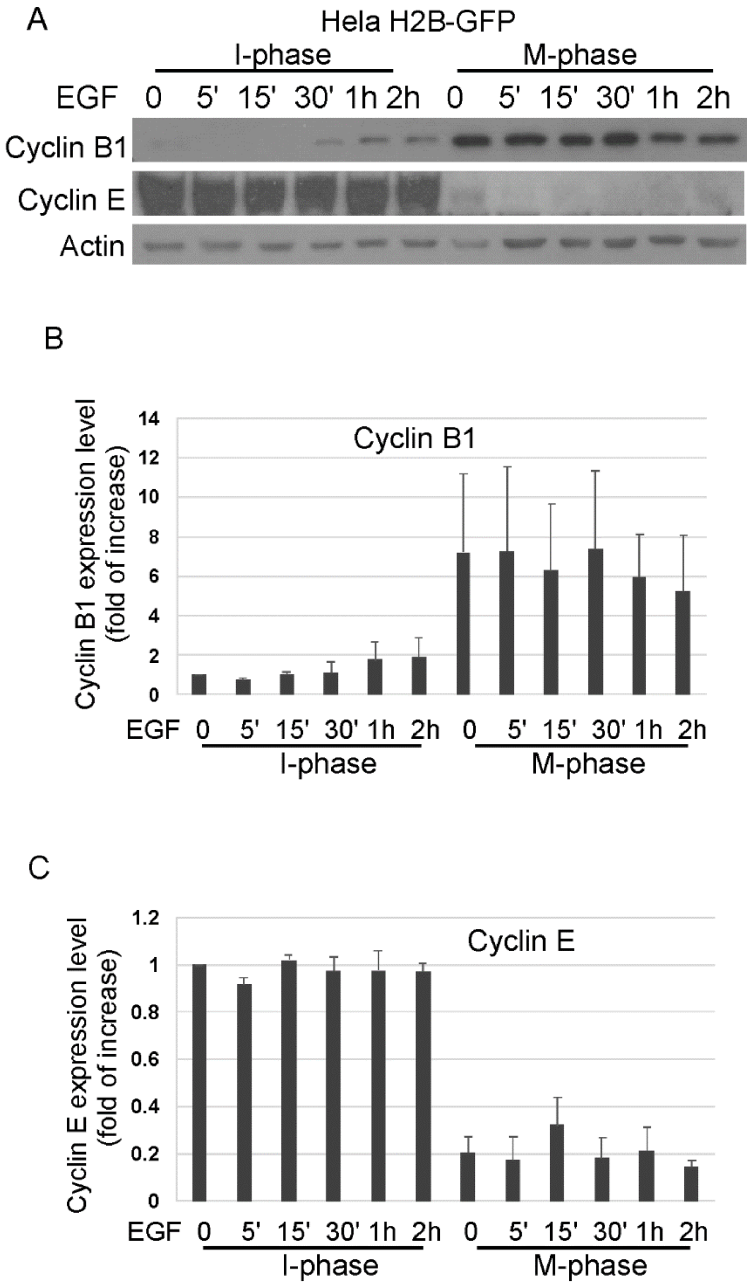


Figure 2.1. Expression of Cyclin B1 and Cyclin E in interphase and mitosis in Hela H2B-GFP cells. Mitotic cells (M-phase) were obtained by treating the cells with nocodazole for 16 hours and cells without nocodazole treatment were used as the interphase (I-phase) cells. Cells both in I-phase and M-phase were stimulated with EGF for the indicated time and the expression of Cyclin B1 and Cyclin E were examined by immunoblotting as described in Experimental procedures. (A) Immunoblotting to show the expression of Cyclin B1 and Cyclin E. (B-C) Quantification of the data from (A) to show the expression of Cyclin B1 (B) and Cyclin E (C). Each value is the average of at least three independent experiments and the error bar is the standard error.

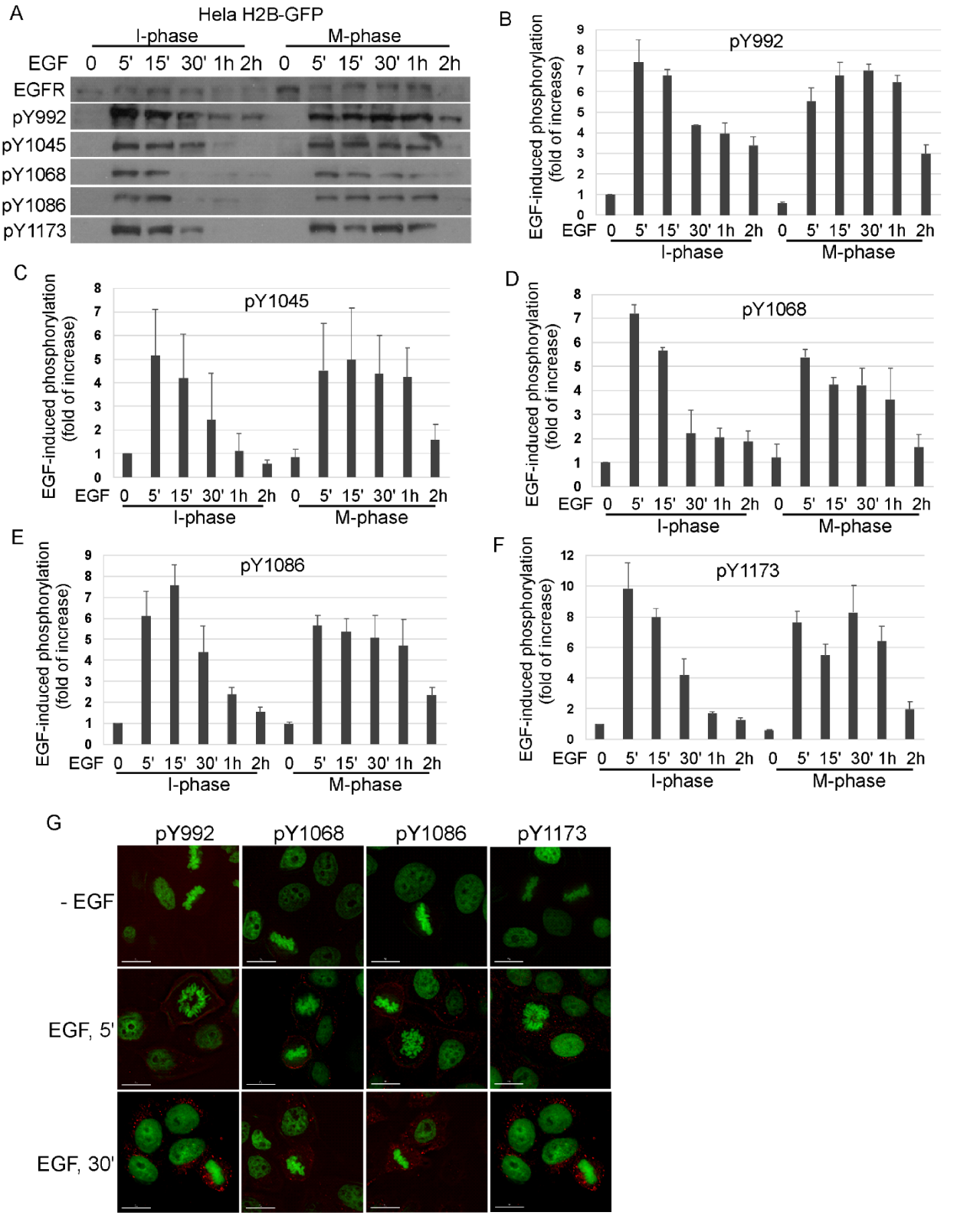

A
Hela H2B-GFP
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
EGFR
pY992
pY1045
pY1068
pY1086
pY1173
B
pY992
EGF-induced phosphorylation (fold of increase)
C
pY1045
D
pY1068
E
pY1086
F
pY1173
G
pY992
pY1068
pY1086
pY1173
- EGF
EGF, 5'
EGF, 30'

Figure 2.2. Characterization of EGFR phosphorylation following EGF stimulation in Hela H2B-GFP cells. Cells in interphase and mitosis were stimulated with EGF for the indicated time and phosphorylation of EGFR at various tyrosine residues were examined by immunoblotting (A-F) and indirect immunofluorescence (G) as described in Materials and Methods. (A) The phosphorylation of EGFR was examined with various antibodies specific for the 5 major tyrosine phosphorylation sites as indicated. (B-F) Quantification of the data from (A). Each value is the average of at least three independent experiments and the error bar is the standard error. **: $p<0.01$. (G) The phosphorylation of EGFR was examined by indirect immunofluorescence. The red indicates the localization of phosphorylated EGFR and the green indicated the cell cycle stage. Controls used IgG instead of primary EGFR antibodies. Size bar: 20 μm.

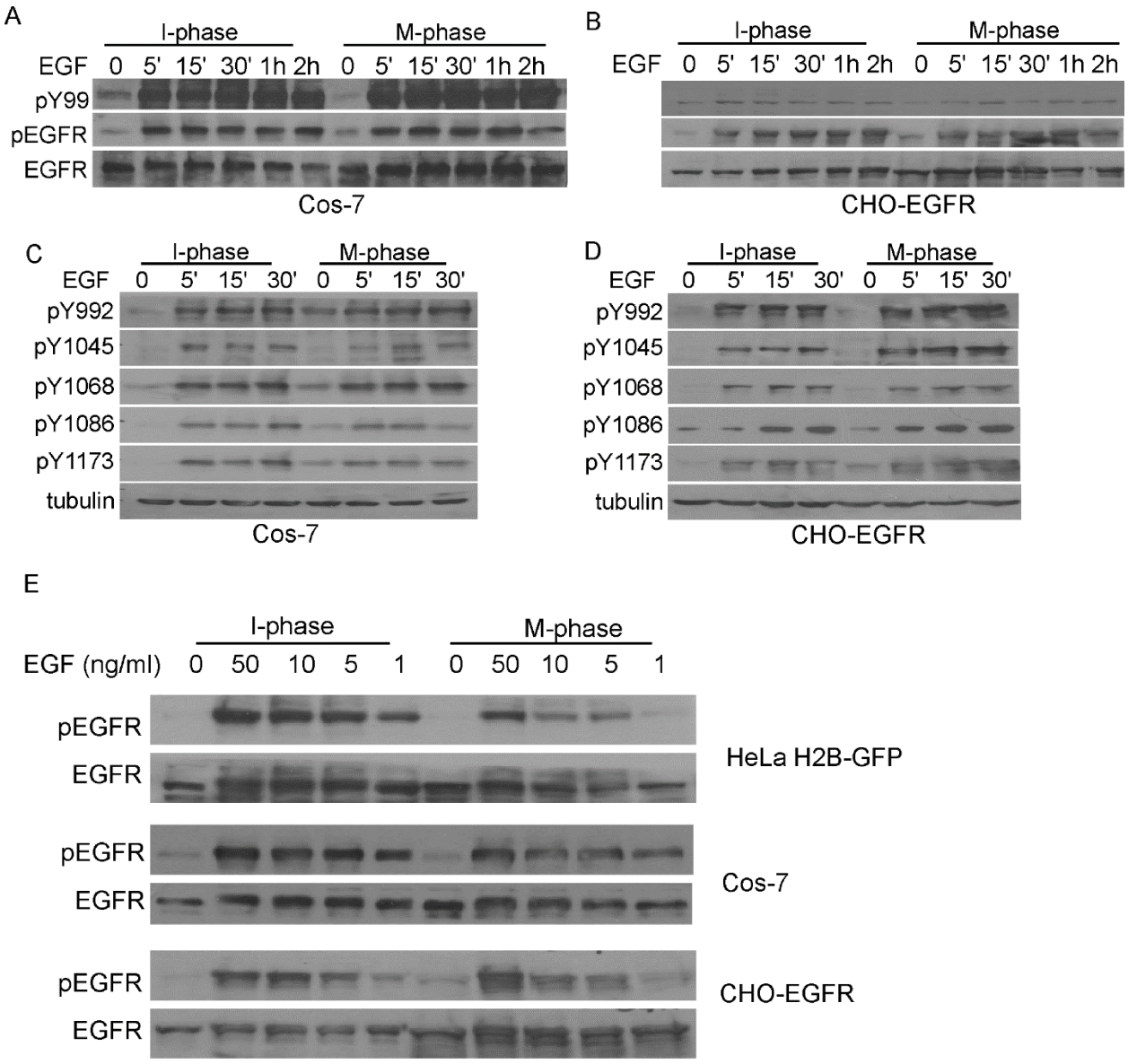


Figure 2.3. Characterization of EGFR phosphorylation following EGF stimulation in Cos7 and CHO-EGFR, and Hela H2B-GFP cells. (A-B) The phosphorylation of EGFR was examined with antibody to phosphotyrosine (PY99) and to phosphore EGFR (pEGFR) in Cos7 cells (A) and CHO-EGFR cells (B). (C-D) The phosphorylation of EGFR was examined with various antibodies specific for the 5 major tyrosine phosphorylation sites as indicated in Cos7 cells (C) and CHO-EGFR cells (D). (E) EGFR activation in response to EGF of various concentrations during interphase and mitosis in Hela H2B-GFP, Cos7 and CHO-EGFR cells. I-phase and M-phase cells were stimulated with EGF of indicated concentrations for 15 min and phosphorylation of EGFR was examined by immunoblotting as described in Materials and Methods.

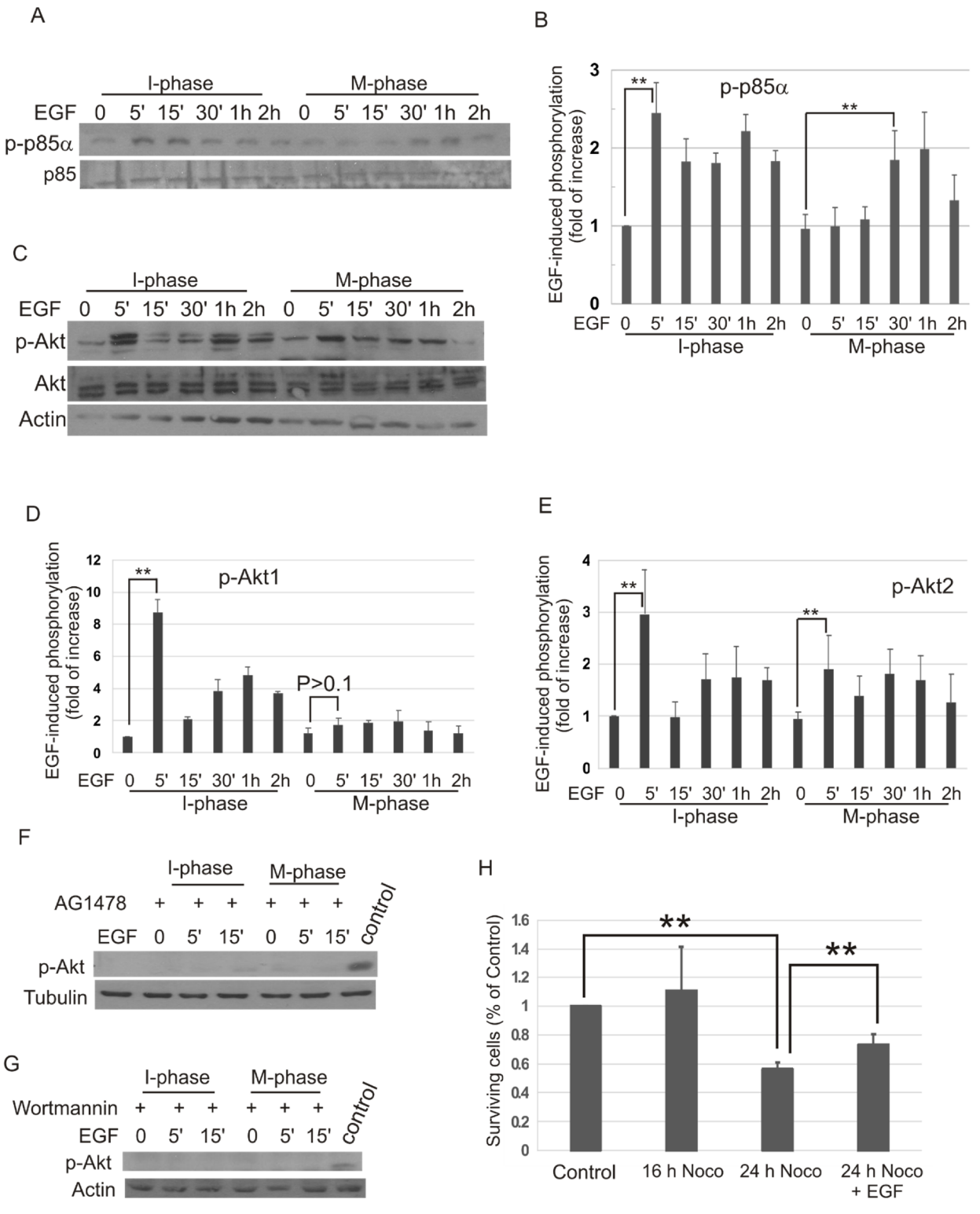

A
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-p85α
p85
B
p-p85α
EGF-induced phosphorylation (fold of increase)
**
**
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
C
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-Akt
Akt
Actin
D
p-Akt1
EGF-induced phosphorylation (fold of increase)
**
P>0.1
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
E
p-Akt2
EGF-induced phosphorylation (fold of increase)
**
**
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
F
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 0 5' 15'
control
p-Akt
Tubulin
G
I-phase
M-phase
Wortmannin + + + + + +
EGF 0 5' 15' 0 5' 15'
control
p-Akt
Actin
H
Surviving cells (% of Control)
**
**
Control
16 h Noco
24 h Noco
24 h Noco + EGF

Figure 2.4. EGF-induced activation of PI3K-AKT pathway and the effects on cell survival during interphase and mitosis in Hela H2B-GFP cells. (A) I-phase and M-phase cells were stimulated with EGF for the indicated time, and the expression and phosphorylation of p85 were examined by immunoblotting as described in Experimental procedures. (B) I-phase and M-phase cells were stimulated with EGF for the indicated time, and the expression and phosphorylation of AKT were examined by immunoblotting. (C-D) Quantification of the data from (B) for pAKT1 (C) and pAKT2 (D). Each value is the average of at least three independent experiments and the error bar is the standard error. (E) Inhibition of EGF-induced AKT phosphorylation by EGFR kinase inhibitor AG1478. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for indicated time. The phosphorylation of AKT was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. (F) Inhibition of EGF-induced AKT phosphorylation by PI3K inhibitor wortmannin. Both I-phase and M-phase cells were treated with wortmannin for 30 min and then stimulated with EGF (50 ng/ml) for the indicated time. The phosphorylation of AKT was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. (G) The effects of EGF on nocodazole-induced cell death during M phase as determined by MTT assay. **: $p<0.01$.

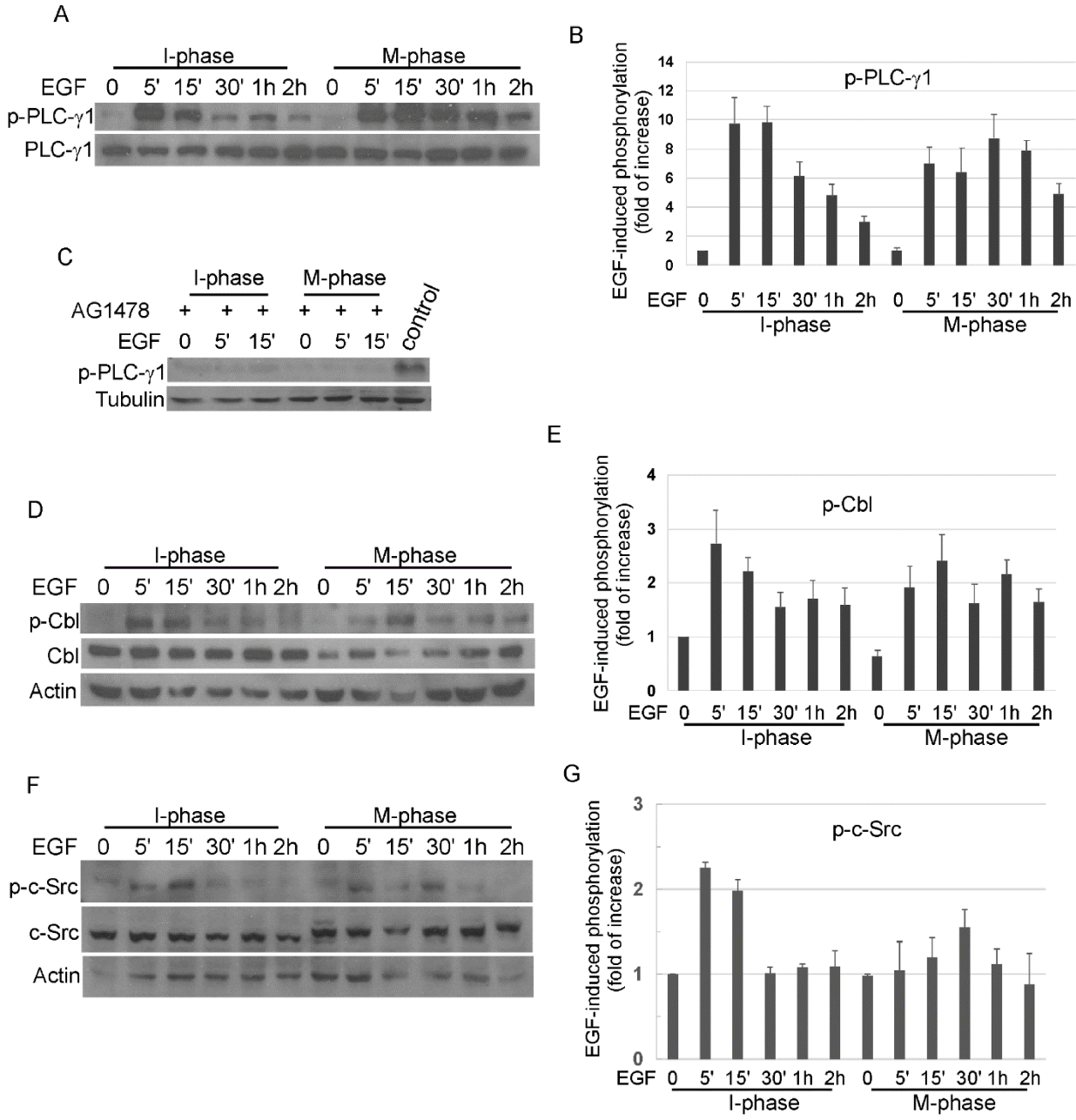

A
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-PLC-γ1
PLC-γ1
B
p-PLC-γ1
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
C
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 0 5' 15'
control
p-PLC-γ1
Tubulin
D
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-Cbl
Cbl
Actin
E
p-Cbl
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
F
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-c-Src
c-Src
Actin
G
p-c-Src
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase

Figure 2.5. EGF-induced activation of PLC-γ1, c-Cbl, and c-Src during interphase and mitosis in Hela H2B-GFP cells. I-phase and M-phase cells were stimulated with EGF for the indicated time and phosphorylation of PLC-γ1, c-Cbl, and c-Src were examined by immunoblotting as described in Experimental procedures. (A) The expression and the phosphorylation of PLC-γ1 were examined by immunoblotting Hela H2B-GFP cells. (B) Quantification of the data from (A) for p PLC-γ1. Each value is the average of at least three independent experiments and the error bar is the standard error. (C) Inhibition of EGF-induced PLC-γ1 phosphorylation by EGFR kinase inhibitor AG1478. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for the indicated time. The phosphorylation of PLC-γ1 was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. (D) The expression and the phosphorylation of c-Cbl were examined by immunoblotting. (E) Quantification of the data from (D) for p-c-Cbl. Each value is the average of at least three independent experiments and the error bar is the standard error. (F) The expression and the phosphorylation of c-Src at Y418 were examined by immunoblotting. (G) Quantification of the data from (F) for p-c-Src. Each value is the average of at least three independent experiments and the error bar is the standard error. **: $p<0.01$.

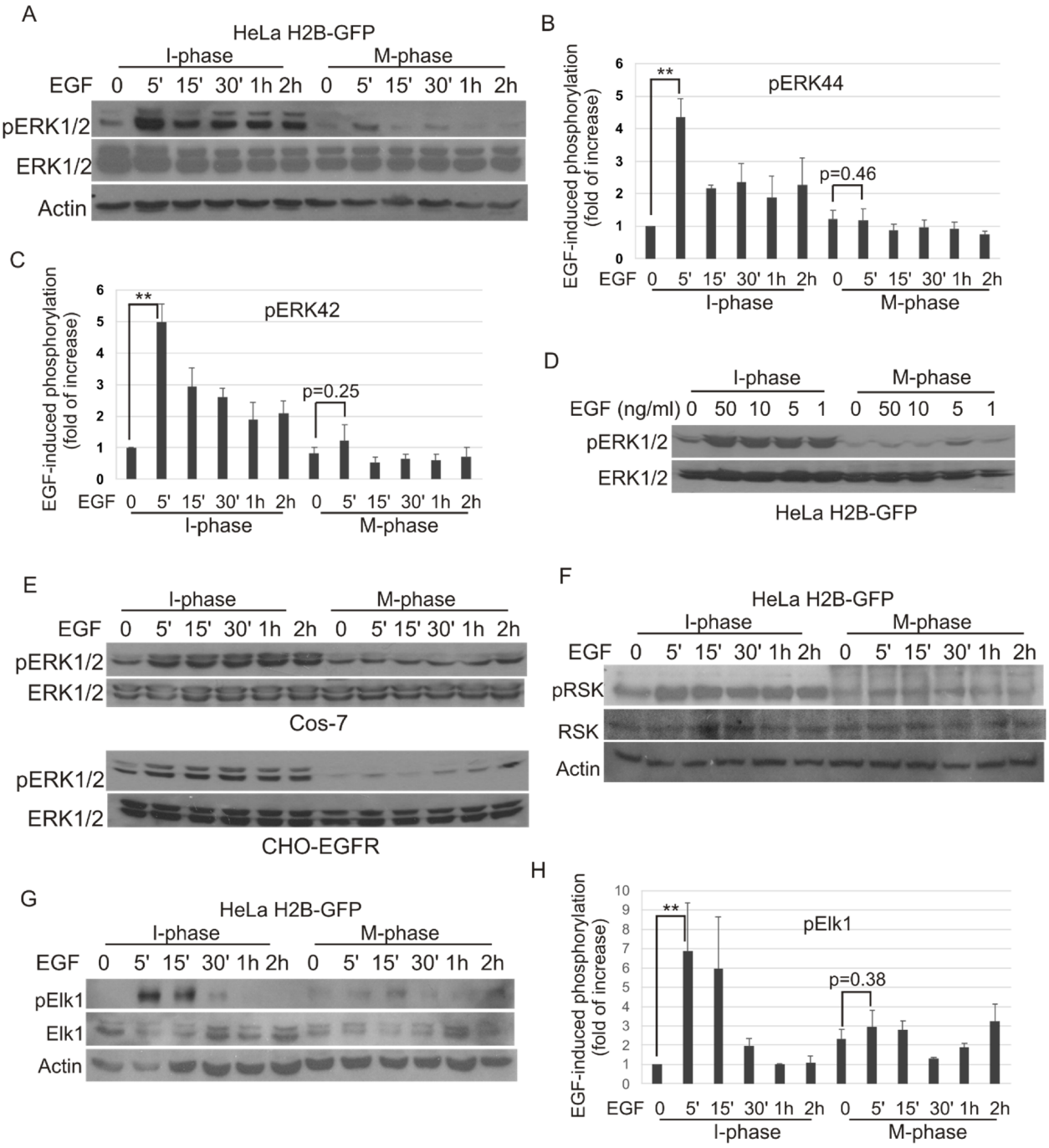

A
HeLa H2B-GFP
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pERK1/2
ERK1/2
Actin
B
pERK44
EGF-induced phosphorylation (fold of increase)
**
p=0.46
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
C
pERK42
EGF-induced phosphorylation (fold of increase)
**
p=0.25
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
D
I-phase
M-phase
EGF (ng/ml) 0 50 10 5 1 0 50 10 5 1
pERK1/2
ERK1/2
HeLa H2B-GFP
E
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pERK1/2
ERK1/2
Cos-7
pERK1/2
ERK1/2
CHO-EGFR
F
HeLa H2B-GFP
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pRSK
RSK
Actin
G
HeLa H2B-GFP
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pElk1
Elk1
Actin
H
pElk1
EGF-induced phosphorylation (fold of increase)
**
p=0.38
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase

Figure 2.6. EGF-induced activation of ERK1/2 and their substrates RSK and Elk-1 during interphase and mitosis in Hela H2B-GFP, Cos7 and CHO-EGFR cells. Interphase (I-phase) and mitotic (M-phase) cells were stimulated with EGF for the indicated time and phosphorylation of ERK1/2 (pERK44 and pERK42) were examined by immunoblotting as described in Experimental procedures. (A) The expression of ERK1/2 and the phosphorylation of ERK1/2 (pERK44 and pERK42) were examined with specific antibodies as indicated in Hela H2B-GFP cells. (B-C) Quantification of the data from (A) for pERK44 (B) and pERK42 (C). Each value is the average of at least three independent experiments and the error bar is the standard error. (D) The phosphorylation of ERK1/2 following the stimulation by EGF of indicated concentrations for 15 min in Hela H2B-GFP cells. (E) The expression of ERK1/2 and the phosphorylation of ERK1/2 (pERK44 and pERK42) were examined with specific antibodies as indicated in Cos7 and CHO-EGFR cells. (F) The expression and the phosphorylation of RSK were examined with specific antibodies as indicated in Hela H2B-GFP cells. (G) The expression and the phosphorylation of Elk-1 were examined with specific antibodies as indicated in Hela H2B-GFP cells. (H) Quantification of the data from (G) for pElk-1. Each value is the average of at least three independent experiments and the error bar is the standard error. **: $p<0.01$.

