Molecular Characterization of the Tumor and Metastasis Suppressor Activity of Plakoglobin in Mutant p53 Expressing Carcinoma Cells

by

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Abstract

Plakoglobin (γ-catenin) is an Armadillo protein family member and a paralog of β-catenin with similar dual cell-cell adhesion and signaling activities. These proteins interact with cadherins at the membrane and mediate cell-cell adhesion. In the cytoplasm, they interact with an array of cellular protein partners to regulate signaling pathways involved in tumorigenesis and metastasis. Recently, our laboratory identified p53 as one of the interacting partners of plakoglobin. p53 is a tumor suppressor and transcription factor that in response to various stress signals activates physiological pathways that regulate cell cycle arrest, DNA repair and apoptosis. More than half of all cancers harbor a mutant form of p53. In addition to the loss of tumor suppressor activity, a number of most frequent mutant p53 proteins acquire oncogenic properties and are known as gain of function mutants.

Here, we first assessed the *in vitro* tumor and metastasis suppressive functions of plakoglobin in high-grade ovarian serous carcinoma cell lines expressing wild type or mutant p53 proteins with different adhesion profiles. We showed that plakoglobin-deficient ovarian cancer cells that express N-cadherin and mutant p53 were highly migratory and invasive, whereas those that express mutant p53 and plakoglobin were not. Exogenously expressed plakoglobin colocalized with cadherins in adhesion complexes, interacted with wild type and mutant p53 proteins and significantly reduced growth, migration and invasion of ovarian cancer cells expressing N-cadherin and mutant p53 *in vitro*.

Next, we mapped the interacting domain of p53 and plakoglobin and showed that p53/plakoglobin interaction was mediated by the DNA binding domain of p53 and

the C-terminal transactivation domain of plakoglobin. We showed that wild type plakoglobin and wild type p53 acted synergistically to significantly reduce *in vitro* growth, migration and invasion of transfectants relative to parental cells. Additionally, the C-terminal of plakoglobin was necessary for its invasion suppressor activity.

We examined the effects of one of the most frequently expressed p53 mutations $p53^{R175H}$ (Arginine 175 to Histidine) on β-catenin accumulation and transcriptional activation and their modifications by plakoglobin co-expression. $p53^{R175H}$ expression in plakoglobin null cells increased total and nuclear levels of β-catenin and its transcriptional activity. Co-expression of plakoglobin in these cells promoted β-catenin's proteasomal degradation, and decreased its nuclear levels and transactivation. Wnt/β-catenin targets, *c-MYC* and *S100A4* were upregulated in $p53^{R175H}$ cells and were downregulated when plakoglobin was co-expressed. The plakoglobin-$p53^{R175H}$ cells also showed significant reduction in their migration and invasion *in vitro*.

Taken together, the experimental evidence from this PhD project strongly suggest that underlying mechanisms for tumor and metastasis suppressor effects of plakoglobin may be its interaction with mutant p53 proteins and down-modulation of β-catenin-TCF axis.

Dedicated

to

My Mother and Father

For their unconditional love and endless support

My grandparents

For being my first teachers

&

My love, Alireza

For his continuous encouragement

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ACTB	Actin beta
Akt	Protein kinase B
APC	Adenomatous polyposis coli
Arm	Armadillo
Bak	BCL2 antagonist/killer 1
Bax	BCL2 associated X, apoptosis regulator
Bcl-2	B-cell lymphoma-2, antiapoptotic
Bcl-XL	B-cell lymphoma-extra large
BID	BH3 interacting domain, proapoptotic
CCND1	Cyclin D1
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CKI	Casein kinase 1
CKII	Casein kinase 2
c-Met	MET proto-oncogene, receptor tyrosine kinase, Hepatocyte growth factor receptor
Co-IP	Co-immunoprecipitation
Cox2	Cyclooxygenase-2
CSK	Cytoskeleton
CT	C-terminal
CTNNB	Catenin (cadherin-associated protein), beta 1

DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DP	Desmoplakin
DSC2	Desmocollins-2
E1b	Adenovirus E1B protein
E2F	E2F transcription factor
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
EMMPRIN	Extracellular matrix metalloproteinase inducer
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
FAK	Focal adhesion kinase
Fas	Fas cell surface death receptor; tumor necrosis factor receptor superfamily member 6
FBS	Fetal bovine serum
GADD45	Growth arrest and DNA damage-inducible
45GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOF	Gain of function
GSK3β	Glycogen synthase kinase 3 beta
HAI-1	Hepatocyte growth factor activator inhibitor type 1
HDAC4	Histone deacetylase 4
Hdm2	Human double minute protein2
HEK293T	Human embryonic kidney cell line

HGS	High grade serous
HGS-OVCA	High grade serous ovarian cancer
IHC	Immunohistochemistry
IOSE	Immortalized ovarian surface epithelium
JUP	Junction plakoglobin
kDa	Kilodalton
Lats1/2	Large tumor suppressor kinase 1/2
LEF	Lymphoid enhancer factor
LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MCF-7	Human breast (mammary gland) cancer cell line; derived from metastatic site: pleural effusion
MDA-MB-231	Human breast (mammary gland) cancer cell line; derived from metastatic site: pleural effusion
MDCK	Madin Darby canine kidney; Canine kidney cell line
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal to epithelial transition
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
mp53	Mutant p53 protein
mRNA	Messenger ribonucleic acid
Mst1/2	Mammalian sterile twenty-like 1/2
MYC	Proto-oncogene C-Myc

NES	Nuclear export signal
NLS	Nuclear localization signal
Nm23	Non-metastatic protein 23
Nm23-H1	NM23 nucleoside diphosphate kinase 1
Nm23-H2	NM23 nucleoside diphosphate kinase 2
NME1	Gene encoding the Nm23 protein
NOXA	Pro-apoptotic protein
NPM	Nucleophosmin
NSCLC	Non-small cell lung carcinoma
NT	N-terminal
OVCA	Ovarian cancer
p21	Cyclin-Dependent Kinase Inhibitor 1A; Cip1
p53	Tumor suppressor p53 protein
PAX	Paired box transcription factor
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PG	Plakoglobin (γ-catenin)
PI3K	Phosphoinositide 3-kinase
PKP	Plakophilin
PML	Promyelocytic leukemia
pRb	Retinoblastoma
PrP(c)	The cellular prion protein

PSK	Penicillin, Streptomycin, Kanamycin
PUMA	p53 upregulated modulator of apoptosis
R175H	Arginine175 to Histidine p53 mutant
Ras	Sarcoma viral oncogene homolog
RT-PCR	Reverse transcriptase polymerase chain reaction
S100A4	S100 Calcium Binding Protein A4
SATB1	Special AT-Rich Sequence Binding Protein 1
SCC9	Tongue squamous carcinoma cell line
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFN	Stratifin; 14-3-3σ
shN-cadherin	Short hairpin RNA against N-cadherin
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Snail1	Snail family transcriptional repressor 1
SOX-4	SRY (Sex Determining Region Y)-Box 4
Src	SRC proto-oncogene, non-receptor tyrosine kinase
SV-40	Simian vacuolating virus 40; simian virus 40t
TA	Transactivation
TAZ	Transcriptional coactivator with PDZ-binding motif
TCF	T-cell factor
TEAD	TEA domain family of transcription factors

THBS1	Thrombospondin 1
TOPFLASH	TCF luciferase reporter plasmid
TP53	Tumor suppressor p53 gene
uPA	Urokinase
WT	Wild type
YAP	Yes-associated protein

Chapter one

Introduction

1.1.Cancer

An estimated 206,200 new cases of cancer will be diagnosed in Canada in 2017 and 80,800 patients will die from the disease [1]. Cancer is not only a life-threatening illness but it imposes a huge financial burden on the health care system. Tumor development is a complex process resulting from an uncontrolled proliferation of cells [2]. Further complication occurs when cancer cells acquire the ability to dissociate from the primary site and spread to the other locations in the body, which is a fatal process called metastasis [3]. Defective cell-cell adhesion is one of the key contributing factors to both initiation and metastatic progression of different types of cancer [4-6].