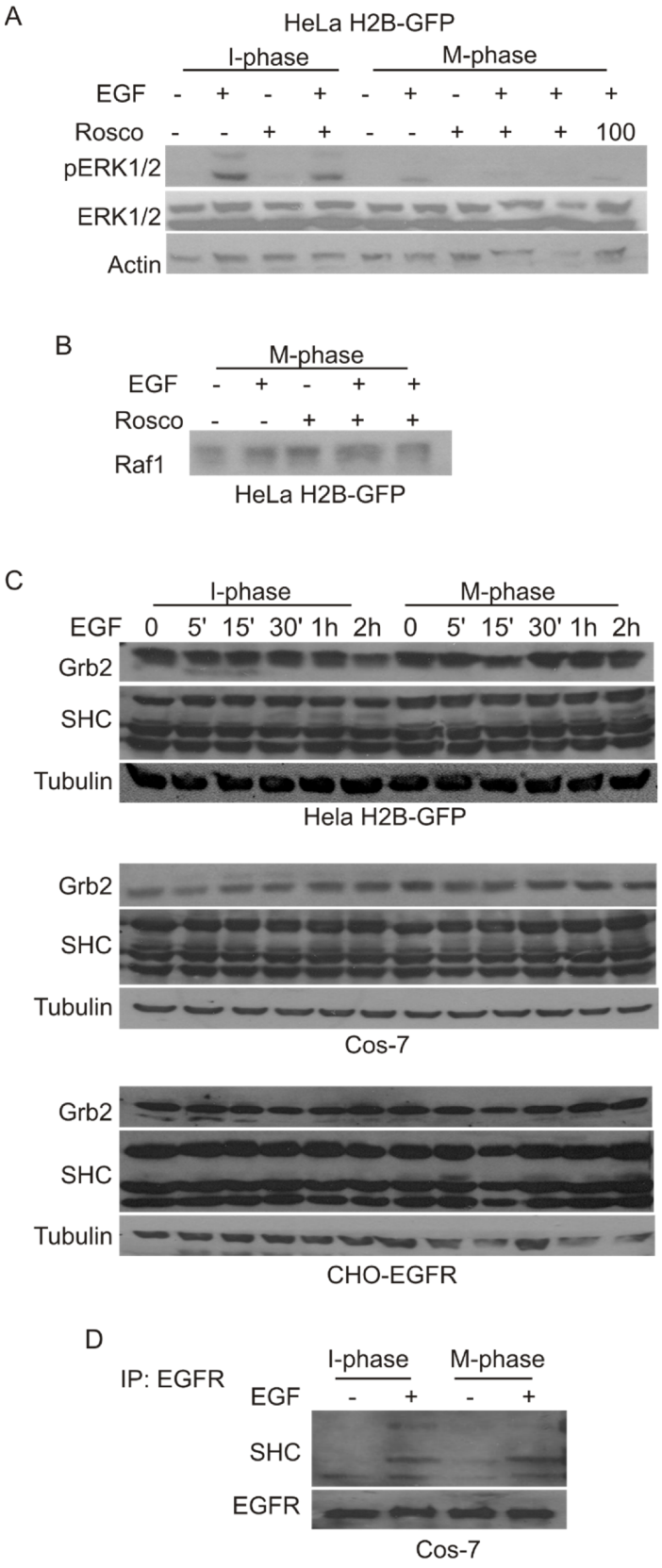

A
HeLa H2B-GFP
I-phase
M-phase
EGF - + - + - + - + + +
Rosco - - + + - - + + + 100
pERK1/2
ERK1/2
Actin
B
M-phase
EGF - + - + +
Rosco - - + + +
Raf1
HeLa H2B-GFP
C
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
Grb2
SHC
Tubulin
Hela H2B-GFP
Grb2
SHC
Tubulin
Cos-7
Grb2
SHC
Tubulin
CHO-EGFR
D
IP: EGFR
I-phase
M-phase
EGF - + - +
SHC
EGFR
Cos-7

Figure 2.7. Activation of ERK signaling pathways during interphase and mitosis in Hela H2B-GFP, Cos7 and CHO-EGFR cells. (A-B) Hela H2B-GFP cells in I-phase and M-phase were treated with EGF with or without rosocovitine (Rosco). The phosphorylation of ERK1/2 and Raf-1 was examined by immunoblotting with indicated antibodies as described in Experimental procedures. (C) The expression of SHC and Grb2 during interphase and M phase in Hela H2B-GFP, Cos7 and CHO-EGFR cells was examined by immunoblotting with indicated antibodies. (D) Co-immunoprecipitation (Co-IP) of EGFR and SHC. For Cos7 cells either in interphase or in mitosis, EGFR was immunoprecipitated with specific antibody and the Co-IPed SHC was examined by immunoblotting.

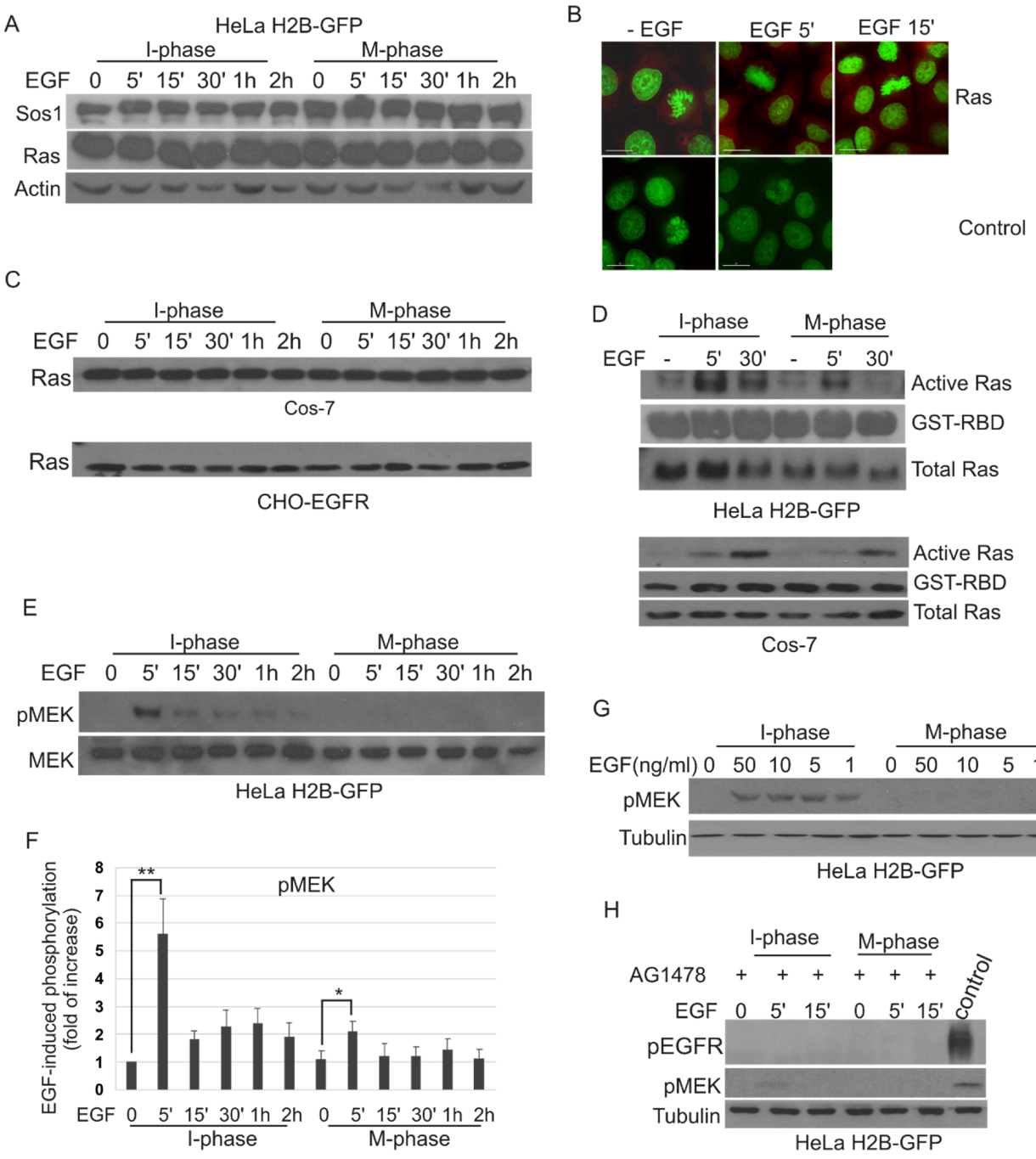

A
HeLa H2B-GFP
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
Sos1
Ras
Actin
B
- EGF
EGF 5'
EGF 15'
Ras
Control
C
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
Ras
Cos-7
Ras
CHO-EGFR
D
I-phase
M-phase
EGF - 5' 30' - 5' 30'
Active Ras
GST-RBD
Total Ras
HeLa H2B-GFP
Active Ras
GST-RBD
Total Ras
Cos-7
E
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pMEK
MEK
HeLa H2B-GFP
F
EGF-induced phosphorylation (fold of increase)
pMEK
**
*
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
G
I-phase
M-phase
EGF(ng/ml) 0 50 10 5 1 0 50 10 5 1
pMEK
Tubulin
HeLa H2B-GFP
H
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 0 5' 15'
control
pEGFR
pMEK
Tubulin
HeLa H2B-GFP

Figure 2.8. EGF-induced activation of Ras and MEK during interphase and M phase in Hela H2B-GFP, Cos7 and CHO-EGFR cells. (A) The expression of Sos1 and Ras in Hela H2B-GFP cells was examined by immunoblotting with indicated antibodies. (B) The subcellular localization of Ras (red) was examined by indirect immunofluorescence in Hela H2B-GFP cells. Controls used IgG instead of primary EGFR antibodies. (C) The expression of Ras in Cos7 and CHO-EGFR cells was examined by immunoblotting with antibody to Ras. The loading control (tubulin) is the same as in Fig. 4C. (D) EGF-induced activation of Ras in Hela H2B-GFP and Cos7 cells. Cell lysates were incubated with GST-RBD conjugated with glutathione beads. The glutathione beads were then subjected to immunoblotting analysis with mouse anti-Ras antibody. GST-RBD and total Ras in the lysates were stained by antibodies to show equal loading. (E) EGF-induced activation of MEK during interphase and M phase in Hela H2B-GFP cells. Cells were stimulated with EGF (50 ng/ml) for the indicated time and phosphorylation of MEK was examined by immunoblotting as described in Experimental procedures. (F) Quantification of the data from (E). Each value is the average of at least three independent experiments and the error bar is the standard error. (G) Activation of MEK in response to EGF of various concentrations in Hela H2B-GFP cells. Both interphase (I-phase) and mitosis (M-phase) cells were stimulated with EGF of indicated concentration for 15 min and the phosphorylation of MEK was examined by immunoblotting. (H) Inhibition of EGF-induced MEK activation by EGFR kinase inhibitor AG1478. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for the indicated time. The phosphorylation of EGFR and MEK was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. **: $p<0.01$ and *: $p<0.05$.

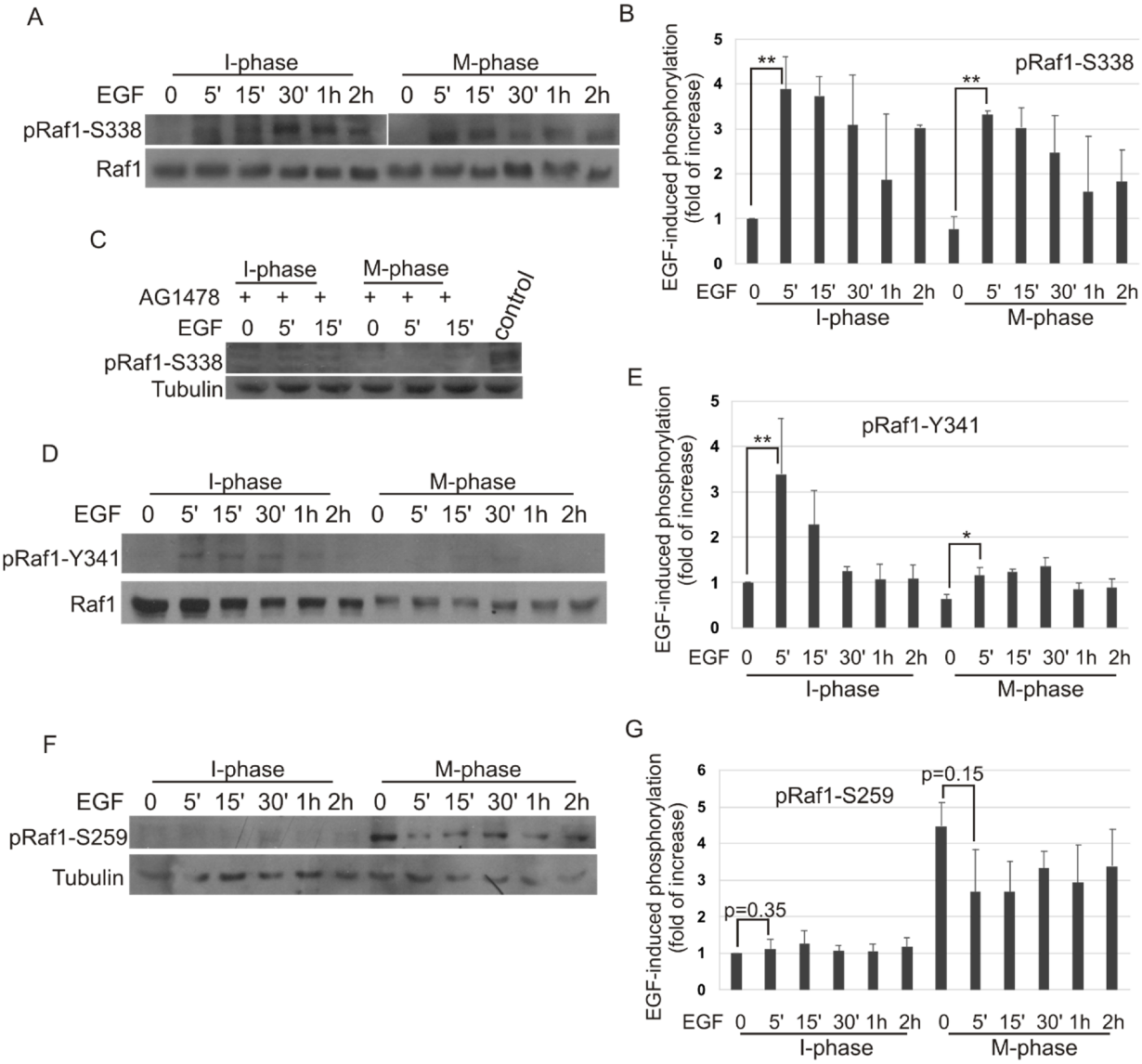

A
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pRaf1-S338
Raf1
B
pRaf1-S338
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
C
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 0 5' 15'
control
pRaf1-S338
Tubulin
D
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pRaf1-Y341
Raf1
E
pRaf1-Y341
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase
F
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pRaf1-S259
Tubulin
G
pRaf1-S259
p=0.15
p=0.35
EGF-induced phosphorylation (fold of increase)
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
I-phase
M-phase

Figure 2.9. EGF-induced activation of Raf-1 during interphase and mitosis in Hela H2B-GFP cells. Cells were stimulated with EGF for the indicated time and phosphorylation of Raf-1 was examined by immunoblotting as described in Experimental procedures. (A) The expression of Raf-1 and the phosphorylation of Raf1-S338 were examined with specific antibodies as indicated. (B) Quantification of the data from (A) for Raf1-S338. Each value is the average of at least three independent experiments and the error bar is the standard error. (C) Inhibition of EGF-induced Raf1-S338 phosphorylation by EGFR kinase inhibitor AG1478. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for indicated time. The phosphorylation of Raf1-S338 was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. (D) The phosphorylation of Raf1-Y341 was examined with by immunoblotting. (E) Quantification of the data from (D) for Raf1-Y341. Each value is the average of at least three independent experiments and the error bar is the standard error. (F) The phosphorylation of Raf1-S259 was examined by immunoblotting. (G) Quantification of the data from (F) for Raf1-S259. Each value is the average of at least three independent experiments and the error bar is the standard error. **: $p<0.01$ and *: $p<0.05$.

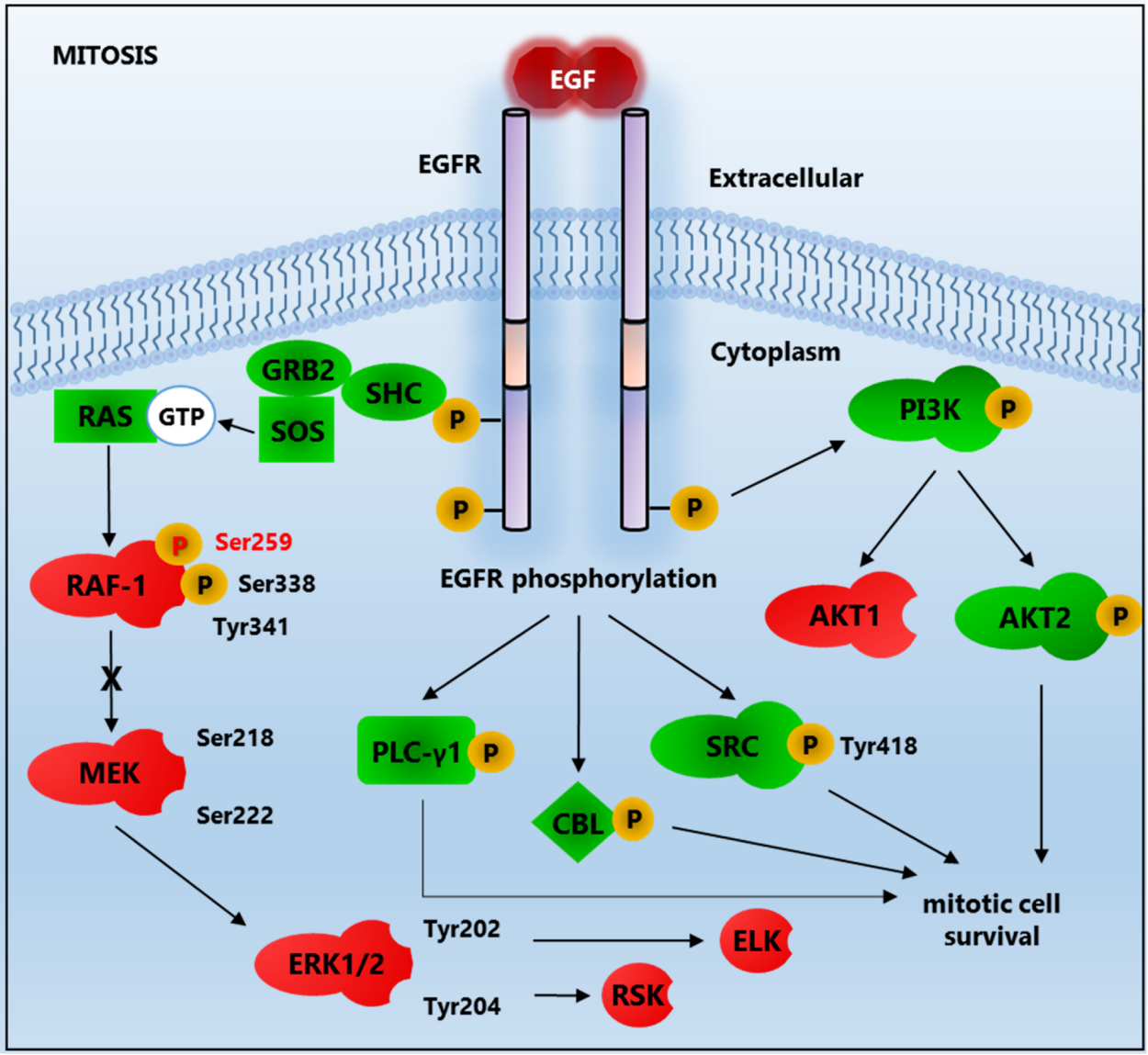
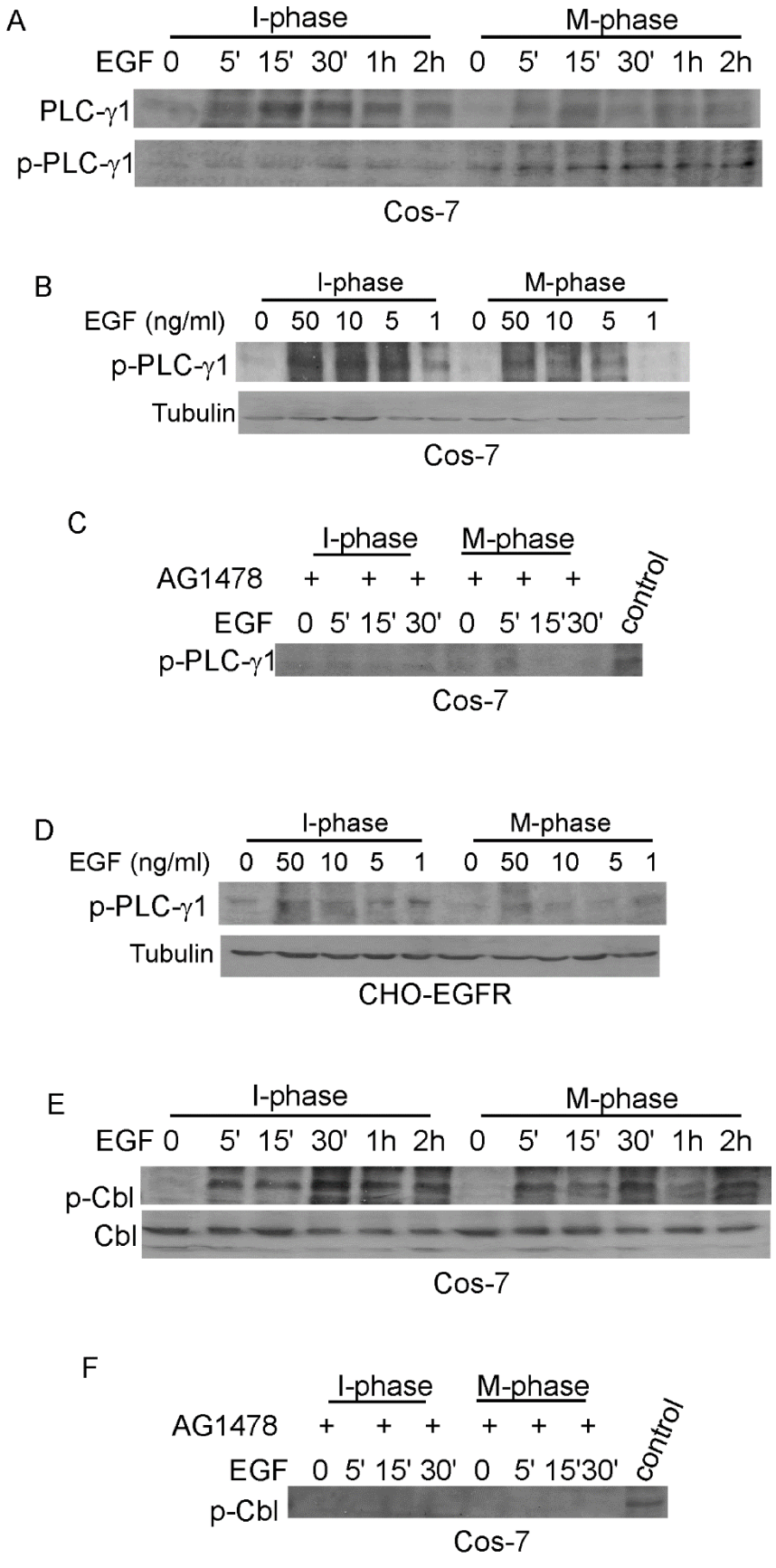
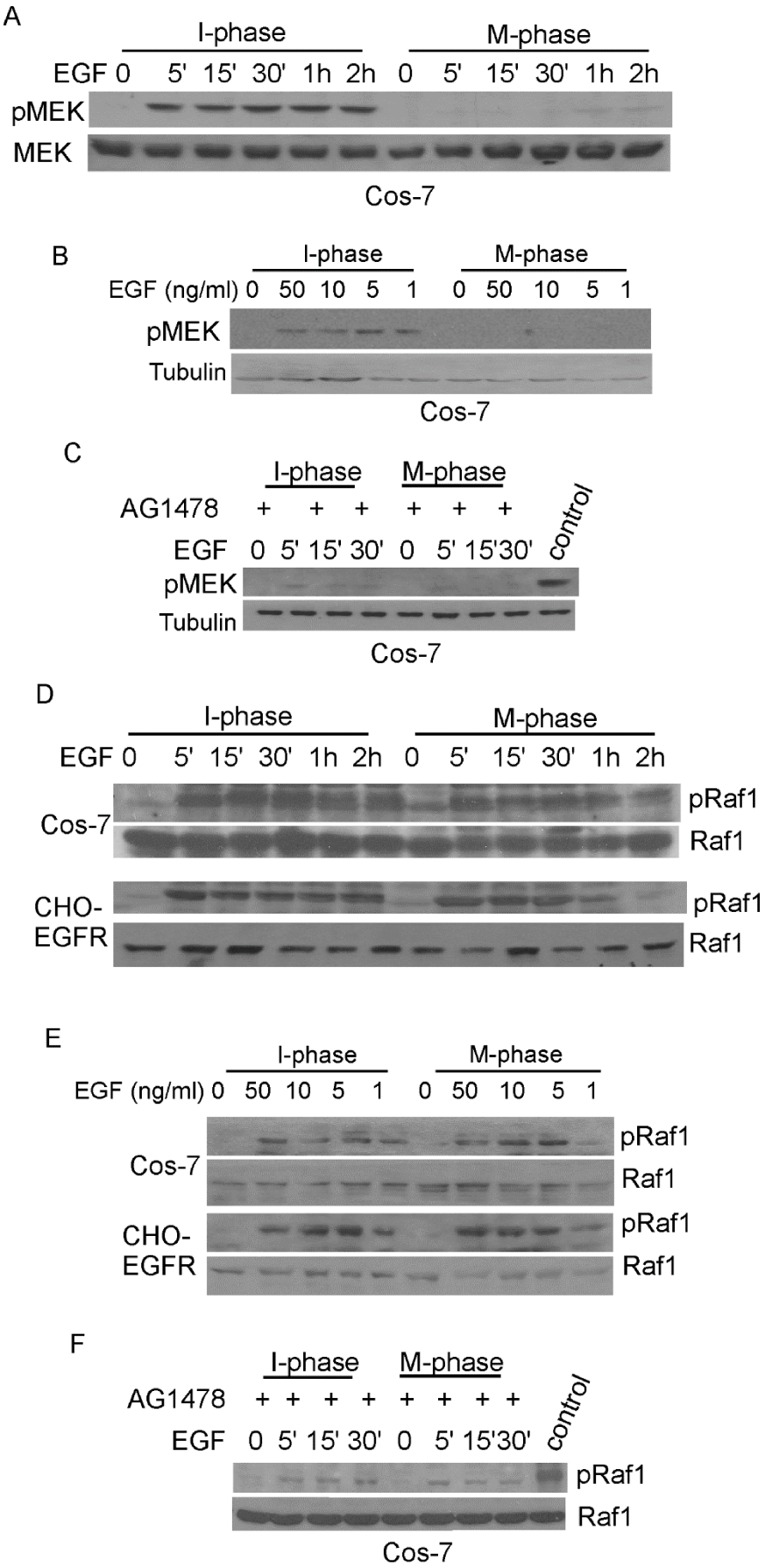


Figure 2.10. Schematic illustration of EGFR signaling pathways during mitosis and interphase. The green color indicates the proteins activated in mitosis and the red indicates the proteins not activated in mitosis, but activated in interphase.


A
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
PLC-γ1
p-PLC-γ1
Cos-7
B
I-phase
M-phase
EGF (ng/ml) 0 50 10 5 1 0 50 10 5 1
p-PLC-γ1
Tubulin
Cos-7
C
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 30' 0 5' 15'30'
control
p-PLC-γ1
Cos-7
D
I-phase
M-phase
EGF (ng/ml) 0 50 10 5 1 0 50 10 5 1
p-PLC-γ1
Tubulin
CHO-EGFR
E
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
p-Cbl
Cbl
Cos-7
F
I-phase
M-phase
AG1478 + + + + + +
EGF 0 5' 15' 30' 0 5' 15'30'
control
p-Cbl
Cos-7

Suppl Figure 2.1. EGF-induced activation of PLC-γ1 and c-Cbl during interphase and M phase in Cos7 and CHO-EGFR cells. I-phase and M-phase cells were stimulated with EGF for the indicated time and phosphorylation of PLC-γ1 and c-Cbl were examined by immunoblotting as described in Materials and Methods. (A) The expression and the phosphorylation of PLC-γ1 were examined by immunoblotting in Cos7 cells. (B) Phosphorylation of PLC-γ1 in response to EGF of various concentrations in Cos7 cells. Both I-phase and M-phase cells were stimulated with EGF of indicated concentration for 15 min and the phosphorylation of PLC-γ1 was examined by immunoblotting. (C) Inhibition of EGF-induced PLC-γ1 phosphorylation by EGFR kinase inhibitor AG1478 in Cos7 cells. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for the indicated time. The phosphorylation of PLC-γ1 was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min. (D) EGF-induced phosphorylation of PLC-γ1 in CHO-EGFR cells. Both I-phase and M-phase cells were stimulated with EGF of indicated concentration for 15 min and the phosphorylation of PLC-γ1 was examined by immunoblotting. (E) The expression and the phosphorylation of c-Cbl were examined by immunoblotting in Cos7 cells. (F) Inhibition of EGF-induced c-Cbl phosphorylation by EGFR kinase inhibitor AG1478 in Cos7 cells. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for indicated time. The phosphorylation of c-Cbl was examined by immunoblotting. Control: M-phase cells treated with EGF (50 ng/ml) for 15 min.


A
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
pMEK
MEK
Cos-7
B
I-phase
M-phase
EGF (ng/ml) 0 50 10 5 1 0 50 10 5 1
pMEK
Tubulin
Cos-7
C
I-phase
M-phase
AG1478 + + + + + +
control
EGF 0 5' 15' 30' 0 5' 15'30'
pMEK
Tubulin
Cos-7
D
I-phase
M-phase
EGF 0 5' 15' 30' 1h 2h 0 5' 15' 30' 1h 2h
Cos-7
pRaf1
Raf1
CHO-EGFR
pRaf1
Raf1
E
I-phase
M-phase
EGF (ng/ml) 0 50 10 5 1 0 50 10 5 1
Cos-7
pRaf1
Raf1
CHO-EGFR
pRaf1
Raf1
F
I-phase
M-phase
AG1478 + + + + + + + +
control
EGF 0 5' 15' 30' 0 5' 15'30'
pRaf1
Raf1
Cos-7

Suppl Figure 2.2. EGF-induced activation of MEK and Raf1 during interphase and M phase in Cos7 and CHO-EGFR cells. (A) EGF-induced activation of MEK in Cos7 cells. Cells in I-phase and M-phase were stimulated with EGF (50 ng/ml) for the indicated time and phosphorylation of MEK was examined by immunoblotting as described in Materials and Methods. (B) Activation of MEK in response to EGF of various concentrations in Cos7 cells. Both I-phase and M-phase cells were stimulated with EGF of indicated concentration for 15 min and the phosphorylation of MEK was examined by immunoblotting. (C) Inhibition of EGF-induced MEK activation by EGFR kinase inhibitor AG1478 in Cos7 cells. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for indicated time. The phosphorylation of MEK was examined by immunoblotting. Control: I-phase cells treated with EGF (50 ng/ml) for 15 min. (D) EGF-induced phosphorylation of Raf1-S338 in Cos7 and CHO-EGFR cells. Cells in I-phase and M-phase were stimulated with EGF (50 ng/ml) for the indicated time and phosphorylation of Raf1-S338 was examined by immunoblotting as described in Materials and Methods. (E) Phosphorylation of Raf1-S338 in response to EGF of various concentrations in Cos7 and CHO-EGFR cells. Both I-phase and M-phase cells were stimulated with EGF of indicated concentration for 15 min and the phosphorylation of Raf1-S338 was examined by immunoblotting. (F) Inhibition of EGF-induced Raf1-S338 phosphorylation by EGFR kinase inhibitor AG1478 in Cos7 cells. Both I-phase and M-phase cells were treated with AG1478 for 30 min and then stimulated with EGF (50 ng/ml) for indicated time. The phosphorylation of Raf1-S338 was examined by immunoblotting. Control: I-phase cells treated with EGF (50 ng/ml) for 15 min.

3 Chapter 3

Regulation of epidermal growth factor receptor endocytosis during mitosis

Note: All experiments were carried out by Ping Wee.

3.1 Chapter Abstract

The overactivation of EGFR is implicated in various cancers. Endocytosis represents a means of EGFR signaling attenuation, by internalizing and deactivating EGFR. We previously found that EGFR endocytosis during mitosis is mediated differently than during interphase. Here, we sought to study the mechanism of the differential mitotic endocytosis that includes slower endocytosis, independence from clathrin, and dependence on EGFR kinase activity. We found that contrary to interphase cells, mitotic EGFR endocytosis is more reliant on the activation of the E3 ligase CBL for endocytosis. At high EGF doses, inhibition of CBL by siRNA or by transfection with dominant negative CBL inhibited EGFR endocytosis of mitotic cells, but not of interphase cells. Moreover, EGFR receptors with mutations in CBL- and GRB2-binding regions also followed a similar pattern. EGF stimulation appeared to induce stronger ubiquitination of mitotic EGFR compared to interphase EGFR. Since EGFR ubiquitination mostly mediates non-clathrin mediated endocytosis (NCE), and CBL mediates this ubiquitination, mitotic EGFR endocytosis appears to occur exclusively through NCE. Low doses of EGF only activates clathrin-mediated endocytosis (CME) during interphase, however during mitosis, we found that NCE was the only active endocytic pathway. Contrary to interphase, CBL and the CBL-binding regions of EGFR were required for mitotic EGFR endocytosis at low doses. This was due to the mitotic ubiquitination of the EGFR even at low EGF doses. As a result of the exclusive activation of NCE during mitosis, mitotic EGFR is only trafficked toward lysosomal degradation. In summary, we report the molecular mechanisms of mitotic EGFR endocytosis. This work may be used to exclusively target pharmacological agents to be retained in cells, instead of being expunged through recycling endocytic pathways.

3.2 Introduction

The overactivation of the epidermal growth factor receptor (EGFR) is a common occurrence in cancers. It imbues cells with increased cell proliferation, and thus higher mitotic indexes [316,317]. Despite an increase in the number of mitotic cells in cancers, little is known about the functional role of the EGFR during mitosis. In addition, mitotic cells are more abundant in cancers treated with the anti-mitotic class of cancer drugs, which arrests cells in mitosis. Therefore, a better understanding of the differences in EGFR signaling and endocytosis between interphase and mitosis may lead to exploitable features for pharmacological targeting.

Mitosis represents a period where the needs and requirements of the cell differ vastly from interphase cells. EGFR signaling has been shown to be regulated differently between interphase and mitotic cells. We and others previously found that the EGFR of mitotic cells can still be activated during mitosis, but that the signal transduction pathways are regulated differently compared to interphase cells [3,279]. We also previously found that EGFR endocytosis of mitotic cells is regulated differently, in that EGFR is endocytosed at a slower rate [4]. At the time, we did not fully decipher the molecular mechanisms behind the differential kinetics. Therefore, in this report, we further studied this phenomenon.

Endocytosis of the EGFR can lead to two distinct fates for the receptor: recycling back to the plasma membrane or lysosomal degradation. As such, the route taken directly influences the total number of receptors available for a subsequent signal transduction response. EGFR recycling has been shown to be mediated by clathrin-mediated endocytosis (CME), whereas non-clathrin mediated endocytosis (NCE) targets receptors for lysosomal degradation [48,163,165].

CME is a mechanism of internalization that is dependent on the recruitment of clathrin to the receptor. Actin polymerization plays a key role in CME [318,319]. During mitosis, CME has been found to be inhibited [320–323]. This may be due to the unavailability of actin for CME, as it is occupied in forming the rigid mitotic cell cortex [171]. Furthermore, multiple components of CME may be occupied in performing mitotic-specific non-endocytic functions, such as clathrin, dynamin, or AP-2 functioning at the spindle apparatus, in centrosomes, or with BubR1 respectively [324–328]. In agreement with this, we previously found mitotic EGFR endocytosis to be clathrin-independent, since siRNA depletion of clathrin heavy chain did not affect mitotic EGFR endocytosis [4]. We therefore hypothesized that mitotic EGFR proceeded exclusively through NCE.

NCE has been described as having potential tumour suppressive characteristics [329]. NCE has also been described as initiating more slowly than CME [48,166,168,205,330,331], which fits with our observed delay in mitotic EGFR endocytosis [4]. Molecularly, EGFR NCE is only activated by physiologically high doses of EGF [48,163,165], which is likely a mechanism evolved to compensate for the easily saturable CME pathway and to prevent excessive EGFR signaling [47]. EGFR NCE has been shown to be mediated by ubiquitination of the receptor, and this ubiquitination has been shown to be limited by the activity of the E3 ligase c-CBL [48,163,165]. Therefore, c-CBL (henceforth CBL) provides a critical negative regulatory control of the EGFR, as it targets the EGFR for endocytosis and degradation. The activation of CBL depends on its binding to the activated EGFR, either by direct interaction with pY1045, or by indirect interaction through the adaptor GRB2, which binds to pY1068 or pY1086 [163,165,190,198].

In this report, we find that EGF-stimulated EGFR endocytosis proceeds exclusively by NCE during mitosis. Since CME is shut down, CBL has a more crucial role in mediating mitotic EGFR endocytosis. Surprisingly, CBL activity appears to be enhanced during mitosis, as even low doses of EGF activate CBL-mediated ubiquitination of the EGFR. As a consequence, during mitosis, activated EGFR may be exclusively trafficked to lysosomes. These findings represent a mechanism by which to target EGFR and endosomes directly to lysosomes, which may be useful for inducing EGFR degradation or the internalization and retention of pharmacological agents into cells [332].

3.3 Materials and Methods

3.3.1 Antibodies and chemicals

Antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.), including: mouse anti-EGFR (sc-373746), anti-pY99 (sc-7020), anti-CBL (sc-170), anti-Ubiquitin (sc-8017), anti-Cyclin B1 (sc-245), and anti-β-Tubulin (sc-5274), rabbit anti-GRB2 (sc-8034) and anti-SHC (sc-967), and goat anti pY1068 (sc-16804) and pY1086 (sc-16804). The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA) and the fluorescence-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Goat anti-mouse immunoglobulin G (IgG) conjugated with agarose were from Sigma (St. Louis, Mo.). EGF was from Upstate Biotechnology.

3.3.2 Plasmid construction

The EGFR-YFP, EGFR-Y1045F-YFP, EGFR-Δ991-YFP, and EGFR-Δ1044-YFP constructs were described previously [187]. The c-CBL-YFP and 70z-CBL-YFP constructs were generous gifts from the Sorkin Lab.

3.3.3 Cell Culture, transfection, and treatment

HeLa, 293T, and MCF-7 cells were growth at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotic/antimycotic solution maintained at 5% CO_2 atmosphere. For transfection, MCF-7 cells in 24-well plates were transfected using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol, and 293T cells in 24-well plates were transfected using calcium phosphate precipitation with BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer [333]. MCF-7 and 293T cells were chosen due to their low levels of endogenous EGFR [334,335]. Small interfering RNA-mediated silencing transfections were done using Cbl siRNA (sc-29241; Santa Cruz Biotechnology, Santa Cruz, Calif) in HeLa cells as per the manufacturer's protocol.

Mitotic cells were collected by gentle mitotic shake-off as previously described [279]. Briefly, cells were arrested in prometaphase by treating cells with nocodazole (200 ng/mL) in serum-free media for 16h. The nocodazole-arrested cells were treated with EGF (2 ng/mL or 50 ng/mL) for 5, 30, and 45 min, or not treated with EGF (0 min). The EGF-containing media was then removed and serum-free media was added. Cells were placed on ice and dislodged by gently tapping the plates for 5 min. The mitotic cell-containing media was centrifuged at 1000 rpm for 5 min. The obtained mitotic cells were then lysed with cold Mammalian Protein Extraction Reagent (M-Per) (Thermo Fisher Scientific Inc, Rockford, IL USA) buffer in the presence of phosphatase and protease inhibitors including 100 mm NaF, 5 mM MgCl2, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM

pepstatin A. To collect lysates for interphase cells, cells were serum-starved for 16h. Cells were then treated with EGFR for 5, 30, and 45 min. To ensure consistency with the mitotic treatment, the cells were also tapped on ice for 5 min to remove mitotic cells and then left on ice for 5 min. The remaining interphase cells were collected by scraping on ice in cold M-Per in the presence of phosphatase and protease inhibitors. For both interphase and mitotic cells, after lysing, the samples were centrifuged at 21,000 ×g and the supernatant was collected for immunoblotting.

3.3.4 Immunoprecipitation and immunoblotting

Immunoprecipitation experiments were carried out as described previously [187]. Interphase or mitotic cells were lysed with immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/mL aprotinin, and 1 μM pepstatin A) for 15 min at 4°C. Cell lysates were then centrifuged at 21,000 ×g. The supernatant, containing 1 mg of total protein, were incubated with 0.8 μg of mouse monoclonal anti-EGFR antibody A-10 (Santa Cruz) for 2 h at 4°C with gentle mixing by inversion. Goat anti-mouse IgG conjugated with agarose was added to each fraction and incubated for 2 h at 4°C with gentle mixing by inversion. Next, the agarose beads were centrifuged, washed three times with immunoprecipitation buffer, and 2× loading buffer was added. The samples were boiled for 5 min at 95°C and loaded for SDS-PAGE for subsequent immunoblotting.

Immunoblotting was performed as previously described [158]. Briefly, protein samples were separated by SDS-PAGE and were transferred to nitrocellulose. The membranes were blocked for non-specific binding, and incubated with primary antibody overnight. The membranes were then probed with HRP-conjugated secondary antibody followed by detection

with enhanced chemiluminescence solution (Pierce Chemical, Rockford, IL) and light detection on Fuji Super RX Film (Tokyo, Japan) on MACHINE.

3.3.5 Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described [4]. Cells were grown on glass coverslips and serum-starved for 16 h. After treatment, the cells were fixed with ice cold methanol for 10 min. The cells were then permeabilized with 0.2% Triton X-100 for 10 min on ice. Next, cells were blocked with 1% BSA for 1 h on ice. Cells were then incubated with primary antibody overnight at 4°C. Primary antibody anti-CBL was used at 1:50, and anti-pEGFR-Y1086 and anti-EGFR were used at 1:200. Cells were then washed three times with PBS, and incubated with rhodamine- or FITC-labeled secondary antibody for 1 h at 4°C. Cells were then washed three times PBS, followed by nuclear staining with DAPI (4′6-diamidino-2-phenylindole) (300 nM). Finally, cells were washed three times and mounted. Images were taken with DeltaVision deconvolution microscopy (GE Healthcare Life Sciences, Buckinghamshire).

Quantification of EGFR internalization was performed using ImageJ as previously described [4]. Briefly, the cells were visualized by differential interface contrast (DIC). For each image, a large polygon (V_L) was drawn along the outer edge of the cell membrane to represent the entire area of the cell. In addition, a small polygon (V_S) was drawn along the inner edge of the cell membrane to represent the cell interior. The V_L and V_S values were calculated for either stains of EGFR, pEGFR, or for YFP (for EGFR-YFP mutants), and membrane EGFR percentage was obtained by the following equation:

$$\text{Membrane EGFR percentage} = \frac{V_L - V_S}{V_L}$$

3.4 Results

3.4.1 CBL interaction with EGFR during mitosis

EGFR expression at the plasma membrane does not change from interphase to mitosis [4,279,280]. Previously, we found that similar to interphase, stimulation of nocodazole-arrested mitotic HeLa cells with high doses of EGF (50 ng/mL) induced the phosphorylation of the EGFR at all major tyrosine residues, including Y992, Y1045, Y1068, Y1086, and Y1173 [279]. Moreover, this also phosphorylated CBL to similar levels.

To confirm mitotic CBL activation by EGF stimulation, we observed CBL localization in mitotic HeLa cells by immunofluorescence microscopy. Immunofluorescence co-staining using anti-EGFR and anti-CBL antibodies revealed that CBL co-localizes to EGFR upon 5 min of 50 ng/mL EGF treatment in both interphase and mitotic cells (Figure 3.1A). Furthermore, immunoprecipitation of EGFR using a monoclonal anti-EGFR antibody of both interphase and mitotic cell lysates showed that mitotic cells stimulated with EGF for 5 mins had higher IPs of CBL with EGFR than interphase cells (Figure 3.1B). Interestingly, CBL IP with EGFR decreased after 30 min EGF in mitotic cells, whereas it increased for interphase cells, and continued increasing at 45 mins EGF. Most surprisingly however, was that ubiquitination of the EGFR was enhanced at all time points studied during mitosis compared to interphase (Figure 3.1B). Since CBL also binds EGFR indirectly through the EGFR adaptor GRB2, we also immunoblotted EGFR immunoprecipitates for GRB2 and SHC. The results showed that during mitosis, GRB2 and SHC also bind to EGFR following EGF stimulation (Figure 3.1B).

In summary, immunofluorescence staining for both EGFR and CBL reveal that both proteins co-localize after EGF stimulation during mitosis. Co-IP experiments also suggest EGF-induced EGFR and CBL interaction. In addition, EGFR is more strongly ubiquitinated by EGF stimulation during mitosis.

3.4.2 Effects of altering CBL activity during mitosis

CME has been shown to be inhibited during mitosis [171,320,321]. Therefore, we sought to discover whether altering CBL activity, the major mediator of NCE, would inhibit EGFR endocytosis. We first silenced CBL in HeLa cells by siRNA transfection and found that transfected mitotic cells had much less EGFR endocytosis following EGF (50 ng/mL) stimulation, as observed by immunofluorescence (IF) staining of activated EGFR (Figure 3.2A). In comparison, transfected interphase cells were little affected. Similarly, MCF-7 cells transfected with EGFR-YFP and the CBL siRNA also inhibited mitotic endocytosis exclusively (Figure 3.2B). To further verify the role of CBL, we used the dominant-negative 70Z-CBL-YFP mutant, which has a deletion of 17 amino acids that disrupts the RING finger structure making it unable to interact properly with ubiquitin-conjugating enzymes (E2 ligases) [330,336]. The 70Z-CBL-YFP protein can still bind to the cytoplasmic tail of activated EGFR [181,189,330,337]. Transfection with 70Z-CBL-YFP significantly inhibited EGF-induced mitotic EGFR endocytosis, but not in interphase cells. (Figure 3.3A). Mitotic cells transfected with 70Z-CBL-YFP retained EGFR at the plasma membrane compared to non-transfected cells, even after 60 minutes of EGF treatment (50 ng/mL). Taken together, downregulating CBL activity decreased mitotic, but not interphase EGFR endocytosis. Therefore, CBL activity appears more important during mitotic EGFR endocytosis than during interphase.

We next sought to see whether CBL overexpression could increase the rate of endocytosis during mitosis. CBL overexpression in HeLa cells did not appear to induce endocytosis at earlier time points, nor increase the rate of EGFR internalization (Figure 3.3B). This is similar to interphase cells, where it was previously reported that overexpression of CBL did not increase the rate of EGFR internalization [181,197].

3.4.3 Role of EGFR C-terminal domains for mitotic endocytosis

We previously showed that mitotic EGFR endocytosis requires EGFR kinase activity [4]. Treatment with the EGFR-tyrosine kinase antagonist AG1418 inhibited mitotic EGFR endocytosis, and washing away AG1478 restored endocytosis [4]. In contrast, interphase EGFR could still undergo endocytosis in the presence of AG1478 [4]. To further explore the role of EGFR kinase activity in the activation of CBL, we blocked EGFR activation with or without AG1478 for 1 h prior to EGF (50 ng/mL) treatment. As before, this treatment prevented EGFR endocytosis during mitosis as visualized by IF [4] (data not shown). We performed Western blots of these samples and blotting with p-CBL antibody showed that similar to interphase cells, AG1478 inhibited EGF-induced CBL tyrosine phosphorylation in mitotic cells. (Figure 3.4A). Therefore, EGFR kinase activity is required for CBL activation.

We next sought to investigate which EGFR domains were important for mitotic endocytosis. We made use of previously constructed YFP-tagged EGFR mutants and truncations: EGFR with Y1045F substitution (Y1045F-YFP, no direct CBL binding), EGFR truncated at 1045 (Δ1044-YFP, no CBL binding), EGFR truncated at 992 (Δ991-YFP, no internalization), and WT (EGFR-YFP) (Figure 3.5F). We transfected these constructs into MCF-7 or HEK 293T cells, since they express low amounts of endogenous EGFR, then observed the effects of EGF

treatment on their plasma membrane localization using indirect immunofluorescence (Figure 3.4B-E & Figure 3.5A-E).

In non-EGF treated MCF-7 cells, all mutants exhibited high plasma membrane localization and low cytoplasmic localization during both interphase and mitosis (Figure 3.5A-E). Treatment of EGF (50 ng/mL) for 30, 45, or 60 mins significantly increased the internalization of interphasic EGFR-YFP, Y1045F-YFP, and Δ1044-YFP, but not Δ991-YFP which never became internalized into the cell. The internalization levels of EGFR-YFP, Y1045F-YFP, and Δ1044-YFP during interphase at all three time points were all similar. In contrast, these mutants responded to EGF treatment differently from each other when cells were in mitosis. Similar to interphasic EGFR-YFP, approximately two-thirds of mitotic EGFR-YFP was internalized following 30 min EGF treatment, with more EGFR-YFP becoming internalized at 45 and 60 min. The internalization of both mitotic Y1045F-YFP and Δ1044-YFP however were impaired when compared to EGFR-YFP. The Y1045F-YFP mutants showed lower levels of mitotic endocytosis compared to EGFR-YFP, but a high proportion of them eventually became endocytosed. However, the Δ1044-YFP mutants had significantly inhibited mitotic endocytosis, with little endocytosis after 60 min of EGF treatment. These results were also observed when the experiments were repeated in 293T cells (Figure 3.4B-E).

Taken together, this data shows that the CBL-binding domains of the EGFR are more important for mitotic EGFR endocytosis than interphase. These results also suggest that GRB2 cooperation for indirect CBL-binding to EGFR dramatically increases mitotic EGFR endocytosis.

3.4.4 Low EGF doses activate mitotic EGFR NCE

The above experiments were all performed using high concentrations of EGF (50 ng/mL). During interphase, low doses of EGF only activates CME, whereas high doses activate both CME and NCE [48,163,165]. By this logic, during mitosis, low doses of EGF should not induce mitotic EGFR endocytosis since CME is mitotically inhibited and only high doses of EGF activates NCE. However, we previously saw that low doses TR-EGF (2 ng/mL) could still lead to their internalization in mitotic HeLa and CHO cells. We therefore decided to address this discrepancy.

We hypothesized that NCE was still active during low dose EGF-induced mitotic endocytosis. To test this, we once again interfered with CBL activity using our EGFR and CBL mutants, and observed endocytosis by IF microscopy. In MCF-7 cells transfected with EGFR-YFP, the low EGF dose (2 ng/mL) induced endocytosis in both interphase and mitotic cells (Figure 3.6). Both the Y1045F-YFP and Δ1044-YFP had impaired mitotic EGFR endocytosis compared to EGFR-YFP. HeLa cells transfected with 70Z-CBL also became impaired for mitotic EGFR endocytosis (Figure 3.7A). As in high dose EGF conditions, low dose EGF and CBL-YFP overexpression in HeLa cells did not appear to induce endocytosis at earlier time points, nor increase the rate of EGFR internalization (Figure 3.7B). Therefore, mitotic EGFR endocytosis stimulated by low dose EGF proceeds similar to high dose EGF.

Since CBL appears to be involved in low EGF dose mitotic EGFR endocytosis, we examined whether this was due to ubiquitination. To this end, we compared the ubiquitination of interphase and mitotic EGFR cells treated with 2 ng/mL or 50 ng/mL EGF for 45 mins (Figure 3.8A). Co-immunoprecipitation of EGFR and immunoblotting for ubiquitin revealed that as before, high dose EGF (50 ng/mL) ubiquitinated mitotic EGFR more than interphase EGFR. Low dose EGF (2 ng/mL) did not induce the ubiquitination of interphase EGFR, as previously

reported [48,163,165]. However, low dose EGF stimulation caused significant EGFR ubiquitination in mitotic cells. Moreover, the binding of CBL to EGFR followed the same pattern as ubiquitination, with CBL again binding to EGFR at low doses during mitosis, but not during interphase.

We also performed similar experiments with different times of EGF stimulation, at 5, 30, and 45 mins (Figure 3.8B). The phosphotyrosine-specific antibody pY99 was used to confirm EGFR phosphorylation. Blotting for ubiquitin revealed that mitotic ubiquitination of EGFR occurs at 5 mins, and is sustained through to 45 mins of low dose EGF stimulation. In contrast, interphase EGFR is less ubiquitinated, especially at later time points. In addition, CBL and SHC are pulled-down with EGFR during both interphase and mitotic. Therefore, it appears that low dose EGF stimulation differentially ubiquitinates mitotic EGFR, and not interphase EGFR.

Since mitotic EGFR is strongly ubiquitinated at low doses of EGF, and ubiquitination has been associated with EGFR degradation, we hypothesized that low EGF doses could lead to EGFR degradation during mitosis. Total cell lysates of interphase and mitotic cells treated with low doses of and total EGFR levels were assayed by Western blot. Whereas interphase EGFR levels remain constant throughout 45 mins of low dose EGF treatment, we found that mitotic EGFR levels drop drastically with time (Figure 3.8C). Taken together, these results suggest that, unlike interphase, low doses of EGF activate CBL-mediated EGFR degradation in mitotic cells. Interestingly, by Western blotting, the CBL band appears smaller in mitotic samples than interphase samples.