Carcinomas are the most common type of cancer that originate from epithelial cells. Epithelial tissues cover the surface of the body and line the internal cavities [7]. The structural integrity and normal functions of these tissues is dependent on proper cell-cell adhesion and interactions mediated by adhesive complexes including adherens junctions and desmosomes [8]. Adherens junctions are ubiquitously formed in both epithelial and non-epithelial tissues [9], whereas desmosomes are intercellular adhesive complexes that hold epithelial cells together and impart tensile strength and resilience to non-epithelial cells that endure mechanical stress such as cardiac muscle and meninges [10]. Regardless of their differences, both adherens junctions and desmosomes are cadherin-based adhesion complexes.

Although originally identified as structural proteins with a “glue-like” function, cadherin-based cell adhesion complexes have subsequently been shown to regulate

signaling pathways through their interactions with an array of functionally diverse proteins, including receptor tyrosine kinases and phosphatases, tumor suppressors and transcription factors [11,12]. Consequently, elements of adhesive complexes play important roles in regulating tumor development and metastasis progression.

1.2. Cadherin mediated cell-cell adhesion

Both adherens junctions and desmosomes are cadherin-based junctional complexes. Adherens junctions are formed when the extracellular domain of E-cadherin dimers in one cell interacts with the extracellular domain of cadherin proteins on the neighboring cell [13,14]. E-cadherin interacts with p120-catenin via its juxtamembrane domain, which stabilizes the cadherin dimer at the membrane [13,14]. The extreme cytoplasmic tail of the E-cadherin interacts with β-catenin or plakoglobin in a mutually exclusive manner, which in turn interact with α-catenin, an actin-binding protein [15,16] (Figure 1.1). Recruitment of actin microfilaments by α-catenin stabilizes cadherin-catenin complex at the membrane [13,14]. Similarly, desmosomes are formed when two desmosomal cadherins, desmoglein and desmocollin, form heterodimers that participate in homotypic interactions with desmosomal cadherin dimers on adjacent cells [17]. The cytoplasmic tail of desmosomal cadherins interacts with plakoglobin and plakophilin, which in turn associate with desmoplakin that recruits intermediate filaments to stabilize desmosomes at the membrane [17] (Figure 1.1).