3.4.5 Endocytic trafficking of mitotic EGFR

The endocytic pathway that the EGFR takes has been shown to influence the fate of the EGFR. CME has been shown to lead to EGFR, whereas NCE targets receptors for lysosomal degradation [163,165]. Since we observed that mitotic EGFR endocytosis proceeds exclusively in an ubiquitin-mediated manner and that total EGFR levels drop following low dose EGF stimulation (Figure 3.8), we hypothesized that mitotic endocytosis should lead exclusively to lysosomal trafficking. To test this, we examined the co-localization of endocytic route markers with EGFR by IF microscopy. The EGFR of both mitotic and interphase cells showed strong co-localization with EEA-1 and RAB5, indicating that the EGFR is trafficked to early endosomes (Figure 3.9). Importantly, EEA-1 and RAB5 did not co-localize with any EGFR at the plasma membrane of either mitotic or interphase cells. Staining with recycling markers (RAB11) and late endosomal markers (LAMP-1 and RAB7) has thus far been unsuccessful, but a working antibody and fixation method has been identified, and will be performed shortly. However, since Western blotting shows that total EGFR levels during mitosis drop dramatically following low dose EGF stimulation compared to interphase (Figure 3.8), we expect that the EGFR undergoes a lysosomal route.

3.5 Discussion

Our results showed that mitotic EGF-induced EGFR endocytosis proceeds exclusively by CBL-dependent NCE (Figure 3.10). NCE plays a major role in the regulation of EGFR fate by targeting it to lysosomes for degradation. Our research has uncovered a temporal period by which to exclusively target EGFR for degradation. This bypasses the receptor recycling pathway that is undesirable if the goal is EGFR attenuation, or if it is to deliver and keep a pharmacological agent into a cell [332]. Targeting mitotic cells is feasible for EGFR-

overexpressing cancer cells, as these cells intrinsically undergo more cell proliferation. In addition, the population of mitotic cells can be increased by treatment with anti-mitotic drugs, such as the commonly used taxanes and vinca alkaloids. Therefore, mitotic cells of EGFR-overexpressing cells can be targeted more directly. Moreover, the FDA-approved EGFR antibody cetuximab has been shown to initiate receptor endocytosis [338]. Whether mitotic EGFR treated with EGFR antibodies are also internalized by NCE remains to be investigated. However, if it does, nano-conjugation of EGFR antibodies to pharmacological agents may provide a targeted approach to treating these cancers.

The study of EGFR NCE thus far has relied on the inhibition of clathrin, as well as the use of high doses of EGF to activate NCE. Our results suggest that mitotic cells offer an alternate system for studying the NCE of the EGFR, even with low doses of EGF. However, as NCE is composed of various different pathways, including caveolin-mediated endocytosis, flotillin-mediated endocytosis, CLIC/GEEK, and FEME pathway [47], the mitotic activity of these pathways need to be elucidated.

3.5.1 The role of CBL in mitotic EGFR endocytosis

The theory that EGFR ubiquitination is absolutely necessary for endocytosis has been a subject of controversy, as previous publications have opposed this idea [169,339–341]. Our research supports the notion that ubiquitination by CBL is important for NCE [48,163]. Furthermore, our research shows strong support for the requirement of CBL and GRB2 binding to the EGFR in order to cause its ubiquitination [163,165]. Our results argue that GRB2-mediated CBL binding is more important than direct CBL-binding during mitosis, as the Δ1044 mutant had significantly more inhibited internalization, whereas the Y1045F mutant had a slight inhibition. Overexpression of CBL did not accelerate nor enhance mitotic EGF-induced EGFR

endocytosis. NCE has been reported to initiate more slowly than CME [48,166,168,205,330,331], and it therefore appears that CBL overexpression is not the limiting factor to the speed of EGFR NCE. Other important mediators of NCE, for example EPS15, EPS15R, and EPSIN [48], or endoplasmic reticulum (ER)-resident protein reticulon 3 (RTN3) and CD147 [329] may warrant investigation. More importantly, the exact mechanism by which the ubiquitination of the EGFR induces internalization is still unknown, and studies to elucidate the precise molecular mechanism would be extremely impactful.

The inactivation of CBL has been shown to display pro-oncogenic features [188,195,196,342,343]. Moreover, common pro-oncogenic EGFR mutations L858R and L858R/T790M have impaired CBL-binding, slower endocytosis, and impaired degradation [344]. EGFRvIII, the most common variant in gliomas, also has a reduced interaction with CBL and thus impaired ubiquitination owing to hypophosphorylation of pY1045 [345,346]. Here, we showed that CBL activity during mitosis is even more important, and its activity is enhanced compared to interphase cells. Evolutionarily, since mitotic cells do not have active CME [320,321], the activation of NCE may have been even more critical during mitosis to suppress EGFR overactivation. A loss of CBL activity, whether by inactivating mutations to CBL or EGFR CBL-binding, would therefore have a more pronounced effect during mitosis, as the EGFR would continue to signal excessively. The functional role of mitotic EGFR activation is still not well known. It is unclear how abnormally sustained EGFR signaling during mitosis affects cellular processes, however, it does appear to help mitotic cancer cells resist nocodazole-mediated cell death [279].

We showed that the EGFR is more strongly ubiquitinated during mitosis at both low and high doses of EGF, suggesting that CBL activity is enhanced during mitosis. How can CBL be

better primed to induce endocytosis, even at low concentrations of EGF during mitosis? It has been shown that CBL also acts as an adaptor in the CME pathway. Since CME is no longer active during mitosis, a possible explanation may be due to increased CBL protein availability, as the cellular pool of CBL no longer needs to divide its time between CME and NCE. Another explanation may be that CBL is modified during mitosis to be better primed for its E3 ligase activity. Interestingly, probing with the CBL antibody revealed that the mitotic CBL band appears smaller compared to interphase (Figure 3.8), although the exact significance is unknown Another alternative possibility may revolve around the DUBs (deubiquitinating enzymes) that deubiquitinate EGFR. Fifteen DUBs have been reported to impact EGFR fate, although some may be deubiquitinating non-EGFR component, such as EPS15 [347]. These DUBs may be shut off during mitosis, causing ubiquitination to persist longer than during the interphase.

3.5.2 Mitosis and EGFR

Mitosis represents a phase of tremendous transition to the cell. It is a critical moment of the cell's life where its genetic material is precisely separated to two daughter cells. To ensure proper chromosome segregation, the mitotic environment must be carefully controlled, as misproper mitosis result in chromosome bridging, lagging, or mis-aggregation, and ultimately aneuploidy [270]. To achieve this, mitotic cells undergo mitotic cell rounding, which is the dramatic transformation of cells from well spread and flattened to spherical and rigid. The mitotic shape is thought to confer cells with a predictable and defined geometry regardless of its external environment so to facilitate chromosome capture and the symmetric segregation of contents [348–350]. The spherical shape and rigid cell cortex however present the EGFR with changes in conditions during mitosis. For example, it is likely that the inhibition of CME during

mitosis is due to the unavailability of actin in participating in endocytosis, as it must form the rigid mitotic cell cortex [171]. Furthermore, mitosis changes the amount of space in the cell, so that they reach a minimal volume during metaphase [351]. It is possible that the molecular interactions necessary for NCE may become facilitated by a smaller cell volume. The more compacted volume does not appear to aid in EGFR activation, as the phosphorylation of the EGFR upon EGF addition is similar between interphase cells and mitotic cells. Therefore, in this situation, the smaller cell volume may aid CBL or GRB2 binding to the EGFR.

Another change to normal interphase EGFR signaling during mitosis is the global phosphorylation of mitotic proteins by mitotic kinases. For example, studies have shown the mitotic phosphorylation of over 1000-6027 proteins, including 14,000-50,000 phosphorylation events depending on the study [352–354]. Interestingly, many phospho-sites overlap between EGF-stimulated cells and mitotic cells [353]. Indeed, various components of the EGFR signaling and endocytic pathways appear to play different roles in mitosis, a phenomenon known as moonlighting [326,355]. This includes important members of EGFR CME, such as clathrin, dynamin, and AP-2 [324–328]. Since these proteins and many others are moonlighting in mitosis-related processes, their availability to participate in EGFR endocytosis during mitosis may be compromised. This may also affect EGFR signaling. It has been shown that EGF-induced AKT activation requires EGFR residence in clathrin coated pits, but not internalization [356,357]. We previously showed that only AKT2, and not AKT1, becomes activated following EGF stimulation during mitosis [279]. Since CME is shut down during mitosis, it can be speculated that the differential activation of AKT during mitosis is a consequence of the inability of clathrin to be involved in mitotic EGFR endocytosis. Therefore, the changes imparted by

global mitotic phosphorylation and mitotic cell rounding cannot be discounted to EGFR signaling, and likely of other signaling receptors as well.

In our study, we made use of the microtubule depolymerizer nocodazole to arrest cells in mitosis. So far, nocodazole is still the most widely used drug for arresting cells in mitosis [353,358–360]. We decided to use nocodazole in our research in order to obtain synchrony between our Western blots, co-IPs, and immunofluorescence experiments, as it has been shown that the sub-stage of mitosis can influence the kinetics of endocytosis [4]. Previous research has showed that factors such as serum starvation, nocodazole, and other mitotic inhibitors could inhibit CME [361]. However, it should be noted that the researchers were evaluating transferrin receptors, which is endocytosed by constitutive endocytosis rather than the ligand-induced mechanism used by EGFR. Furthermore, our previous study that showed that clathrin downregulation by siRNA had no effect on mitotic EGFR endocytosis was performed without the use of nocodazole [4]. We have also previously shown that 16 h nocodazole treatment does not lead to significant cell apoptosis [279].

However, nocodazole is a microtubule depolymerizer, and it is possible that it may interfere with components of the endocytic pathway. It has been reported that nocodazole blocks the transport from early to late endosomes, as this trafficking may involve microtubules. Transport of material from early to late endosomes was shown to be inhibited by nocodazole for fluid-phase endocytosis of dextran and constitutive endocytosis of transferrin in HeLa cells [362,363]. Moreover, in MEK cells, nocodazole reduced the percentage of EGFR colocalizing to LAMP-2-containing vesicles [364]. However, the microtubule stabilizer paclitaxel promotes EGFR degradation to lysosomes in A549 cells, apparently due to a spatially shorter route for the EGFR to lysosomes [365]. However, nocodazole treatment does not change the distribution of

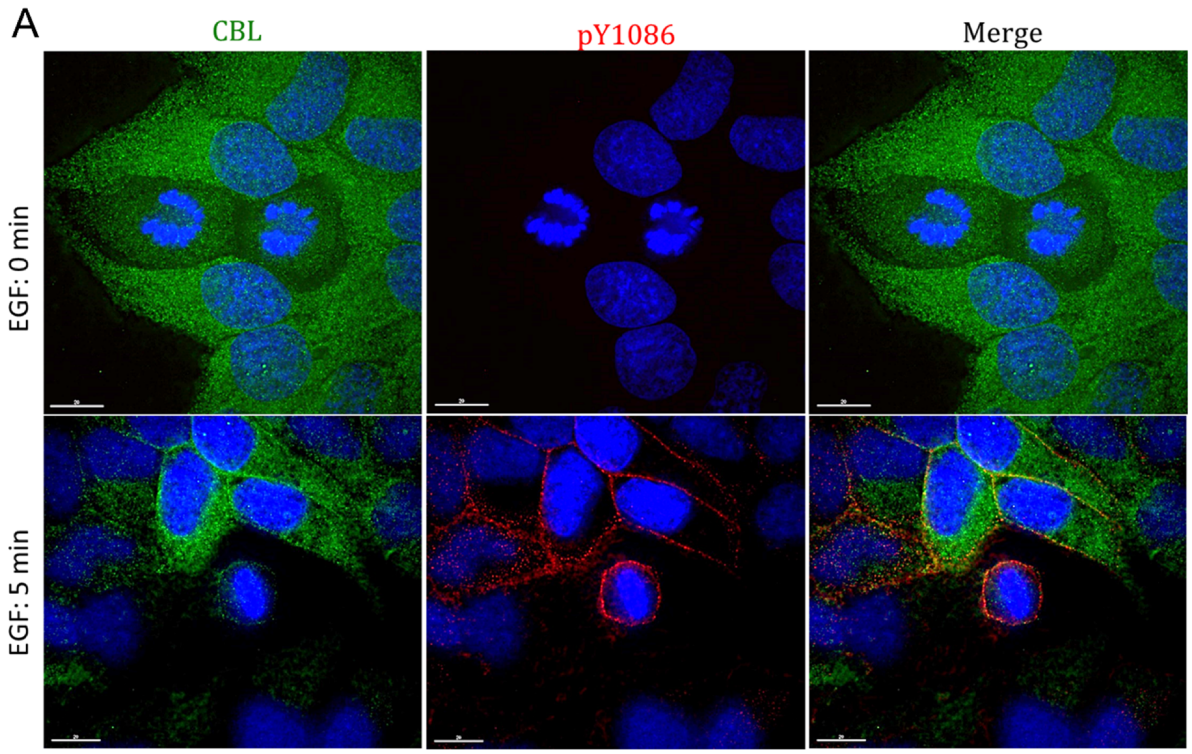
lysosomal membranes [366]. Regardless, it may therefore be possible that the stronger ubiquitination seen on the EGFR is due to the inability of ubiquitinated EGFR to be trafficked to lysosomes for degradation. Our results may therefore be more representative of drug-arrested mitosis.

3.5.3 Conclusion

The EGFR uses various signaling pathways to achieve numerous pro-oncogenic cellular outcomes. Endocytosis downregulates EGFR signaling by internalizing active receptors away from the cell surface. In this way, endocytosis controls EGFR signaling, spatially and temporally, making it an indispensable part of receptor signaling. Therefore, the interplay between EGFR signaling and endocytosis critically determines cellular outcome.

Our research showed that mitotic EGFR endocytosis proceeds exclusively by NCE. The inhibition of CBL therefore severely impedes mitotic EGFR endocytosis, as EGFR ubiquitination is necessary for this endocytosis. Furthermore, as mitotic EGFR endocytosis only proceeds by NCE, the cargo becomes targeted for lysosomal degradation. This period of the cell's natural progression may therefore be leveraged to ensure lysosomal trafficking.

3.6 Figures


A
CBL
pY1086
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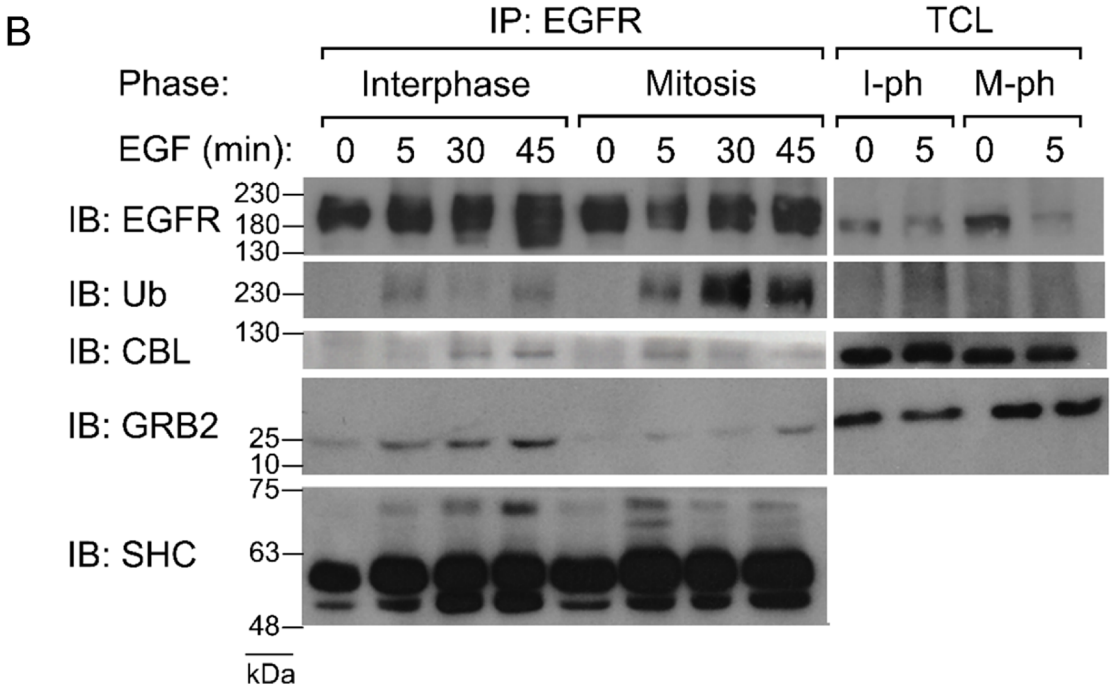

B
IP: EGFR
TCL
Phase:
Interphase
Mitosis
I-ph
M-ph
EGF (min):
0 5 30 45 0 5 30 45 0 5 0 5
IB: EGFR
IB: Ub
IB: CBL
IB: GRB2
IB: SHC
230
180
130
230
130
25
10
75
63
48
kDa

Figure 3.1. CBL is activated by EGF-stimulation during mitosis. A) Direct immunofluorescence images of HeLa cells stained with CBL (green), EGFR pY1086 (red), and DAPI (blue). Cells were treated with EGF (50 ng/mL) for the indicated times. B) Co-immunoprecipitation of EGFR from asynchronous (interphase) or nocodazole-arrested (mitosis) HeLa cells. EGF (50 ng/mL) was used to treat cells for the indicated times. Immunoblotting was performed with the specified antibodies. Mitotic EGFR is more strongly ubiquitinated than interphase. Total cell lysates (input) are also shown. Results are representative of at least two biological replicates.

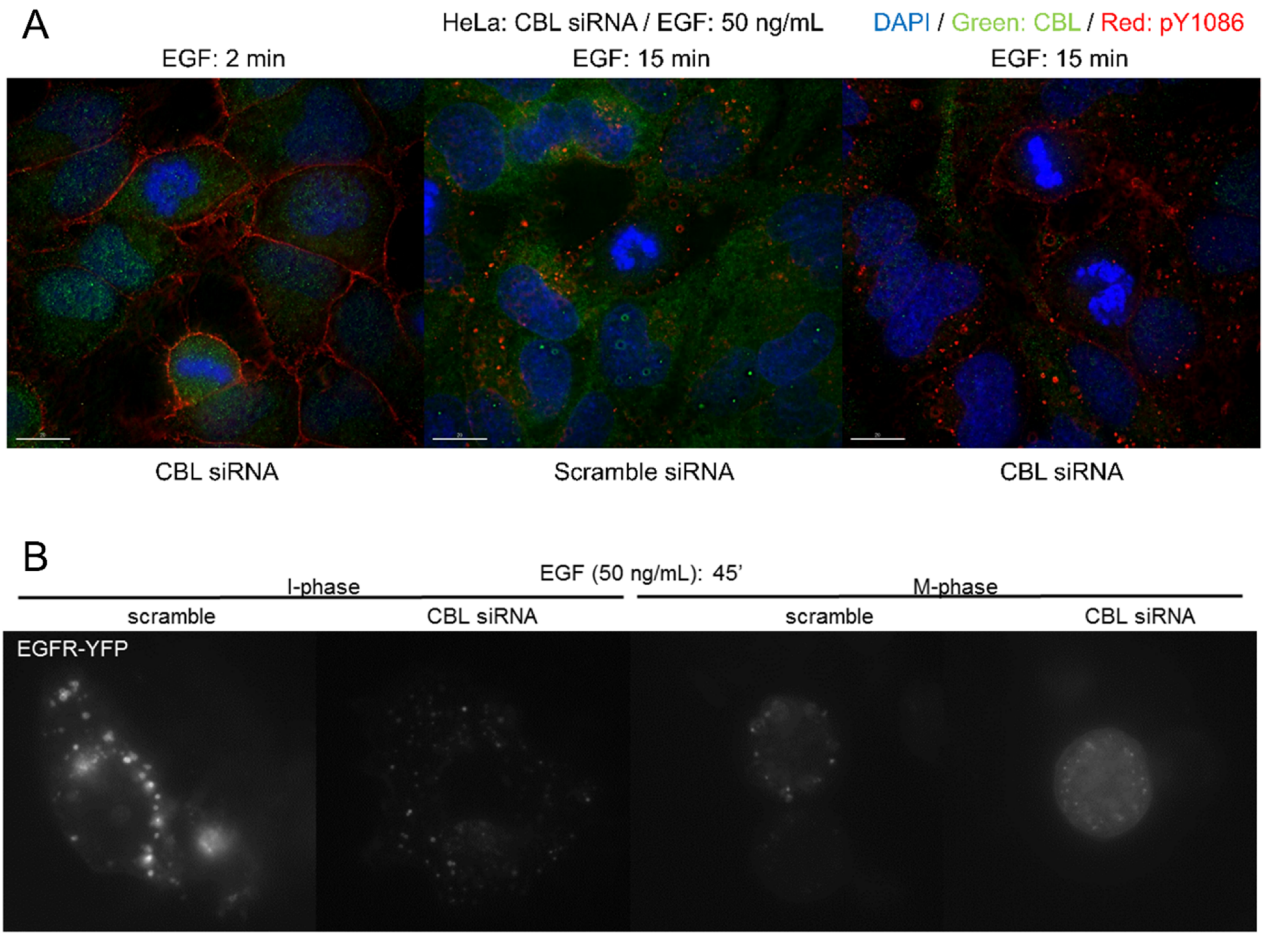


Figure 3.2. siRNA downregulation CBL inhibits mitotic endocytosis. Indirect immunofluorescence to observe EGFR endocytosis in cells treated with CBL siRNA or with scramble siRNA in: A) HeLa cells stained for CBL (green) and EGFR pY1086 (red) and treated with EGF (50 ng/mL) for 15 mins; and B) MCF-7 cells transfected with EGFR-YFP and treated with EGF (50 ng/mL) for 45 mins. MCF-7 cells were treated with nocodazole (200 ng/mL) for 16 h.

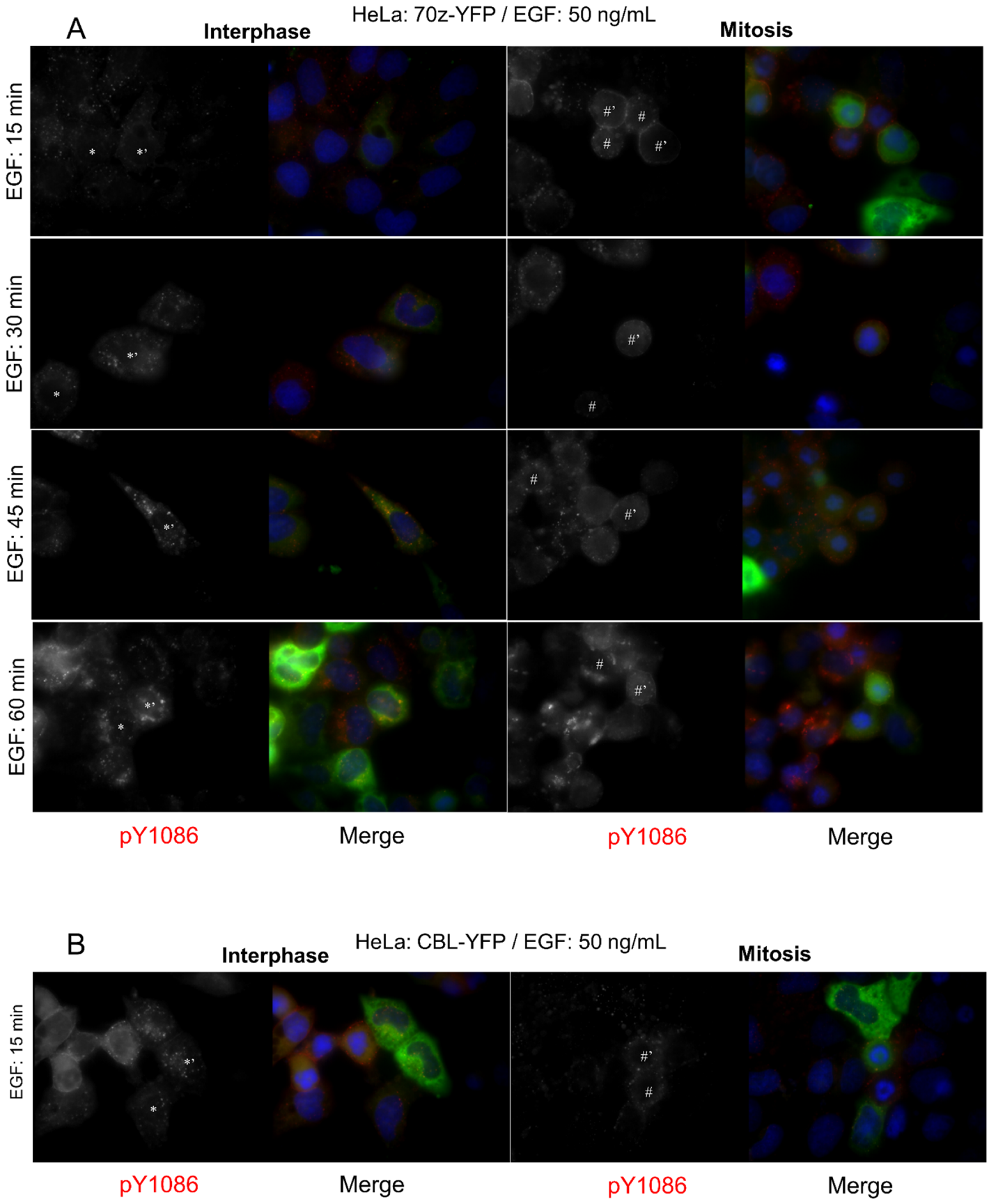
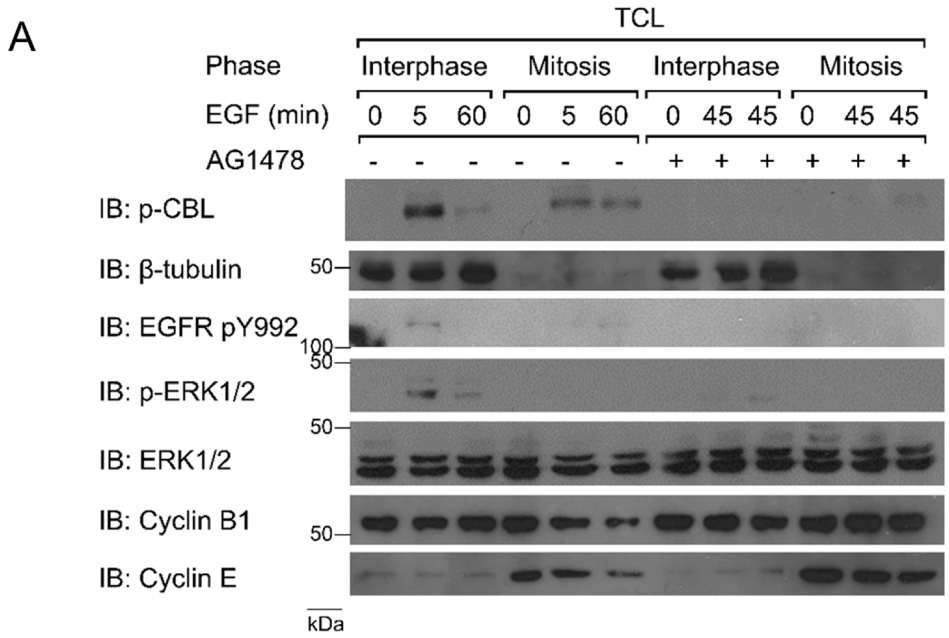
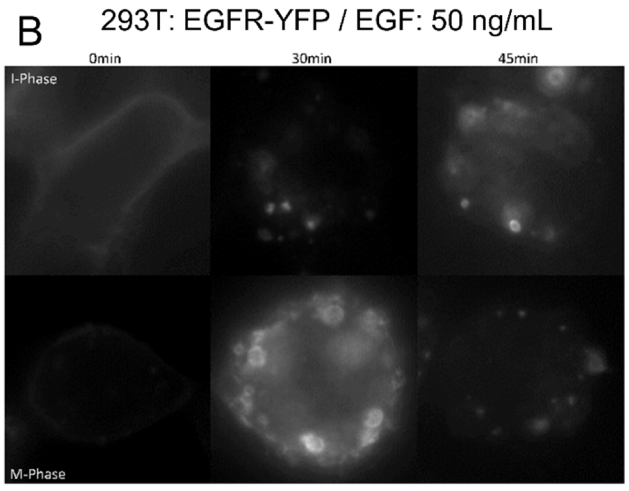
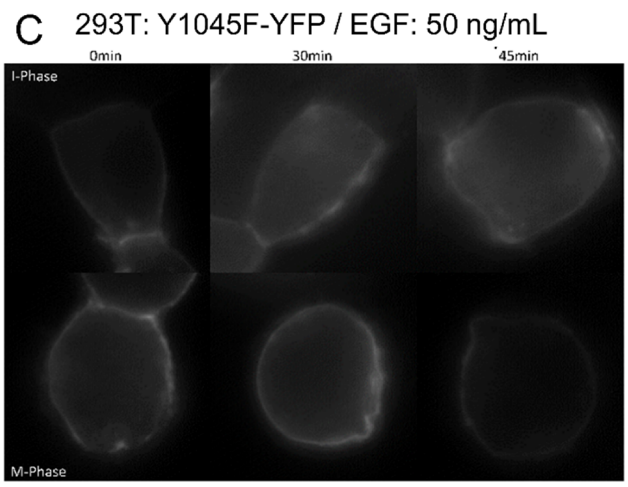
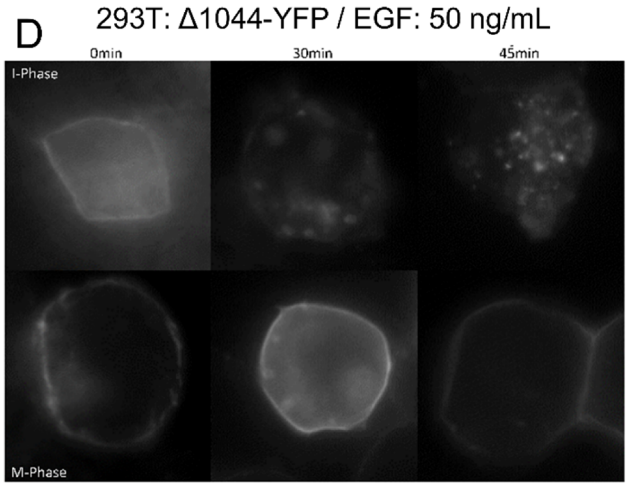

A
HeLa: 70z-YFP / EGF: 50 ng/mL
Interphase
Mitosis
EGF: 15 min
EGF: 30 min
EGF: 45 min
EGF: 60 min
pY1086
Merge
pY1086
Merge
B
HeLa: CBL-YFP / EGF: 50 ng/mL
Interphase
Mitosis
EGF: 15 min
pY1086
Merge
pY1086
Merge

Figure 3.3. The effects of downregulation and overexpression of CBL. Indirect immunofluorescence to observe EGFR endocytosis in HeLa cells transfected with: A) Dominant-negative CBL (70z-YFP) and B) c-CBL-YFP overexpression. 70z-YFP transfection inhibits mitotic EGFR endocytosis, but not in interphase cells. Cells were treated with EGF (50 ng/mL) for the indicated times and were stained for pY1086 (red), and DAPI (blue). The * represents interphase cell, # represents mitotic cell, and ’ represents transfected cell. Cells were treated with nocodazole (200 ng/mL) for 16 h.


A
TCL
Phase
Interphase
Mitosis
Interphase
Mitosis
EGF (min)
0 5 60 0 5 60 0 45 45 0 45 45
AG1478
- - - - - - + + + + + +
IB: p-CBL
IB: β-tubulin
IB: EGFR pY992
IB: p-ERK1/2
IB: ERK1/2
IB: Cyclin B1
IB: Cyclin E
50
100
50
50
50
kDa


B
293T: EGFR-YFP / EGF: 50 ng/mL
0min
30min
45min
I-Phase
M-Phase


C
293T: Y1045F-YFP / EGF: 50 ng/mL
0min
30min
45min
I-Phase
M-Phase


D
293T: Δ1044-YFP / EGF: 50 ng/mL
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30min
45min
I-Phase
M-Phase

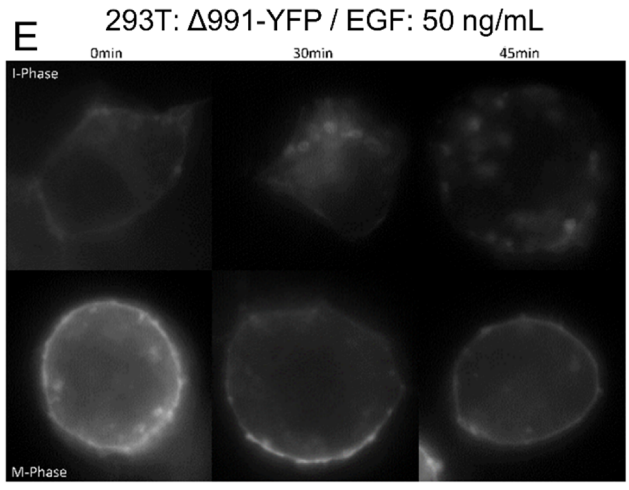
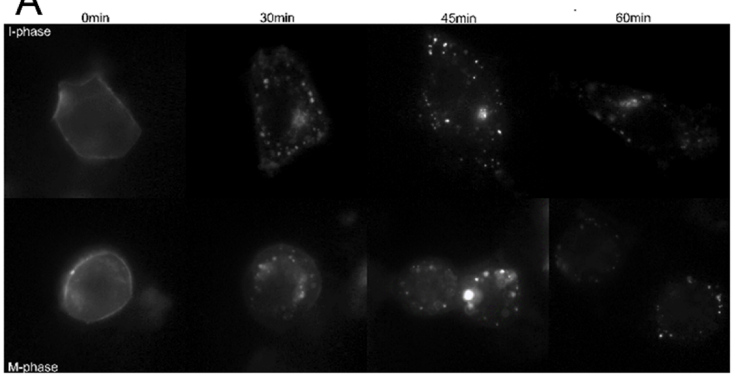

E
293T: Δ991-YFP / EGF: 50 ng/mL
0min
30min
45min
I-Phase
M-Phase

Figure 3.4. The role of EGFR kinase activation in EGFR endocytosis. A) The effects of AG1478 on EGFR and CBL phosphorylation. Asynchronous (I-phase) and nocodazole-arrested (M-phase) HeLa cells were pre-treated with AG1478 1 hour prior to EGF treatment, then treated with EGF (50 ng/mL) for the indicated times. To study the role of EGFR C-terminal domains, 293T cells were transfected with with B) EGFR-YFP (positive control), C) EGFR-Y1045F-YFP (no direct CBL binding), D) EGFR-Δ1044-YFP (no CBL binding), and E) EGFR-Δ991-YFP (negative control). Cells were treated with EGF (50 ng/mL) for the specified times and observed by indirect immunofluorescence. Cell cycle phase of cells were determined by DNA morphology (not shown). Cells were treated with nocodazole (200 ng/mL) for 16 h.

A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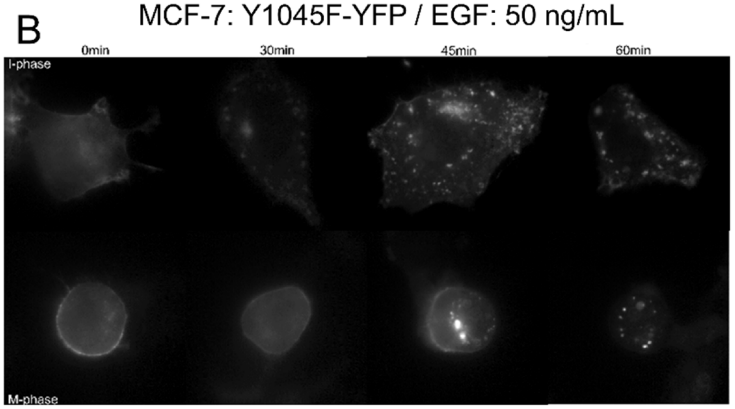
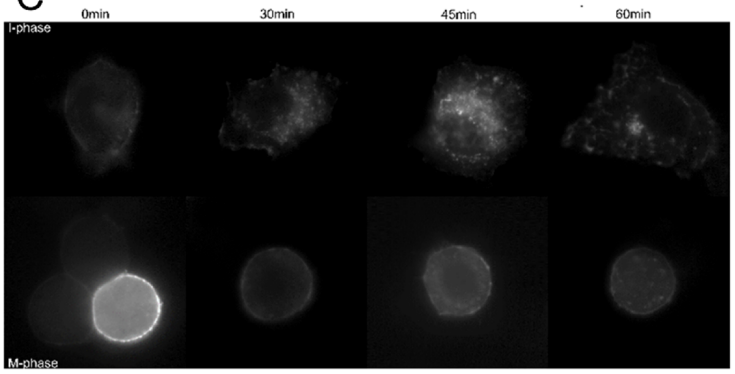
0min
30min
45min
60min
I-phase
M-phase

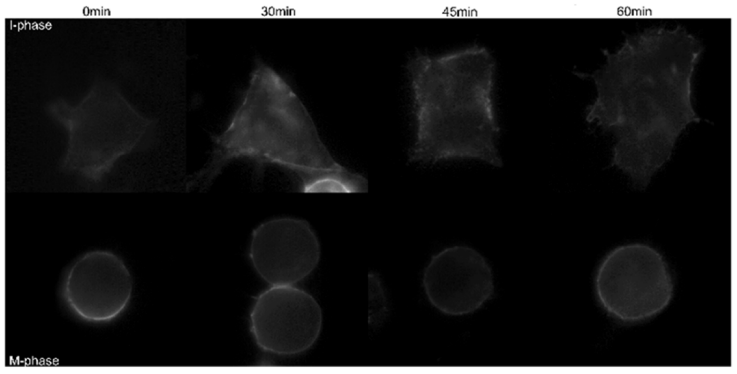
B


MCF-7: Y1045F-YFP / EGF: 50 ng/mL
0min
30min
45min
60min
I-phase
M-phase

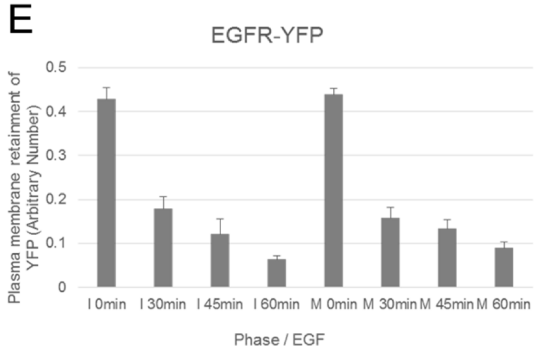
C

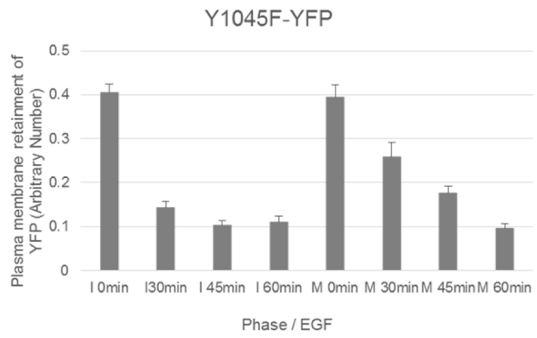

MCF-7: Δ1044-YFP / EGF: 50 ng/mL
0min
30min
45min
60min
I-phase
M-phase

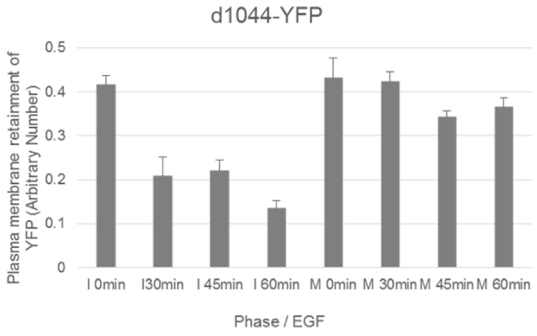
D


MCF-7: Δ991-YFP / EGF: 50 ng/mL
0min
30min
45min
60min
I-phase
M-phase

E


EGFR-YFP
Plasma membrane retainment of YFP (Arbitrary Number)
0.5
0.4
0.3
0.2
0.1
0
I 0min
I 30min
I 45min
I 60min
M 0min
M 30min
M 45min
M 60min
Phase / EGF


Y1045F-YFP
Plasma membrane retainment of YFP (Arbitrary Number)
0.5
0.4
0.3
0.2
0.1
0
I 0min
I30min
I 45min
I 60min
M 0min
M 30min
M 45min
M 60min
Phase / EGF


d1044-YFP
Plasma membrane retainment of YFP (Arbitrary Number)
0.5
0.4
0.3
0.2
0.1
0
I 0min
I30min
I 45min
I 60min
M 0min
M 30min
M 45min
M 60min
Phase / EGF

F

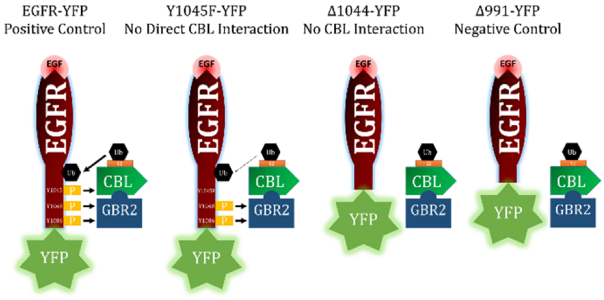

EGFR-YFP
Positive Control
Y1045F-YFP
No Direct CBL Interaction
Δ1044-YFP
No CBL Interaction
Δ991-YFP
Negative Control
EGF
EGFR
Ub
CBL
GBR2
YFP

Figure 3.5. Necessity of EGFR's CBL-binding domains for EGFR endocytosis. MCF-7 cells were transfected with A) EGFR-YFP (positive control), B) EGFR-Y1045F-YFP (no direct CBL binding), C) EGFR-Δ1044-YFP (no CBL binding), and D) EGFR-Δ991-YFP (negative control). Cells were treated with nocodazole (200 ng/mL) for 16 h and with EGF (50 ng/mL) for the specified times and observed by indirect immunofluorescence. Cell cycle phase of cells were determined by DNA morphology (not shown). E) Quantification of plasma membrane retainment of YFP for A)-C) for at least 10 cells (see Materials and Methods). F) Illustration of EGFR mutants used and their ability to bind CBL.

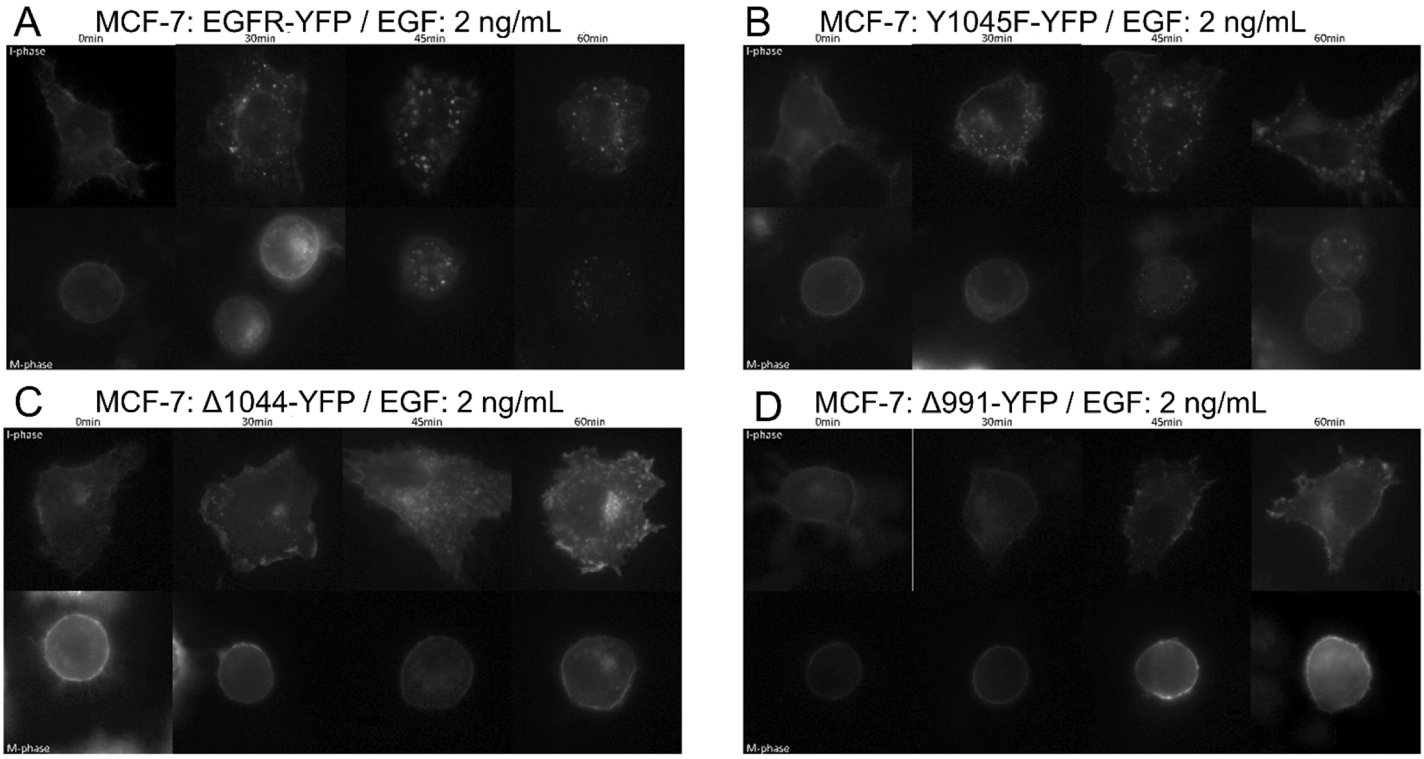


Figure 3.6. Effects of low EGF dose on mitotic EGFR endocytosis. Cells were treated with EGF (2 ng/mL) to only activate CME for the specified times and observed by indirect immunofluorescence. Cell cycle phase of cells were determined by DNA morphology (not shown). MCF-7 cells were transfected with A) EGFR-YFP (positive control), B) EGFR-Y1045F-YFP (no direct CBL binding), C) EGFR-Δ1044-YFP (no CBL binding), and D) EGFR-Δ991-YFP (negative control). Cells were treated with nocodazole (200 ng/mL) for 16 h.

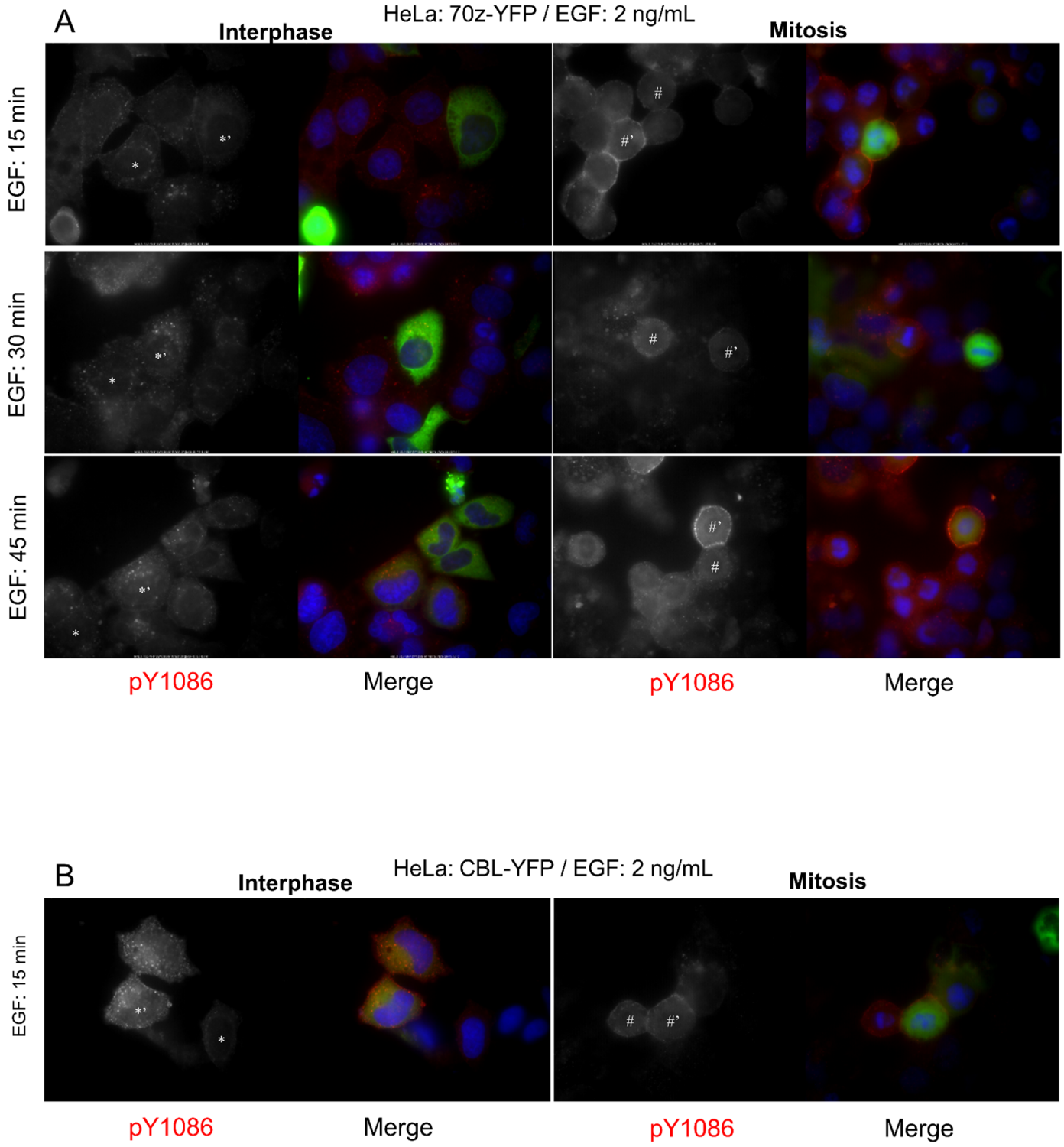


Figure 3.7. Effects of low EGF dose on endocytosis in cells with CBL alterations. HeLa cells were transfected with E) Dominant-negative CBL (70z-YFP) and F) CBL-YFP and treated with low dose EGF (2 ng/mL) for the indicated times. Cells were stained with EGFR pY1086 (red) and DAPI (blue). The * represents interphase cell, # represents mitotic cell, and ’ represents transfected cell. Cells were treated with nocodazole (200 ng/mL) for 16 h.

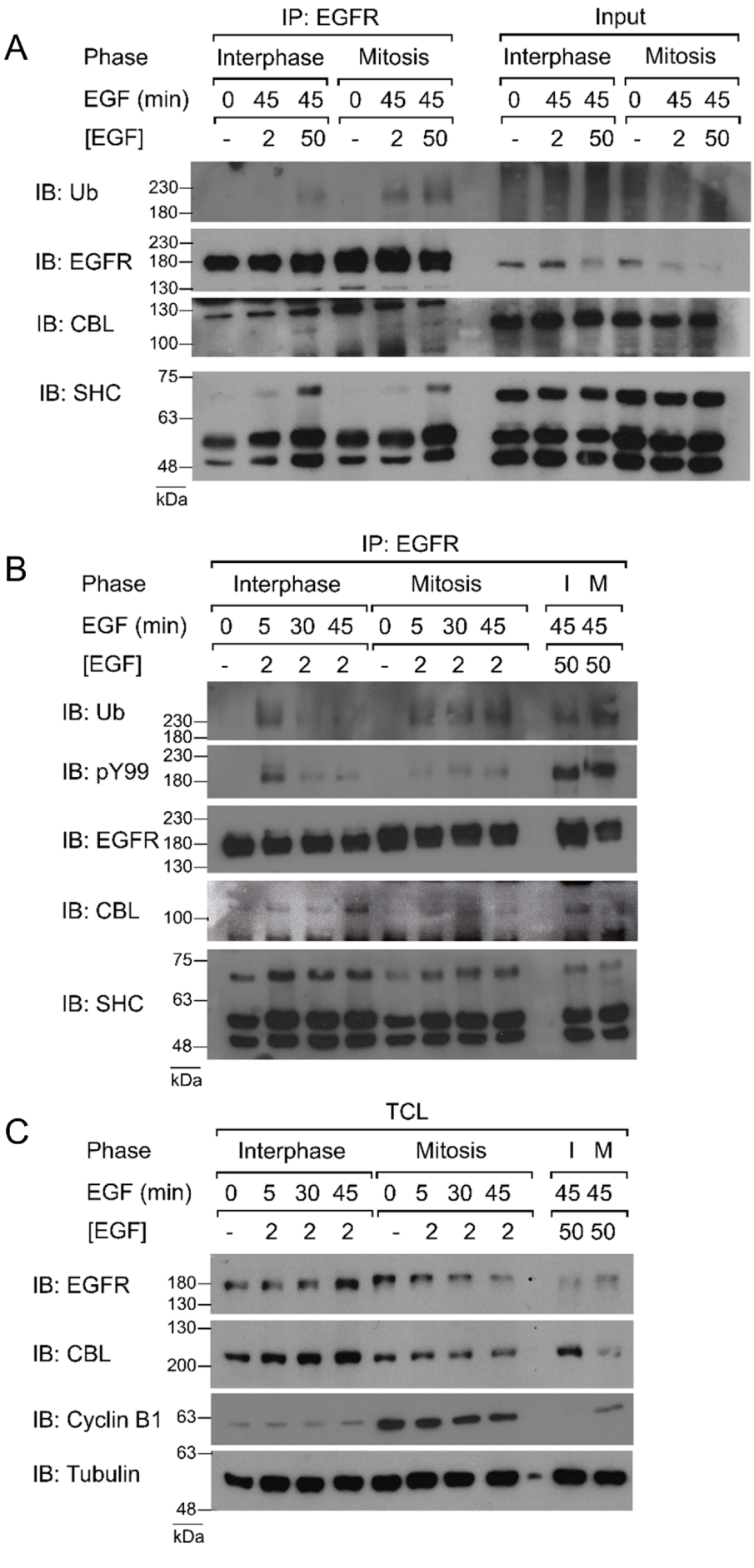

A
IP: EGFR
Input
Phase
Interphase
Mitosis
Interphase
Mitosis
EGF (min)
0 45 45 0 45 45
0 45 45 0 45 45
[EGF]
- 2 50 - 2 50
- 2 50 - 2 50
IB: Ub
230
180
IB: EGFR
230
180
130
IB: CBL
130
100
IB: SHC
75
63
48
kDa
B
IP: EGFR
Phase
Interphase
Mitosis
I M
EGF (min)
0 5 30 45 0 5 30 45 45 45
[EGF]
- 2 2 2 - 2 2 2 50 50
IB: Ub
230
180
IB: pY99
230
180
IB: EGFR
230
180
130
IB: CBL
100
IB: SHC
75
63
48
kDa
C
TCL
Phase
Interphase
Mitosis
I M
EGF (min)
0 5 30 45 0 5 30 45 45 45
[EGF]
- 2 2 2 - 2 2 2 50 50
IB: EGFR
180
130
IB: CBL
130
200
IB: Cyclin B1
63
IB: Tubulin
63
48
kDa

Figure 3.8. Ubiquitination and CBL-binding of EGFR during low EGF doses. Co-immunoprecipitation of EGFR from asynchronous (interphase) or nocodazole-arrested (mitosis) HeLa cells. A) Cells were treated with EGF for 45 mins using low and high dose EGF (2 and 50 ng/mL). B) Cells were treated with low dose EGF (2 ng/mL) for 0, 5, 30, or 45 mins. High dose EGF treatments for 45 minutes are included for reference. C) Immunoblotting of total cell lysate (TCL) with the specified antibodies. Results are representative of at least two biological replicates.

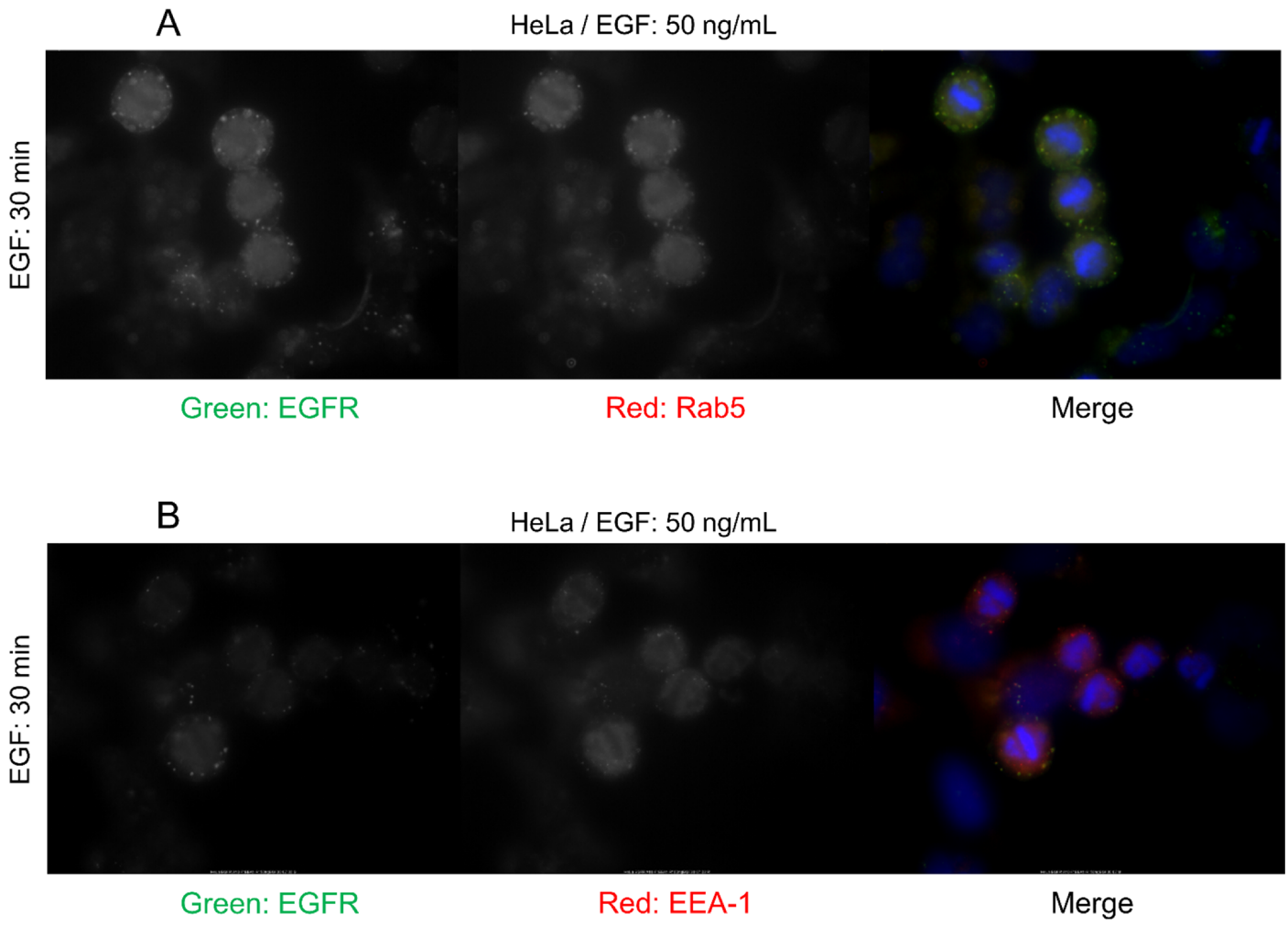


Figure 3.9. Mitotic EGFR is sorted to early endosomes. HeLa cells were treated with nocodazole (200 ng/mL) and EGF (50 ng/mL) for 30 mins and EGFR co-localization with early endosome markers was observed by indirect immunofluorescence. Cells were stained with EGFR (green), DAPI (blue) and A) RAB5 (red) or B) EEA-1 (red).

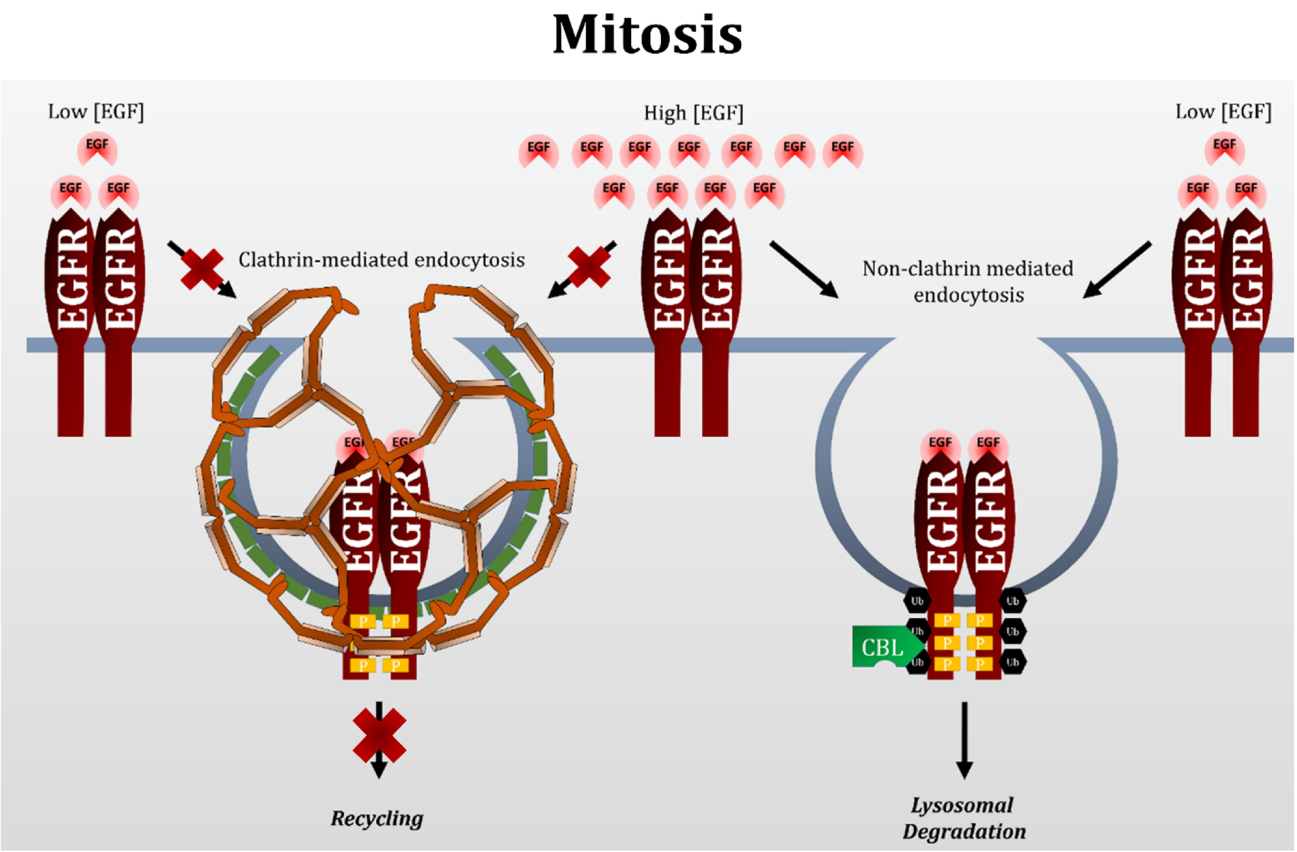


Figure 3.10. Model of EGFR endocytosis during mitosis. In interphase cells, low EGF doses (>2 ng/mL) only activates clathrin-mediated endocytosis (CME), leading to receptor recycling. High EGF doses (>20 ng/mL) also activates CME, but can also activate non-clathrin mediated endocytosis (NCE) due to the dose-dependent activation of CBL and EGFR ubiquitination. NCE leads to lysosomal degradation of EGFR. In mitotic cells, CME is shut off. Therefore, EGFR endocytosis must proceed by NCE. Both low and high concentrations of EGF activate NCE during mitosis, and this may be because contrary to interphase cells, low EGF concentrations can activate CBL and EGFR ubiquitination. Therefore, mitotic EGFR endocytosis leads exclusively to lysosomal degradation.

4 Chapter 4

Regulation of epidermal growth factor receptor cell signaling during the cell cycle

Note: All experiments were carried out by Ping Wee.

4.1 Chapter Abstract

Progression through the cell cycle causes changes in the cell's signaling pathways that can alter EGFR signal transduction. To fully understand EGFR signaling, it is important to decipher these changes. Here, I performed drug and drug-free assays for EGFR and EGFR-mediated pathways during the cell cycle. Using various drugs, I synchronized cells to G1, S-phase, G2, prometaphase, metaphase, and anaphase/telophase. Drug-free assay of EGFR involved the use of imaging flow cytometry. I validated these assays and used them to assay EGFR signaling. S-phase EGFR signaling appeared dampened, compared to G1 and G2 phases. No difference in EGFR signaling was observed between mitotic subphases. This work represents the optimization of methods to study the EGFR during various cell cycle phases, and may be used in to future to find differences in the signal transduction of various proteins during different cell cycle phases.

4.2 Introduction

Cell cycle progression brings about tremendous changes within the cell, complete with different needs and requirements for the cell at that moment. At each step of the cell cycle, kinases including cyclin-dependant kinases (CDKs) phosphorylate their specific but wide-ranging set of substrates, leading not only to changes to their substrate, but also to changes in their pathways. Mass spectrometry studies of the phosphoproteome of cells synchronized to different cell cycle phases reveal massive waves of cell cycle-dependant phosphorylations, especially during mitosis [305,352,367,368]. Whether a protein of interest or one its important downstream effectors are affected by cell cycle progression are important considerations. For example, this cell cycle regulation of a protein can affect the interpretation of results when looking at a total cell population. It can also affect the efficacy of certain targeted therapies, as their intended target may respond differently depending on cell cycle phase. Importantly, a hallmark of cancer, including those with epidermal growth factor receptor (EGFR) overactivation, is their ability to sustain chronic proliferation, therefore continuously dividing cells and their proteins will be more impacted by cell cycle effects.

The EGFR has been heavily studied for its role in affecting the cell cycle. A pulse of EGF stimulation during early G1 and another at late G1 have been shown to be sufficient for driving cells past the Restriction Point of the cell cycle, the point at which the cell must proceed through the cell division program [252,253]. During mitosis, we and others have observed significant changes to EGFR-mediated signaling pathways [2,3,279,352], to EGFR endocytosis kinetics [4], and to EGFR function [270,294]. However, much work remains to be done in characterizing the EGFR function during the entire cell cycle.

Here, I tested methods to evaluate EGFR activation and signaling pathways throughout the different phases of the cell cycle in HeLa cells. The HeLa cell line is the most commonly used mammalian model system for cell cycle research. The entire cell cycle of the HeLa cell lasts approximately 24 hours, with mitosis lasting 40-60 min. A HeLa cell contains 1.4×10^5 EGFR molecules, a number that is close to many transformed and untransformed cells [281]. Pharmacological inhibition was used to arrest cells in the different phases, in order to collect them and assay them by Western blotting. Synchronization in S phase was performed using the widely used double thymidine block. Synchronization in G2 was performed using the CDK inhibitor roscovitine after S phase synchronization release. Mitotic synchronization relied on the use of the popular reversible microtubule depolymerizer nocodazole [369], which synchronized cells in prometaphase. Metaphase cells were obtained by releasing cells from nocodazole block and treating with MG132, a proteasome inhibitor. Anaphase/telophase cells were obtained by prometaphase release followed by treatment with blebbistatin, a myosin II inhibitor [359]. Mitotic cells growing in cell culture were separated from interphase cells using the mitotic shake-off method, which uses mechanical agitation to detach lowly adherent mitotic cells. Synchronization of HeLa cells in G1 was performed by collecting cells that were serum starved. Verification of purity and yield of the synchronization methods was performed by indirect immunofluorescence. Verification was also performed by flow cytometry.

In order to verify these results without the use of pharmacological agents, imaging flow cytometry was used. Imaging flow cytometry combines the quantitative power of flow cytometry together with microscopy. Combined with analysis using the IDEAS software, this allows the measurement of staining intensities while distinguishing between cell cycle phases, and even between the sub-phases of mitosis.

4.3 Materials and Methods

4.3.1 Antibodies and Chemicals

ERK (sc-94), p-ERK Thr202/Tyr204 (sc-7383), AKT (sc-8312), p-AKT Ser473 (sc-33437-R), pEGFR Tyr1086 (sc-16804), EGFR (sc-03), p-Histone 3 Ser10 (sc-8656-R), β-tubulin (sc-5274) primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Hercules, CA) and the fluorescence-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). The cell culture reagents were from Invitrogen (Carlsbad, CA). Mammalian Protein Extraction Reagent (M-Per) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL USA). Unless otherwise specified, all other chemicals were from Sigma (St. Louis, MO)

4.3.2 Cell Culture and Treatment

The cell lines that were used include HeLa, HeLa cell line stably expressing H2B-GFP (HeLa H2B-GFP), and CHO cell lines stably expressing a YFP-tagged, wild-type EGFR (CHO-EGFR) [301]. The HeLa H2B stable cell line is a generous gift from Dr. Wahl (The Salk Institute for Biological Studies). It has been shown that the H2B-GFP fusion protein is incorporated into nucleosomes without affecting cell cycle [302].

All cells were grown at 37°C in growth medium (Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics) and were maintained in a 5% CO2 atmosphere. For CHO cell lines, G418 was added to a final concentration of 500 μg/ml. For the HeLa H2B-GFP cell line, blasticidine was added as a supplement to a concentration of 2 μg/ml to maintain the trans-gene expression.

To collect lysates, cells were synchronized as described for their specific phases. Cells were then treated with EGF or left untreated (0 min). EGF was used at a final concentration of 50 ng/ml. For attached cells (G1, S, G2), cells were collected by scraping on ice in cold Mammalian Protein Extraction Reagent (M-Per) (Thermo Fisher Scientific Inc, Rockford, IL USA) buffer in the presence of phosphatase and protease inhibitors including 100 mm NaF, 5 mM MgCl2, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A. For mitotic cells (prometaphase, metaphase, anaphase/telophase), mitotic shakeoff was performed. Briefly, after EGF treatment, cells were washed with cold DMEM and plates were placed on ice and knocked against the sidewall of ice buckets for 5 mins. The cells were then transferred to centrifuge tubes and centrifuged at 1000 × g for 5 minutes. Ice-cold PBS was added to each plate and shaken until centrifugation was complete to collect remaining cells. The media from the centrifuge tube was then aspirated and the remaining were added to the tube and centrifuged at 1000 × g for 5 minutes. The obtained mitotic cells were then lysed with M-Per in the presence of phosphatase and protease inhibitors. For both interphase and mitotic cells, M-Per was incubated for 5 minutes on ice, and lysis was completed by adding 500 mM Tris-HCl (pH 7.5), 10% Nonidet P-40 and incubating for 15 mins at 4°C on a rocker. Cells were then centrifuged at 14,000 × g at 4°C for 15 mins. The supernatant protein samples were then collected.

4.3.3 Immunoblotting

Immunoblotting was performed as previously described [158]. Briefly, protein samples were separated by SDS-PAGE and then were transferred onto nitrocellulose and probed with primary antibodies. The primary antibodies were detected with a HRP conjugated secondary

antibody followed by enhanced chemiluminescence development (Pierce Chemical, Rockford, IL) and light detection on Fuji Super RX Film (Tokyo, Japan).

4.3.4 Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously [4]. Briefly, cells were grown on glass coverslips and serum starved for 16 h. After treatment, the cells were fixed by methanol and visualized by DeltaVision deconvolution microscopy (GE Healthcare Life Sciences, Buckinghamshire). Cells were counted based on morphology of the cell and of the DNA.

4.3.5 Flow Cytometry

Flow cytometry analysis was used to measure DNA content. G0/G1 cells have 2N DNA, S-phase cells have 2-4N DNA, and G2/M cells have 4N DNA. Imaging flow cytometry was used to further distinguish between each stage of mitosis.

To verify yield and purity of pharmacological agents, I performed the described synchronization protocols. For interphase cells, after completing cell cycle arrest, the media was aspirated and washed with PBS. The cells were then trypsinized, PBS was added to a volume of 5 mL, and the cells were detached by spraying with a Pasteur pipette. The cells were collected in a pre-cooled tube, and centrifuged at 1000 × g for 5 minutes. The supernatant was aspirated and cells were re-suspended in 0.5 mL PBS. The cells were then transferred into 4.5 mL of ice-cold 100% MeOH. For mitotic cells, after completing mitotic shake-off, the cells were re-suspended in 0.5 mL PBS, then transferred to 4.5 mL of ice-cold 100% MeOH. For both interphase and mitotic cells, fixation was performed for 20 minutes at -20°C. Cells were centrifuged at 1000 × g for 5 mins, and re-suspended in 1 mL of Flow Cytometry Blocking Buffer (TBS, 0.01% Triton

X-100, 4% BSA) and incubated on ice for 10 mins. Cells were again centrifuged at 1000 × g for 5 mins, and re-suspended in 1 mL of Flow Cytometry Blocking Buffer (TBS, 0.01% Triton X-100, 4% BSA) and primary antibody and incubated on ice for 1 hour. Cells were then centrifuged and washed with 1 mL ice cold PBS twice. Cells were again centrifuged at 1000 × g for 5 mins, and re-suspended in 1 mL of Flow Cytometry Blocking Buffer (TBS, 0.01% Triton X-100, 4% BSA) and secondary fluorescent-conjugated antibody and incubated on ice for 1 hour. The cells were again washed twice with PBS, and centrifuged. The cells were then re-suspended in 200 µL PBS with DRAQ5 (final concentration of 5 µM). The cells were transferred to a flow cytometry tube and incubated for 15 mins at room temperature. The samples were then processed by flow cytometry.

For non-synchronized cells used for imaging flow cytometry, after EGF treatment of non-serum starved cells, the media was aspirated and washed with PBS. The cells were then trypsinized, PBS was added to a volume of 5 mL, and the cells were detached by spraying with a Pasteur pipette. The cells were collected in a pre-cooled tube, and centrifuged at 1000 × g for 5 minutes. Staining was performed as above.

4.3.6 Cell Synchronization

4.3.6.1 G1 Synchronization

G1 synchronization was performed by serum starvation for 16 hours prior to EGF treatment and collection.

4.3.6.2 S-Phase Synchronization: Double Thymidine Block

The double thymidine block is a highly effective and widely used protocol for synchronization of cells in early S-phase (see Note 2). Excess thymidine inhibits the formation of dCTP, an essential precursor of DNA, and thus halts DNA replication [370].

For S-phase synchronization, first HeLa cells were seeded at 2.0×10^6 HeLa cells in 100-mm plates and grown overnight to ~40% confluency. The cells were then treated with thymidine (final concentration of 2 mM) in growth media for 16 hours. Cells were washed with PBS and fresh growth media was added to release them back into the cell cycle for 8 hours. Cells were incubated again with thymidine for 16 hours.

4.3.6.3 Late G2 Synchronization: Roscovitine

Roscovitine inhibits CDKs by competing with ATP at the ATP binding sites of various CDKs, including CDK1, CDK2, CDK5, and CDK7 [371]. Here, we release cells from the S phase block, allow them to grow into G2, and use roscovitine to prevent the CDK1/cyclin B1 complex (also known as maturation promoting factor) from activating, which prevents cells from entering mitosis.

For G2 synchronization, first S-phase synchronization by double thymidine block was performed. The cells were then released into the cell cycle by washing with PBS three times and incubated with growth medium for 7 hours. The growth media was then aspirated and incubated with warm DMEM with roscovitine (final concentration of 50 μM) for 4 hours.

4.3.6.4 Prometaphase Synchronization: Nocodazole

The microtubule depolymerizing agent nocodazole may be the most commonly used agent for inducing mitotic prometaphase arrest. It has a high affinity to tubulin, and prevents tubulin-composed spindle microtubules from interacting properly with the kinetochores of

chromosomes [372]. These chromosomes are therefore not brought to metaphase plate, and cannot proceed past the spindle assembly checkpoint (SAC) [369]. Here, we used lower concentrations of nocodazole than in those used in Chapter 2 and 3, so that release into metaphase and anaphase/telophase can be more easily performed.

For prometaphase synchronization, first S-phase synchronization by double thymidine block was performed as above. The cells were then released into the cell cycle by washing with PBS three times and incubated with growth medium for 9 hours. The growth media was then aspirated and incubated with warm DMEM with nocodazole (final concentration of 20 ng/mL) for 5 hours.

4.3.6.5 Metaphase Synchronization: MG132

The spindle assembly checkpoint (SAC) ensures all chromosomes are properly aligned prior to commencing anaphase [373]. Upon satisfying the requirements of the SAC, the anaphase-promoting complex (APC) is turned on, and initiates the proteasomal degradation of cyclin B and securin [374–376]. This proteasomal activity allows sister chromatids to separate and transition from metaphase to anaphase. To synchronize cells in metaphase, the proteasome inhibitor MG132 is added to cells in prometaphase, allowing chromosomes to proceed past the SAC, but preventing cells from proceeding into anaphase [305].

For metaphase synchronization, first prometaphase synchronization by nocodazole was performed as above. The cells were then released into the cell cycle by washing with PBS three times and incubated with warm DMEM for 70 mins.

4.3.6.6 Anaphase/Telophase Synchronization: Blebbistatin

Anaphase/Telophase are the most difficult phases to synchronize due in part to their short duration (20-30min). There are no drugs known to completely block cells in anaphase or telophase. However, the myosin II inhibitor blebbistatin may be used to extend the duration of anaphase and telophase [359]

For synchronization in anaphase/telophase, first prometaphase synchronization by nocodazole was performed as above. The cells were then released into the cell cycle by washing with PBS three times and incubated with warm DMEM for 20 mins. Blebbistatin was then added to a final concentration of 50 μM and incubated for 40-55 mins.

4.4 Results

4.4.1 Interphase Synchronization

EGFR signaling during the interphase sub-phases has never been studied in detail. Here, we sought to characterize differences in EGFR signaling between G0/G1, S-phase, and G2. We synchronized cells in G1 by serum starvation, in S-phase by double thymidine block, and in G2 using roscovitine (see Materials and Methods) (Figure 4.1). We collected the cells for flow cytometry analysis.

To verify the synchronization efficiency, I used a combination of flow cytometry and immunofluorescence microscopy. Flow cytometry revealed that asynchronized cells results in 50% of cells in G0/G1, 37% of cells in S-phase, and 13% of cells in G2 (Figure 4.2). Serum starvation resulted in nearly 70% of cells in G0/G1 (Figure 4.2A). Double thymidine block resulted in significant enrichment of cells in S-phase of nearly 75% (Figure 4.2B). G2 synchronization also led to enrichment in G2/M of nearly 40% (Figure 4.2C). For G2

synchronized cells, immunofluorescence microscopy performed in parallel with flow cytometry sample collection showed that very few cells were in mitosis (Figure 4.2D&E). This was in contrast with cells that were not treated with roscovitine, which showed an enrichment of cells in mitosis, even 2 hours after thymidine release (Figure 4.2E).

I performed Western blot analysis of cells synchronized in G0/G1, S-phase, G2, and prometaphase to examine the activation of the EGFR as well as the pathways it mediates (Figure 4.3). Interestingly, S-phase EGFR was less phosphorylated at pY1086 compared to G0/G1, G2, and prometaphase cells. Downstream, ERK1/2 was also less phosphorylated at S-phase compared to G1 and G2, although it was more phosphorylated than during prometaphase, which we previously showed to be inhibited. Preliminary data also showed that p-AKT (S473) was less phosphorylated during S-phase. Immunoblotting for p-CBL, p-PLC-γ1, and p-RAF-1 Y341 gave less appreciable differences between G1, S, G2, and mitosis (Figure 4.4). Since p-EGFR and p-ERK1/2 appear to be less phosphorylated during S-phase, these results appear contradictory.

4.4.2 Mitotic Synchronization

Next, to explore EGFR signaling during mitosis, I synchronized cells into prometaphase, metaphase, and anaphase/telophase. In order to determine the efficiency of cell synchronization for the mitotic subphases, I first used IF microscopy. I counted 500 cells per slide for each synchronization condition and determined the efficiency of synchronization by characterizing cells as either in interphase, prophase, prometaphase, metaphase, anaphase, telophase, or cytokinesis, based on chromosome morphology and overall cell morphology (Figure 4.5A&B). The prometaphase synchronization procedure yielded 58% of cells in prometaphase, with the rest of cells mostly in interphase. The metaphase synchronization procedure yielded 42% of cells in metaphase, 12% of cells in prometaphase, and the rest mostly in interphase. Finally, the

anaphase/telophase synchronization procedure yielded many cells in anaphase/telophase, however, I have not yet quantified the number. Regardless, cells were either treated or not treated with EGF and collected using the mitotic shake-off method, which leaves interphase cells behind, and were subjected to Western blots.

Western blots showed that EGFR can be phosphorylated by EGF stimulation in artificially arrested prometaphase, metaphase, and anaphase/telophase (Figure 4.5C). ERK however, was never phosphorylated in response to EGF stimulation in any of the mitotic phases (Figure 4.5C).

Although ERK is not activated during the subphases of mitosis, we had previously seen that some mitotic EGFR pathways, along with EGFR itself, were still activated. Interestingly, EGFR endocytosis was previously shown to occur at different rates during different phases of mitosis. I attempted to use live imaging to see whether EGF stimulation of cells at different phases of mitosis caused any observable physiological effects in terms of time for progression through mitosis, spindle assembly, or cell morphology. Unfortunately, cells grown under our live imaging microscope's set-up would not allow them to progress normally through mitosis, despite numerous attempts at optimization (data not shown).

4.4.3 Imaging Flow Cytometry for EGFR Pathway Characterization Throughout the Cell Cycle

The methods discussed above for both interphase and mitotic subphase synchronization are heavily reliant on pharmacological agents, which raises the question as to whether the pharmacological agents used may react with EGFR pathways. In order to perform these

experiments without the use of pharmacological synchronization agents, I chose to use imaging flow cytometry.

Imaging flow cytometry combines both flow cytometry and immunofluorescence microscopy. For every event that is read quantitatively by conventional flow cytometry, a picture is also taken of the cell with all of the qualitative information that can be extracted from traditional IF microscopy. Combining this information together for analysis can provide high-throughput qualitative and quantitative data.

For my purposes, imaging flow cytometry can be used to examine EGFR signaling of a specific cell cycle phase from a population of asynchronized cells (ie. without the use of pharmacological synchronization agents) growing in a dish. A dish of asynchronized HeLa cells at approximately 70% confluency, based on flow cytometric readings of the cells' DNA content, are made up of G0/G1 cells at ~50%, S-phase cells at ~37%, and G2/M cells at ~13% (Figure 4.6). Therefore, each dish of asynchronized samples contains many cells from each cell cycle phase, as we typically collect 10,000 events per sample to obtain statistical significance for analysis. In addition, as an important improvement on conventional flow cytometry, the software can be set up to identify mitotic cells as prometaphase, metaphase, anaphase, or telophase cells based on the aspect ratio/spot count parameters, as described previously [377]. Nuclear aspect ratio (y-axis) measures the aspect ratio (relationship between height and width). If the chromosomes are more spread out (ie prometaphase), it has a higher aspect ratio. If they are more condensed, it has a lower aspect ratio (metaphase). Nuclear spot count (x-axis) measures the number of "big spots." This is used to distinguish ana/telo cells, since they have two spots, compared to one in prometaphase and metaphase. Essentially, based on the IF picture of the cell, the program recognizes the intensity of the chromosomes within an area of the cell. Therefore,

imaging flow cytometry can distinguish between all of the cell cycle phases, and by probing with the appropriate antibody, it can provide quantitative and qualitative data on the expression level or of the localization of specific proteins during each specific cell cycle phase.

I started by using propidium iodide (PI) as a nuclear stain and set up the software to distinguish between G0/G1, S, G2, prometaphase, metaphase, or anaphase/telophase. The parameters set showed a good ability to separate cells based G0/G1, S, and G2/M, as expected from conventional flow cytometry, but also for the mitotic subphases (Figure 4.7). However, when I began to stain with other antibodies, it became apparent that the PI colour had a high bleedthrough into other channels (data not shown). I therefore compared PI with another nuclear stain DRAQ5 and found that DRAQ5 showed minimal bleedthrough into other channels. Using DRAQ5, I was able to successfully co-stain with pEGFR Y1086. I did not have the chance to write the code to distinguish between the mitotic subphases for the DRAQ5 stain, however, I was able to compare the amounts of pEGFR Y1086 between G0/G1, S, and G2/M. In contrast to the results obtained in Section 1.1., this method did not show S-phase EGFR repression (Figure 4.8). Using imaging flow cytometry, the means of FITC-pY1086 intensity increased with the cell cycle, with G0/G1 = 307K, S-phase = 376K, and G2/M = 425K. Although these results are preliminary and still require optimization and repeats, an explanation for the concurrent increase in phosphorylated EGFR with cell cycle progression may be that as the cell grows bigger throughout the cell cycle, it also produces and expresses more EGFR on the cell surface. Other EGFR pathway antibodies should be optimized for the purpose of imaging flow cytometry, with the top priorities being EGFR, p-ERK, and p-AKT. Moreover, the use of the mitotic marker p-Histone 3 Ser10, which distinguishes G2 cells from mitotic cells, can be used as a filter (Figure 4.9).

4.5 Discussion

Here, I performed drug and drug-free assays for EGFR and EGFR-mediated pathways during the cell cycle. The drug-dependent synchronization of cells has the advantage of ensuring cells are specifically at one phase of the cell cycle. It is also useful for obtaining a high yield of the desired cells, and is especially useful or Western blot analysis. However, disadvantages include the potential for drug off-target effects. Furthermore, the purity of the synchronization may still be improved, especially for G2 synchronization. Mitotic cells can be synchronized, however it is not yet possible to synchronize cells in prophase, cytokinesis, and anaphase and telophase separately. The use of imaging flow cytometry removes the limitations of using drugs, and can be used to obtain many replicates of each cell cycle phase at once since many cells are assayed.

For imaging flow cytometry, one of the problems that would need to be overcome would be to find suitable antibodies that properly reflect the phosphorylation of a protein. For example, the global phosphorylation of proteins during mitosis creates many more phosphorylated proteins than during interphase. By Western blot, non-specific phosphorylations can be more easily discounted, based on the size of the protein. However, when visualizing by IF, non-specific staining is much harder to distinguish. For example, by IF, the p-ERK gives a strong signal throughout the cell, even though by Western blot, it is obvious that p-ERK is not present [3,279]. Therefore, the antibodies used would need verification and optimization prior to use.

Another notable possibility for studying EGFR signaling during cell cycle phases would be using the FUCCI (fluorescence ubiquitination cell cycle indicator) system, which expresses

certain fluorescent proteins based on the cell cycle phase [378,379]. This allows the easy indication of the cell cycle by immunofluorescence microscopy. Combining FUCCI with imaging flow cytometry may provide even more convincing quantitative measures of proteins.

The data I obtained for the dampened S-phase response is controversial. EGFR and ERK1/2 were less activated, however, p-CBL and p-PLC-γ1 were activated similar to G1 and G2 cells (Figure 4.3-4.4). Moreover, imaging flow cytometry did not reveal differences in p-EGFR staining between cell cycle phases. However, if S-phase EGF-induced EGFR activation is in fact inhibited, the next step would be to find out why. For example, the cell may need to prevent the overactivation of transcription factors, which could collide with DNA replication machineries [380]. Specifically inducing a wave of transcription during S-phase and examining the faithfulness of DNA replication would be a potential future experiment to test this.

4.6 Figures

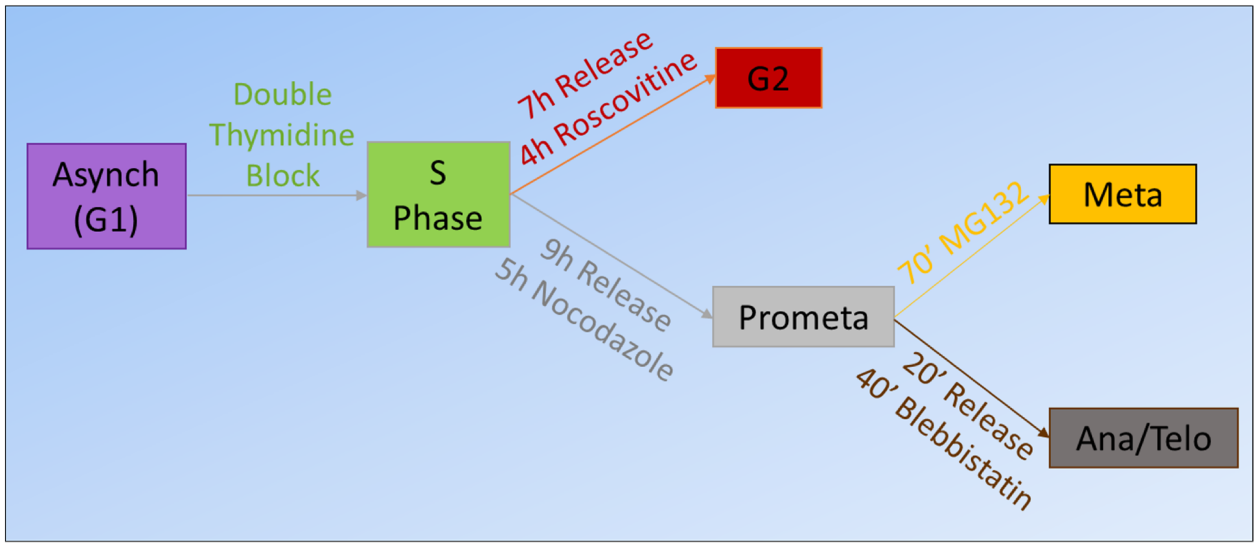


Figure 4.1. Cell synchronization protocols. G1 cells were obtained by serum starvation. S-phase cells were obtained by the often-used and highly efficient double thymidine block. G2 cells were obtained by double thymidine block followed by subsequent CDK inhibition by roscovitine for 3 hours prior to G2 exit. For the mitotic subphases, cells were first synchronized in prometaphase. Prometaphase cells were synchronized first by double thymidine block, released back into the cell cycle for 9h, and nocodazole block for 5h. Metaphase cells were obtained by following the prometaphase synchronization protocol, but adding a release from nocodazole into MG132-containing (a proteosome inhibitor) medium for 20 minutes. Anaphase and telophase cells were obtained again by following the prometaphase synchronization protocol, but released into serum free media for 20 minutes, followed by blebbistatin (myosin inhibitor) treatment for 20 or 50 minutes respectively. Determination of the efficiency of the synchronizations was performed by both flow cytometry and immunofluorescence (IF) microscopy where appropriate.

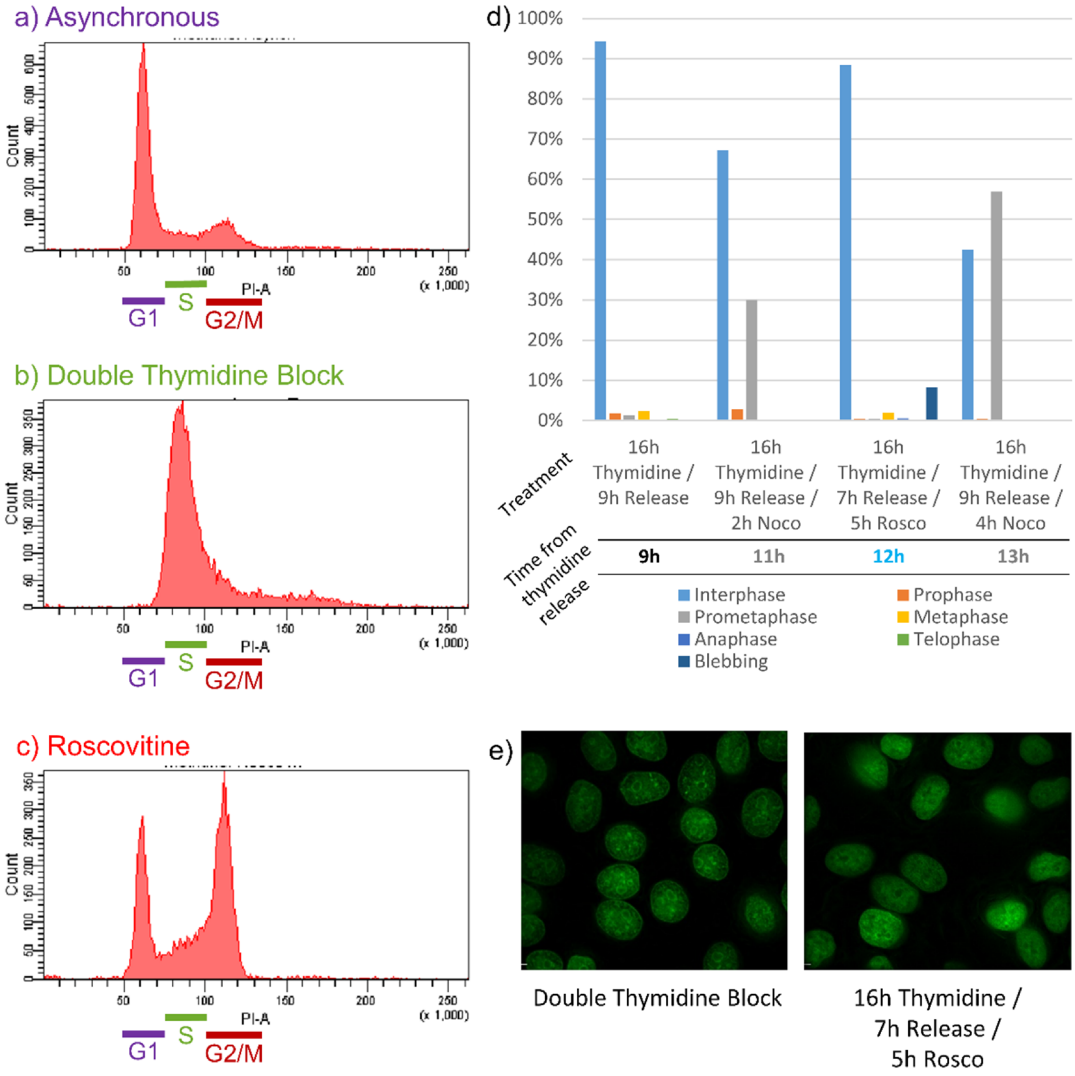
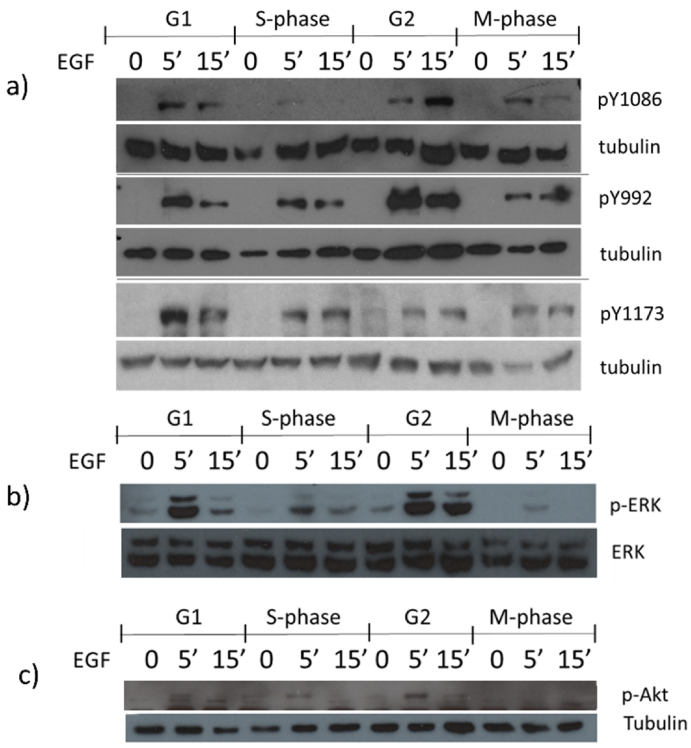
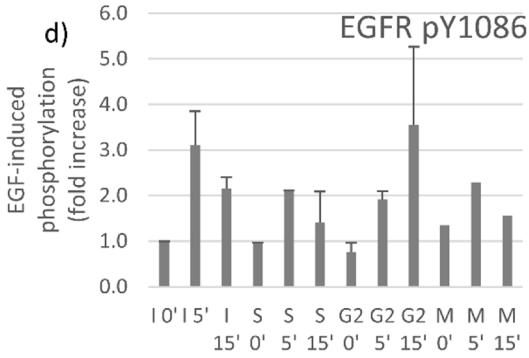
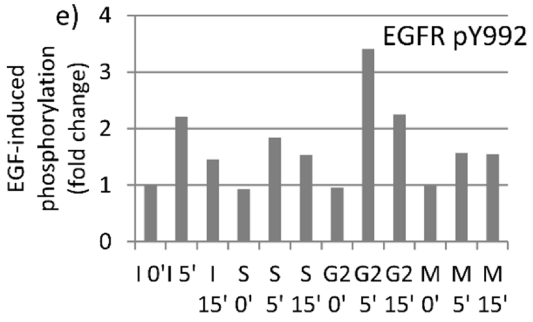
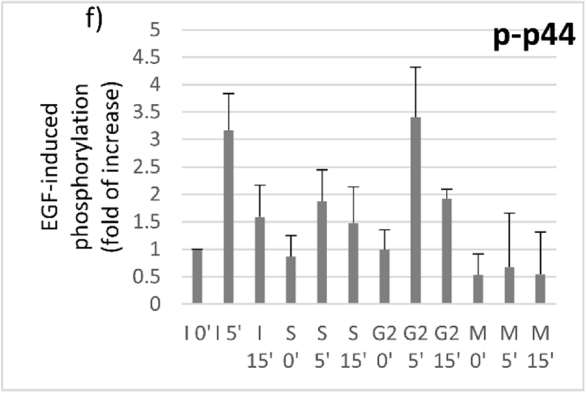


Figure 4.2. Cell cycle analysis of interphase-synchronized HeLa cells. Flow cytometry revealed the methods for synchronizing cells into G1 (no treatment) (a), S (b), or G2 (c) resulted in significant enrichment of cells in each phase. Nuclei were stained with Propidium Iodide (PI). (d) Quantification of cells by IF reveals roscovitine treatment is effective at preventing cells from entering mitosis. (e) Representative IF pictures of HeLa H2B-GFP cells synchronized by into S or G2.


a)
G1
S-phase
G2
M-phase
EGF 0 5′ 15′ 0 5′ 15′ 0 5′ 15′ 0 5′ 15′
pY1086
tubulin
pY992
tubulin
pY1173
tubulin
b)
G1
S-phase
G2
M-phase
EGF 0 5′ 15′ 0 5′ 15′ 0 5′ 15′ 0 5′ 15′
p-ERK
ERK
c)
G1
S-phase
G2
M-phase
EGF 0 5′ 15′ 0 5′ 15′ 0 5′ 15′ 0 5′ 15′
p-Akt
Tubulin


d)
EGFR pY1086
EGF-induced phosphorylation (fold increase)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
I 0' I 5' I 15' S 0' S 5' S 15' G2 0' G2 5' G2 15' M 0' M 5' M 15'


e)
EGFR pY992
EGF-induced phosphorylation (fold change)
4
3
2
1
0
I 0' I 5' I 15' S 0' S 5' S 15' G2 0' G2 5' G2 15' M 0' M 5' M 15'


f)
p-p44
EGF-induced phosphorylation (fold of increase)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
I 0' I 5' I 15' S 0' S 5' S 15' G2 0' G2 5' G2 15' M 0' M 5' M 15'

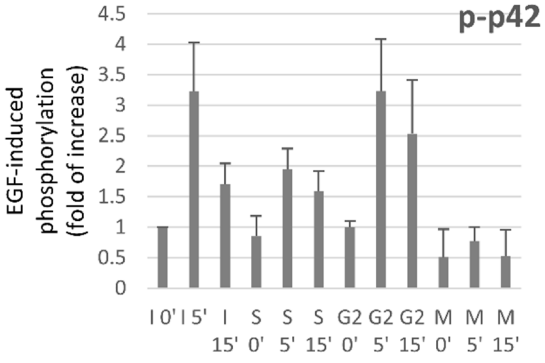

p-p42
EGF-induced phosphorylation (fold of increase)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
I 0' I 5' I 15' S 0' S 5' S 15' G2 0' G2 5' G2 15' M 0' M 5' M 15'

Figure 4.3. Western blotting of EGFR, ERK, and AKT during G1, S, G2, and M. The activation of (a) EGFR, (b) ERK, and (c) AKT for HeLa cells synchronized in G1, S-Phase, G2, and M-phase (prometaphase) was assessed by immunoblotting using phospho-specific antibodies. Cells were treated with EGF (50 ng/mL) for 5 or 15 min, or untreated (0 min). Quantification of (a) (n = 3) and (b) (n = 3) are shown in **(d)-(f)**.

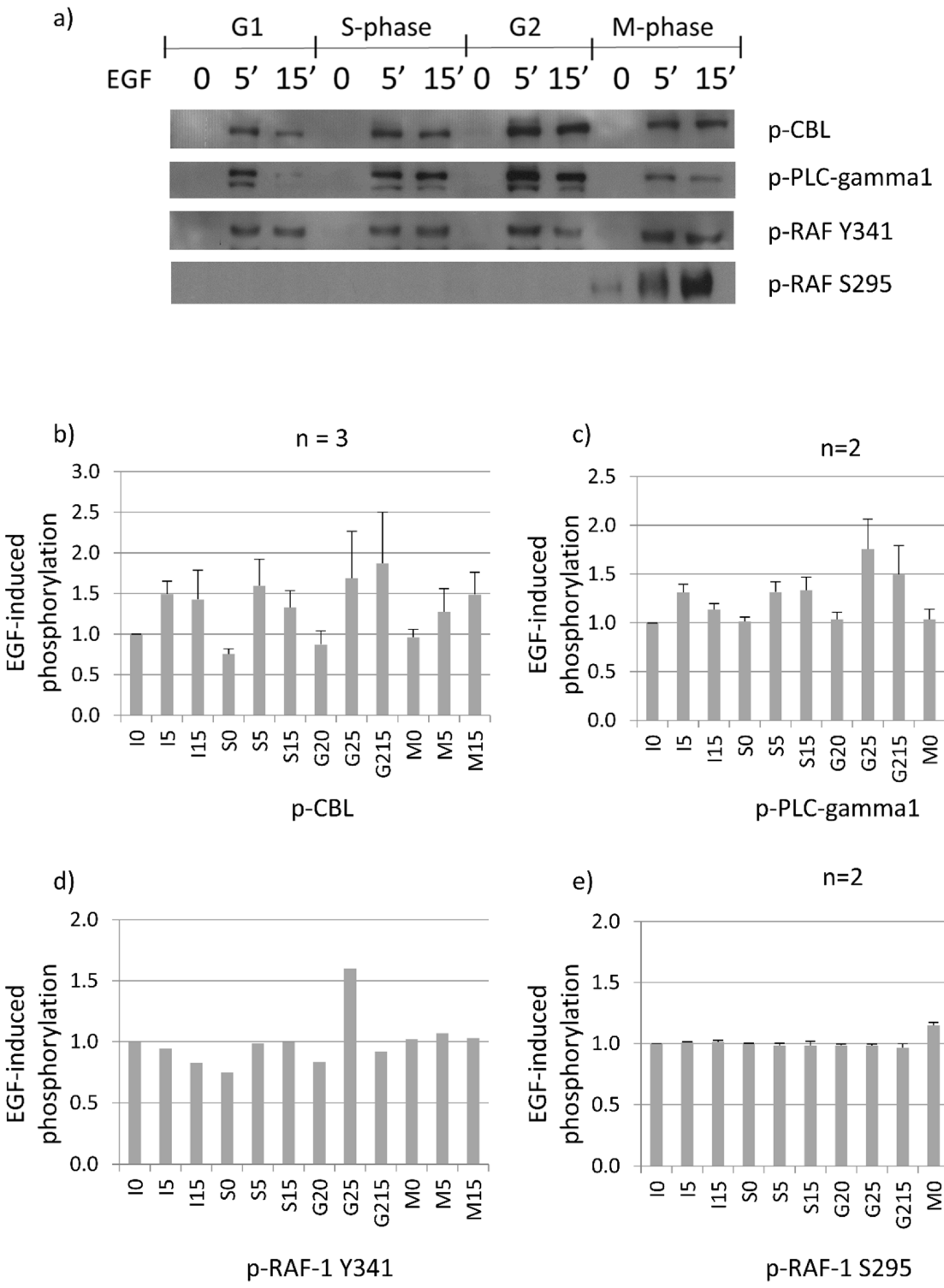
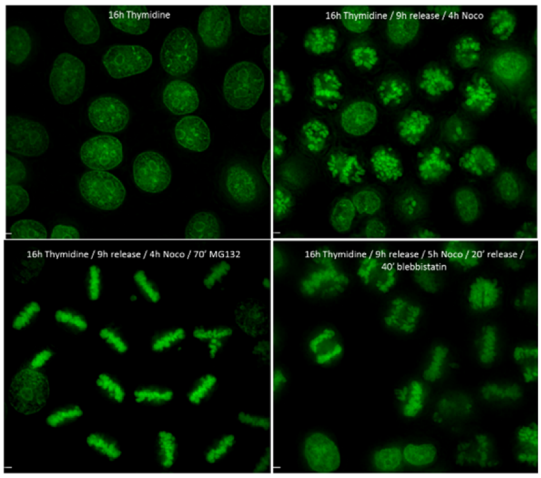
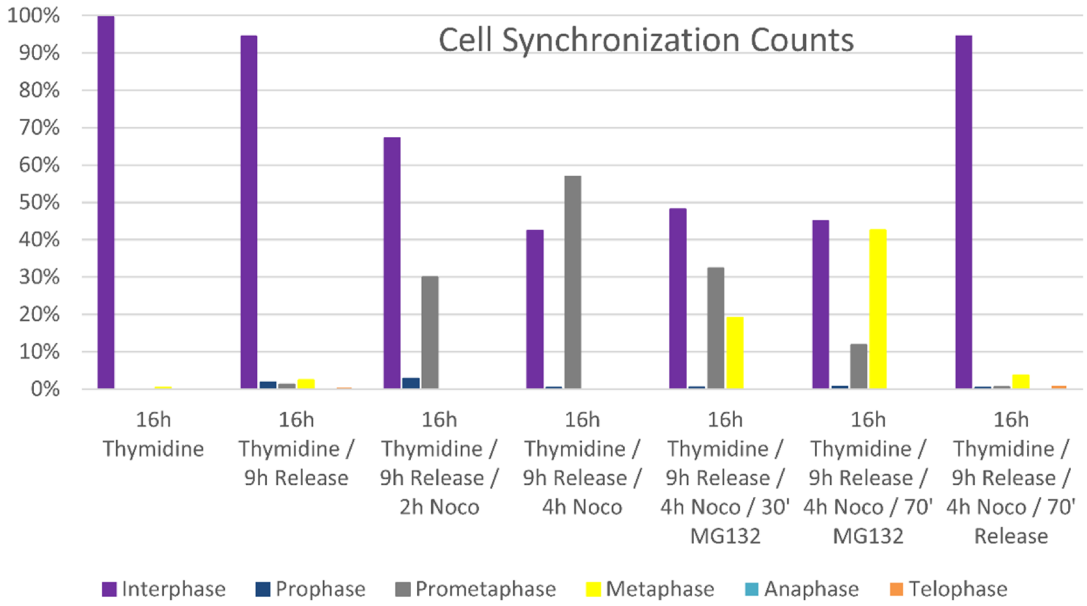

a)
G1
S-phase
G2
M-phase
EGF 0 5' 15' 0 5' 15' 0 5' 15' 0 5' 15'
p-CBL
p-PLC-gamma1
p-RAF Y341
p-RAF S295
b)
n = 3
EGF-induced phosphorylation
3.0
2.5
2.0
1.5
1.0
0.5
0.0
I0 I5 I15 S0 S5 S15 G20 G25 G215 M0 M5 M15
p-CBL
c)
n=2
EGF-induced phosphorylation
2.5
2.0
1.5
1.0
0.5
0.0
I0 I5 I15 S0 S5 S15 G20 G25 G215 M0 M5 M15
p-PLC-gamma1
d)
EGF-induced phosphorylation
2.0
1.5
1.0
0.5
0.0
I0 I5 I15 S0 S5 S15 G20 G25 G215 M0 M5 M15
p-RAF-1 Y341
e)
n=2
EGF-induced phosphorylation
2.0
1.5
1.0
0.5
0.0
I0 I5 I15 S0 S5 S15 G20 G25 G215 M0 M5 M15
p-RAF-1 S295

Figure 4.4. Western blotting of other EGFR-mediated proteins during G1, S, G2, and M. The activation of (a) p-CBL, p-PLC-gamma1, p-RAF Y341, and p-Raf S259 for HeLa cells synchronized in G1, S-Phase, G2, and M-phase (prometaphase) was assessed by immunoblotting using phospho-specific antibodies. Cells were treated with EGF (50 ng/mL) for 5 or 15 min, or untreated (0 min). Quantification of (a) are shown in (b)-(e).

a)


16h Thymidine
16h Thymidine / 9h release / 4h Noco
16h Thymidine / 9h release / 4h Noco / 70' MG132
16h Thymidine / 9h release / 5h Noco / 20' release / 40' blebbistatin

b)


Cell Synchronization Counts
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
16h Thymidine
16h Thymidine / 9h Release
16h Thymidine / 9h Release / 2h Noco
16h Thymidine / 9h Release / 4h Noco
16h Thymidine / 9h Release / 4h Noco / 30' MG132
16h Thymidine / 9h Release / 4h Noco / 70' MG132
16h Thymidine / 9h Release / 4h Noco / 70' Release
Interphase
Prophase
Prometaphase
Metaphase
Anaphase
Telophase

c)

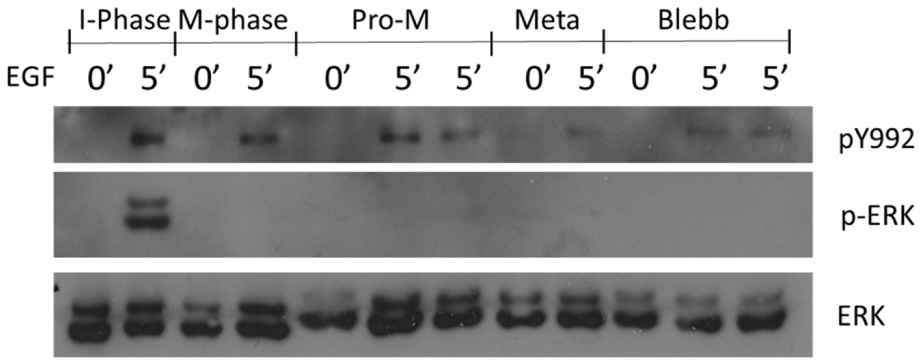
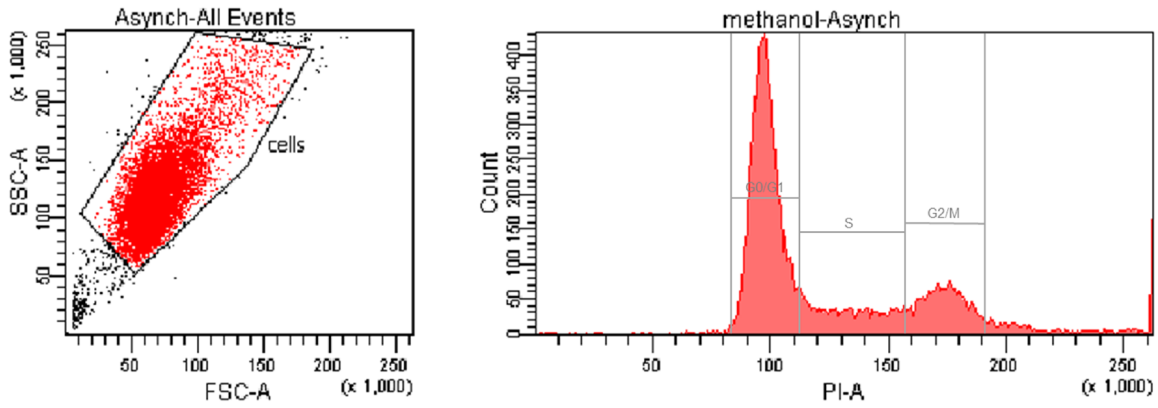

I-Phase
M-phase
Pro-M
Meta
Blebb
EGF
0’ 5’ 0’ 5’ 0’ 5’ 5’ 0’ 5’ 0’ 5’ 5’
pY992
p-ERK
ERK

Figure 4.5. Synchronization of cells in the phases of mitosis. (a) HeLa H2B-GFP cells were synchronized in prometaphase, metaphase, and anaphase/telophase using the described techniques and confirmed by immunofluorescence microscopy. (b) Quantification of cells from synchronization. 500 cells were counted in each condition and classified based on chromosome morphology. (c) EGFR and ERK activation during various phases of the cell cycle were assessed by immunoblotting using phospho-specific antibodies.



G0/G1 Cells	50%
S Cells	37%
G2/M Cells	13%

Figure 4.6. Figure 6. Flow cytometry assessment of asynchronous culture of HeLa cells. G0/G1 cells are present at ~50%, S-phase cells at ~37%, and G2/M cells at ~13%. Cells were labeled with propidium iodide.

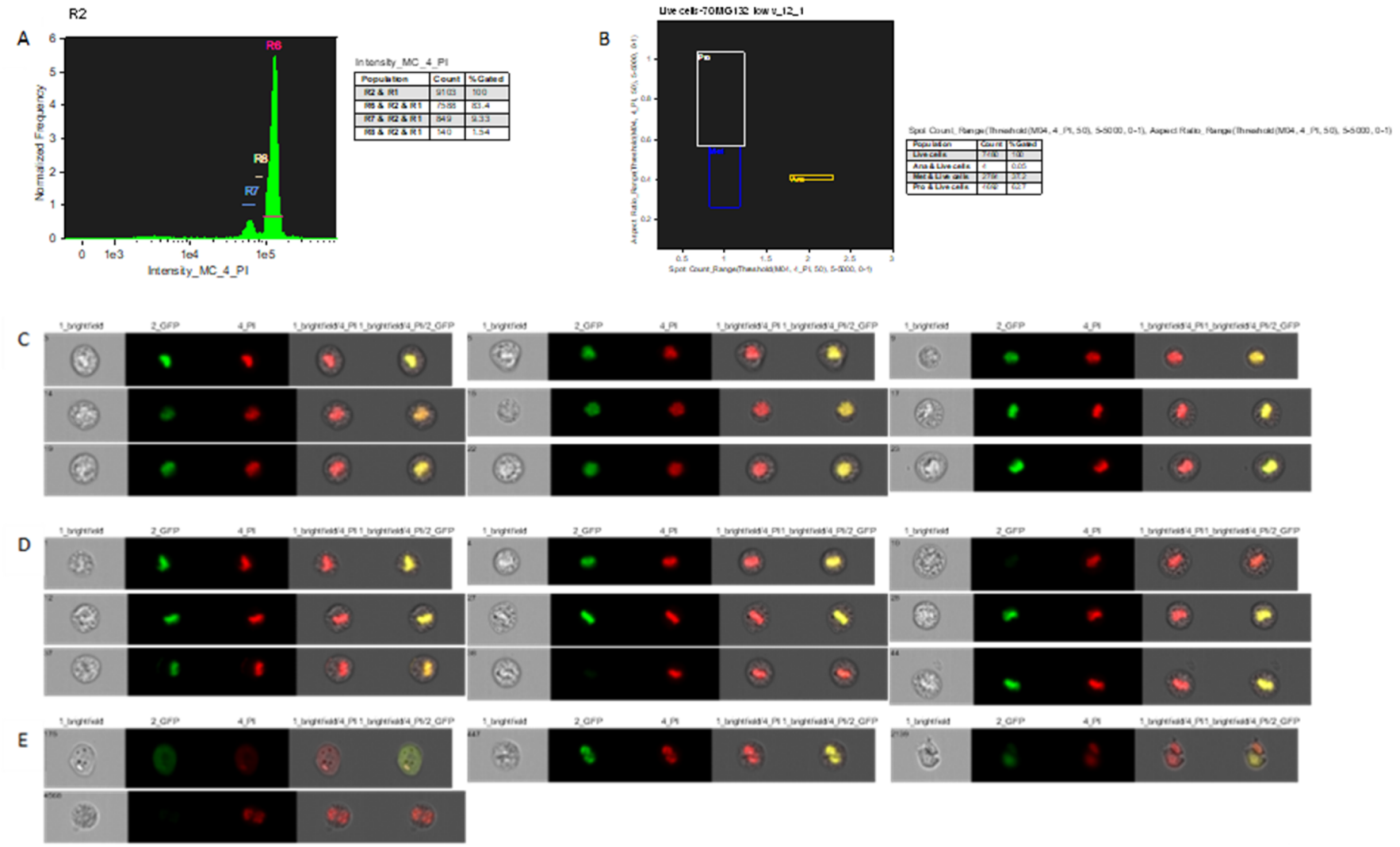


Figure 4.7. Example of imaging flow cytometry results. Cells were synchronized to metaphase (see Figure 4.1). (a) Profile of cells based on DNA content. (b) Spot count and Aspect Ratio parameters set to identify prometaphase, metaphase, or anaphase/telophase cells. (c) Prometaphase cells automatically retrieved from *White Pro* area in (b). (d) Metaphase cells automatically retrieved from *Blue Met* area in (b). (e) Anaphase/ telophase cells automatically retrieved from *Gold Ana* area in (b).

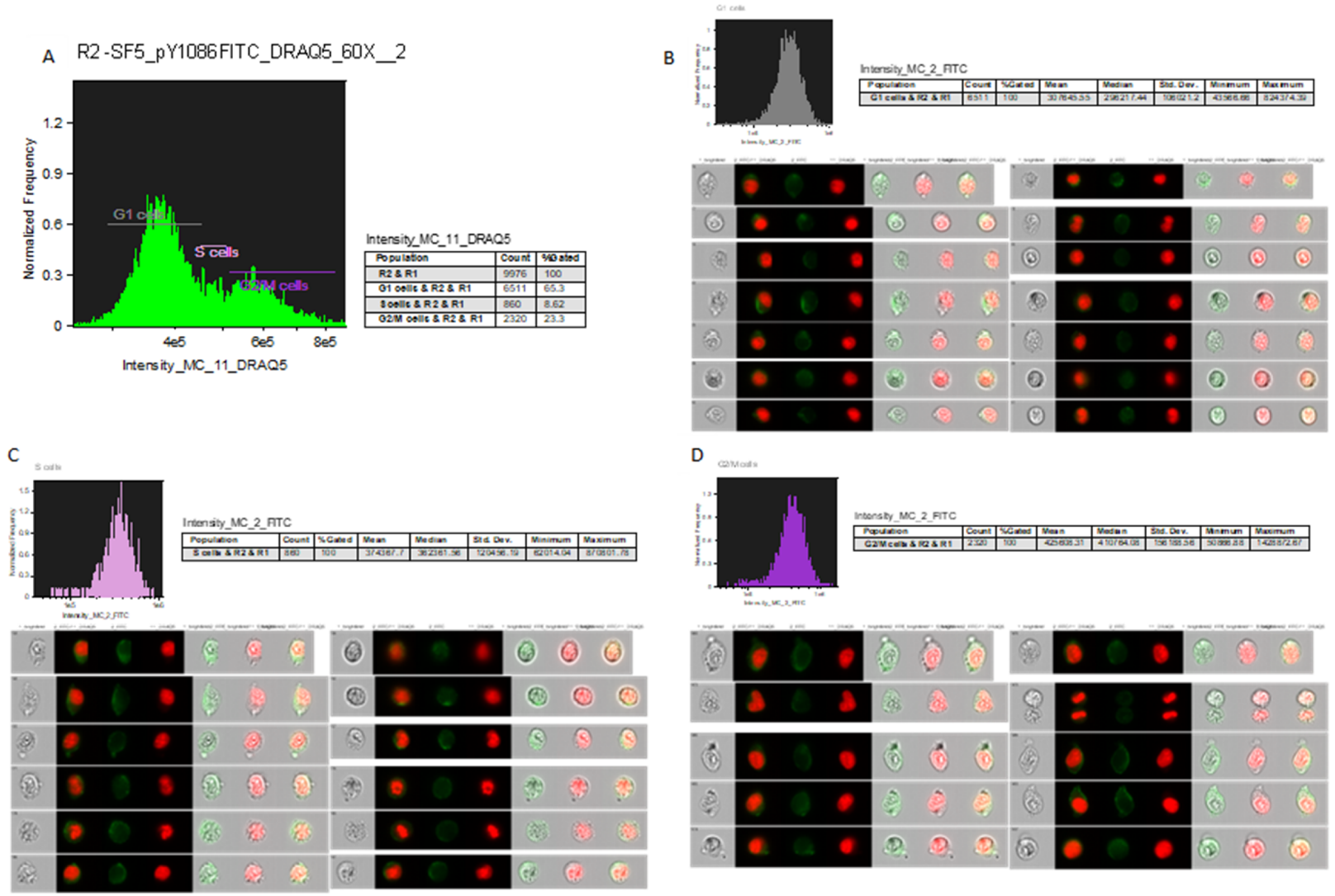


Figure 4.8. Imaging flow cytometry of HeLa cells stained for pEGFR Y1086. Cells were asynchronous and stained for pEGFR Y1086 (FITC-green) and DNA (PI-red). (a) Profile of cells based on DNA content. (b) Intensity of FITC-pEGFR in G0/G1 cells. (c) Intensity of FITC-pEGFR in S cells. (d) Intensity of FITC-pEGFR in G2/M cells.

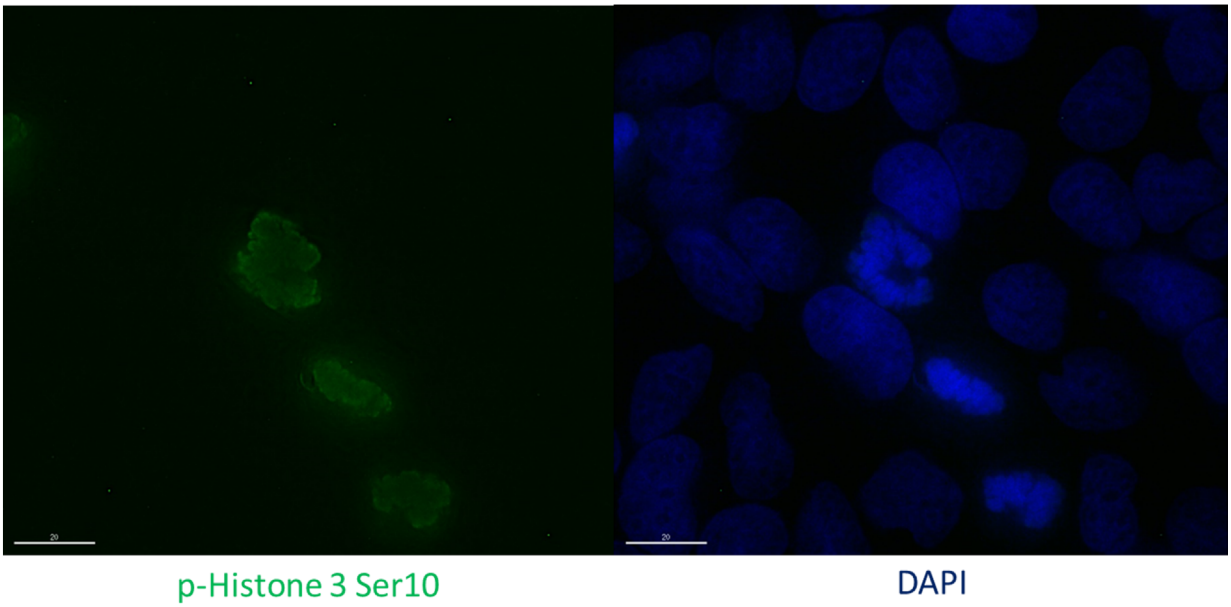


Figure 4.9. p-Histone 3 Ser10 as a marker for mitotic cell. p-Histone 3 Ser10 antibody can be used in imaging flow cytometry to rapidly distinguish between G2 cells and mitotic cells.

5 Chapter 5

Discussion and Future Directions

5.1 Significance

This thesis mainly focused on characterizing the differences in EGFR signaling and endocytosis between mitotic and interphase cells. EGFR signaling during mitosis was found to specifically activate some proteins and pathways, while avoiding others. For example, ERK1/2 and AKT1 were not activated by EGF stimulation during mitosis. However, PLC-γ1, RAS, SRC, AKT2, PI3K, and CBL were all activated. I also found that mitotic EGFR endocytosis proceeds exclusively through NCE, unlike interphase cells where CME is the first line of response to EGF stimulation. Lastly, I established methods for examining EGFR signaling and endocytosis during cell cycle phases.

This sustainment of chronic proliferation is arguably the most fundamental trait of cancer cells [299]. Normal cells carefully regulate their entry and progression into the cell cycle through their compliance with checkpoints in the cell cycle. Cancer cells however often deregulate cell cycle control signaling pathways, so to continuously progress through the cell cycle. Overactive EGFR signaling or that of molecules in its signaling network are well known mechanisms by which cancer cells aberrantly propagate mitogenic signaling. The majority of EGFR signaling has been studied in the context of the G1 phase, as these signaling networks are well known for inducing entry into the cell cycle. However, the activities of the EGFR and its signaling network often show dramatic changes in function throughout the cell cycle, and these changes have not been well characterized. My work here helps us grasp a fuller picture of the EGFR throughout the cell cycle. It helps understand how the activation of the EGFR during each cell cycle phase can affect the cell, and conversely how the conditions of each cell cycle phase affect the output of EGFR signaling. Aside from characterizing these changes, this work could potentially be exploited for informing pharmacological choices.

5.1.1 Pharmacological Benefits

After better elucidating the functional significance, whether we can exploit these differences for pharmacological benefits should follow. Several routes are possible to achieve this.

There are many approved and pending pharmacological targets within the EGFR signaling pathway. For example, EGFR, ERBB2, RAF, MEK1/2, ERK1/2, PI3K, AKT1/2, mTOR, JAK2, and PKC are all drug targets [381,382]. In situations of overactive EGFR, the combination of EGFR inhibitors with inhibitors of the above proteins has been suggested, with special interest in the PI3K-AKT-mTOR arm and MEK-ERK arm of signaling [383]. For example, the combination of PI3K inhibitor with EGFR TKIs better restores drug sensitivity to resistant NSLCL cells [384,385]. The differential activation of these pathways would need to be considered for mitotic cells that make up the tumor at any time. For example, according to our research, MEK or ERK inhibitors would do little to increase the cell death of mitotic cells in the context of MEK and ERK signaling, since these proteins are not even activated by EGFR during mitosis [3,279]. For AKT inhibition, the precise role of the AKT isoforms would first need to be explored before understanding the effects of AKT inhibition of mitotic cells with overactive EGFR. However, since we know that AKT2, and not AKT1 is activated during mitosis, we can imagine that the combination of AKT2 inhibition will be more effective for mitotic cells, and will make a more specific treatment, especially with further combinations with mitotic inhibitors. Therefore, this knowledge can help us inform drug treatments.

Another avenue that the work in this thesis could improve pharmacological targeting is that since we found that mitotic cells internalize EGFR exclusively through NCE, this fact could be used to better deliver drugs into the cell. EGFR endocytosis has been hypothesized as a mechanism of drug deliver. For example, targeting overexpressed EGFR with drug-conjugated EGFR monoclonal antibodies creates a more specific target than the drug along. CME, the predominant form of EGFR internalization during interphase, usually results in the recycling of EGFR back to the PM. Through this pathway, the internalization and retainment of the drug is more difficult. However, during mitosis, NCE exclusively internalizes and retains the internalized agent. In addition, the targeting of internalized drugs to lysosome degradation is a desirable event [332]. Therefore, the combination of an anti-mitotic drug together with a drug-conjugated EGFR monoclonal antibody may hold promise.

5.2 Future Directions

My research has contributed to the characterization of the signaling and endocytosis of the EGFR during mitosis in higher depth than previously. Next would be to fully understand the functional relevance of all of the differences I have observed between mitosis and interphase. My MTT assay has shown that EGFR signaling during mitosis can contribute to higher cell survival from mitotic arrest-mediated cell death. Precisely how this occurs would need to be investigated. For example, it has been shown that following mitosis, some daughter cells can immediately re-enter the cell cycle while others are quiescent. This phenomenon was explained to be due to the daughter cell's "memory" of mitogenic signals in the mother cell, in that any Cyclin D1 mRNA induced by mitogens throughout the cell cycle could be transferred from the mother cell to the daughter cell [386]. Therefore, EGFR signaling during mitosis may make cells more primed to begin cell proliferation in the subsequent G1.

The functional significance of mitotic EGFR signaling and endocytosis would also need to be shown in animal models, such as in mice, however, technical complexities to study mitotic cells in animals would need to be addressed. For example, what cells would be studied? How would EGF be delivered? How would we specifically study mitotic cells in these animals? There are indeed many solutions to these questions, however, intensive trial-and-error would likely be needed prior to using mice as a model study system.

In Chapter 2, we saw that many proteins, such as MEK1/2, ERK1/2, and AKT1, could not be activated by EGF stimulation during mitosis. Evolutionarily, the cell must have found it necessary to prevent their mitotic activation. An interesting future avenue would be to observe the effects of constitutively activating these proteins during mitosis. This improper activation may lead to some pro-oncogenic functions during mitosis that have not been studied.

This thesis explored a wide variety of pathways, and as such, many future directions are possible. The following sections describe some specific future directions, with each hypothesis arising from the data generated in this thesis.

5.2.1 ERMs

The plasma membrane lipids have been shown to be important in the proper completion of mitosis [387,388]. The activation of PI3K and PLC-γ1 by EGFR during mitosis therefore present interesting aspects to explore. Both proteins alter PM phosphoinositides by depleting the pool of PIP_2: PI3K by phosphorylating it to PIP_3, and PLC-γ1 by hydrolysis to IP_3 and DAG. Therefore, the EGFR may be able to modulate some important mitotic physiological or signaling functions. One family of proteins that is dependent on PIP_2 for proper localization to the cell cortex to function are the ERMs (ezrin, radixin, moesin) [389]. Interestingly, ERM activity has

been shown to be required for proper mitotic cell rounding (MCR), by providing the mitotic cell cortical rigidity [349,390–392] and proper spindle pole orientation, and stability [393]. PLC activity has been shown ERM release from the cell membrane, leading to ERM dephosphorylation and inactivation [394].

A chemical activator of PLC, m-3M3-FBS, was recently shown to stimulate the dephosphorylation of ERM proteins in HeLa cells [395]. Since EGF stimulation appears to activate PLC-γ1 during mitosis, it may be possible that active PLC-γ1 could hydrolyze and deplete PIP_2 levels in the plasma membrane of mitotic cells, and that without PIP_2 at the plasma membrane, the ERMs would be unable to localize to the cell cortex to perform their important MCR actions. Conceivably, EGF activation of PLC-γ1 during mitosis might actually cause defects in mitotic cell rounding, which would seem to defy conventional logic for the role of the EGFR in cancer cells.

To test this theory, I've performed a few preliminary experiments, I treated interphase and mitotic cells with or without EGF, and with or without the PLC-γ1 inhibitor U71322, and performed a Western blot, blotting with an anti-p-ERM antibody (Figure 5.1A). In nocodazole-arrested mitotic cells without U71322, EGF stimulation appeared to cause ERM dephosphorylation. However, in the presence of U71322, the ERMs remained phosphorylated. This suggested that PLC-γ1 may indeed dephosphorylate ERM proteins during mitosis. In order to examine whether ERM proteins became delocalized from the cell membrane following EGF treatment, I observed cells stained with anti-p-ERM using indirect microscopy. EGF treatment of mitotic cells did not exhibit discernible cytoplasmic ERM proteins by IF, however, the sharpness of the anti-p-ERM stain in their cell membrane appeared to decrease following 5 minute treatment of either EGF or m-3M3-FBS (Figure 5.1B).

This preliminary data shows that PLC-γ1 activity over the ERMs during mitosis has the potential to affect MCR. In the same vein, PI3K activity may also affect ERM localization. ERM localization during mitosis may be alternatively examined by subcellular fractionation. Physiologically, the effect of mis-localized ERM by EGF, PLC-γ1, or PI3K during mitosis could also be explored by observing the mitotic spindle, assaying for mitotic rigidity, or the height of mitotic cells.

Aside from the ERM proteins, PLC-γ1's canonical signaling pathway operates through the formation of IP3 and DAG, which subsequently activate PKC (protein kinase C), an important family of kinases. Future studies could focus on characterizing IP3 and DAG release resulting from EGF treatment in mitotic cells, as well as PKC activation. Whether PLC-γ1 participates in increasing survival of mitotic cells through this pathway may also be studied. In addition, the intracellular calcium release initiated by IP3 may also be studied. Calcium has recently been shown to help mediate a type of EGFR NCE [329]. Whether this is related to the EGFR NCE we studied in Chapter 3 would be interesting to study.

5.2.2 RhoA

RhoA has been shown to be another important regulators of mitotic cell rounding (MCR) [396–398]. It has been show that EGF can upregulate RhoA through the activation of ERK [399–401]. I have shown that EGF does not activate ERK during mitosis, therefore, this may be a mechanism to avoid aberrant RhoA overactivation during mitosis.

In order to test this hypothesis, an experiment for RhoA activation during mitosis following EGF treatment using a RhoA activation assay could be performed, for example by Rhotekin GST-pulldown assay [402] as used previously in our lab. A next step could be to

express a constitutively active ERK or MEK, and to see whether mitotic RhoA will be deactivated and whether it causes consequences to mitotic cell rounding.

RhoA is also a negative regulator of EGFR CME. Activation of the RhoA effector ROCK phosphorylates endophilin A1 at Thr14 [403]. Endophilin A1 makes up the EGFR-CBL-CIN85 complex that helps form the clathrin coat [404]. Phosphorylation of endophilin A1 prevents its recruitment to the complex and reduces the level of EGFR endocytosis [403]. Since we found that mitotic CME is inhibited, the constant activation of RhoA during mitosis may conceivably play a part in the inhibition.

5.2.3 CDK Inhibitors

The CDK inhibitor $p21^{CIP1/WAF1}$ could be of interest in the future, since $p21^{CIP1/WAF1}$ can arrest cells at G1/S, S, and G2/M [405]. For example, during G1, $p21^{CIP1/WAF1}$ inhibits the CYCLIN D-CDK 4/6 complex and E2F, during S-phase inhibits PCNA leading to DNA replication blockade, and during G2/M inhibits the CYCLIN B1-CDK2 complex [405]. EGFR pathway members RAS, ERK, and AKT can deactivate p21 function [406], meaning activated EGFR could potentially lift $p21^{CIP1/WAF1}$ repression at any phase of the cell cycle. This would mean an expanded role to the EGFR's contribution to tumorigenesis. An experiment to test this would be to induce cell cycle arrest at stage with active $p21^{CIP1/WAF1}$ and seeing whether EGFR activation can lead to cell cycle progression. Activation of $p21^{CIP1/WAF1}$ could be assayed by lack of phosphorylation at Thr145, as this phosphorylation leads to the cytoplasmic localization of $p21^{CIP1/WAF1}$. This activation could also be visualized by $p21^{CIP1/WAF1}$ localization under IF.

5.2.4 Protein Synthesis

During mitosis, the translation of mRNA is repressed by 25-30% compared to interphase, including cap-dependent translation [407,408]. This includes eIF4E-mediated translation. The eIF4E (eukaryotic initiation factor 4) binds to the mRNA and recruits ribosomal assembly components. Interestingly, eIF4E is indirectly activated by both ERK1/2 and AKT activity, through the activation of MNK1 and the inactivation of 4E-BP1 respectively [409,410]. I showed that AKT2 is activated by EGF stimulation of cells during mitosis. Whether this is sufficient to activate eIF4E-mediated protein synthesis could be explored. In addition, whether constitutively active ERK1/2 or AKT1 activity could cause improper activation of eIF4E, and whether there are consequences to the cell could be examined. For example, new protein synthesis of mitotically-arrested cells could be a mechanism by which cells undergo mitotic slippage. Proteins downstream of ERK1/2 and AKT, such as the aforementioned MNK1 and 4E-BP1 proteins could be studied.

Furthermore, we did not study the effects of EGF stimulation on mTOR activation during mitosis. The mTOR arm of signaling is one of the most important downstream pathways of AKT and RAS. The mTOR complex receives stimulatory signals from growth factors, as well as from nutrient inputs through amino acids, glucose, and oxygen availability, and regulates cell growth and autophagy [117]. mTOR has been shown to initiate the translation of specific mRNAs, translation elongation, and ribosome biogenesis [411]. Due to the complexity of inputs that affect mTOR activity, we did not study it in Chapter 2 since the use of nocodazole could potentially impact mTOR signaling. However, by imaging flow cytometry, it will be possible to better study mTOR, as drug synchronization would not be required and the growth conditions of cells in different cell cycle phases would be kept constant. The activation of mTOR is usually assayed by

4E-BP1 and S6K phosphorylation, and would be logical next steps to explore. How autophagy is regulated could also be studied.

5.3 Figures

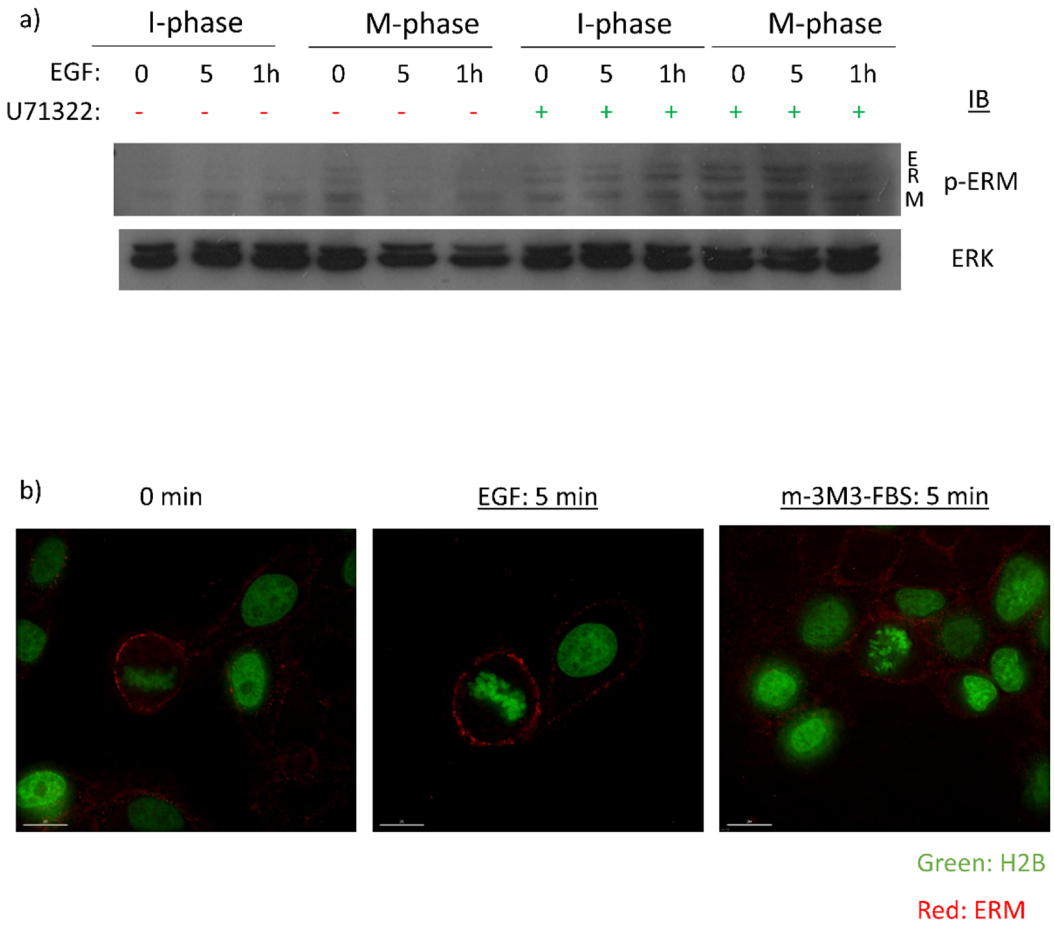


Figure 5.1. Effect of EGF stimulation and PLC-γ1 activation on ERM proteins. A p-ERM Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (41A3) rabbit monoclonal antibody (Cell Signaling, cat: #3149) was used for detection (gift from Dr. Sarah Hughes). (a) EGF-induced ERM dephosphorylation in mitosis. Interphase and nocodazole-arrested mitotic HeLa H2B-GFP cells were stimulated with EGF for the indicated times, with or without U71322, a PLC inhibitor. (b) ERM localization was visualized by indirect IF.

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