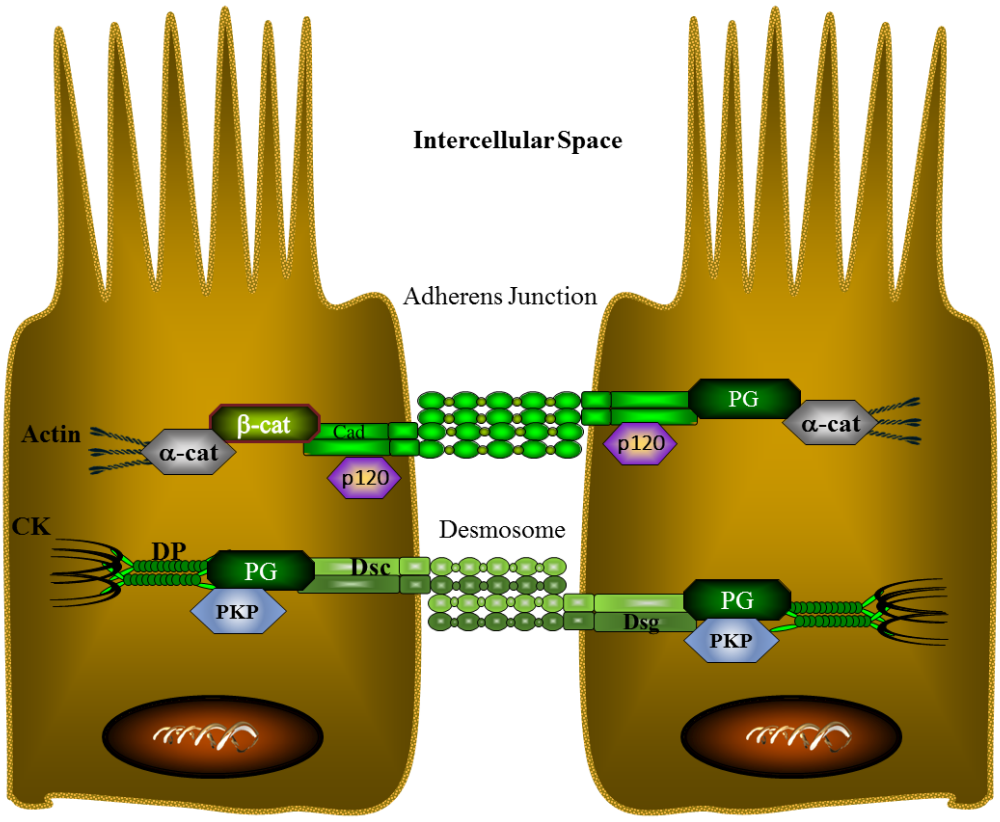

Figure 1.1. Cell junctional complexes in epithelial tissues. Adherens junction and desmosomes are the two main cell-cell adhesion complexes in epithelial cells. At the adherens junction, the extracellular domain of the E-cadherin dimer interacts with E-cadherin proteins on the adjacent cells. E-cadherin interacts with p120-catenin via its juxtamembrane domain whereas it interacts with β-catenin and plakoglobin in a mutually exclusive manner via its extreme cytoplasmic tail. In turn β-catenin and plakoglobin interact with α-catenin that binds to actin microfilaments. At the desmosomes, desmosomal cadherins, desmoglein and desmocollin form a heterodimer. The cytoplasmic tails of the heterodimers interact with plakoglobin and plakophilin, which in turn bind to desmoplakin that recruits the cytokeratin intermediate filaments. α-cat, α-catenin; β-cat, β-catenin; Cad, E-cadherin; CK, cytokeratin; Dsc, desmocollins; Dsg, desmoglein; DP, desmoplakin; p120, p120-catenin; PG, plakoglobin; PKP, plakophilin

Modified from Pasdar, M.

1.3. Plakoglobin (gamma-, γ-catenin)

Plakoglobin is an 83 kDa protein that is encoded by the *JUP* gene located on chromosome 17q21 [18]. Plakoglobin is an Armadillo protein family member and a paralogue of β-catenin with dual adhesive and signaling functions [19,20]. As a cell adhesion protein plakoglobin participates in the formation of adherens junctions and desmosomes [21,22]. Both cytoplasmic and membrane-associated pools of plakoglobin interact with different protein partners to regulate their levels and localization [21,22]. Furthermore, the soluble pool of plakoglobin can interact with other cellular proteins and regulate pathways involved in tumorigenesis and metastasis [21,22]. Plakoglobin also translocates to the nucleus and regulates gene expression [21,22].

1.3.1. Initial discovery of plakoglobin

Plakoglobin was first identified in the membrane-associated desmosomal plaques [23]. Further investigations showed that this protein was a cytoplasmic component of both desmosomes and adherens junctions [18,23]. Subsequent co-immunoprecipitation studies validated its role as an adhesion protein that associated with the desmosomal cadherin desmoglein [24]. Further studies demonstrated that plakoglobin had a cytoskeleton associated pool that interacted with desmoglein and E-cadherin, as well as a cadherin-independent cytoplasmic pool. While the insoluble pool of plakoglobin is serine phosphorylated and is distributed along the lateral membrane, the soluble pool is serine, threonine and tyrosine phosphorylated and remains in the cytoplasm [25].

1.3.2. Plakoglobin and regulation of cell-cell adhesion

The most extensively studies function of plakoglobin is the regulation of cell-cell adhesion. Plakoglobin has an indispensable role in the proper assembly and stability of desmosomal plaques. Plakoglobin double knockout mice have been used to investigate whether plakoglobin deficiency affects embryonic development. Loss of plakoglobin in embryos resulted in lethality due to severe heart defects at embryonic day 10.5 to 12.5 (E10.5 to E12.5). Heart failure occurred as the consequence of the disruption of desmosome assembly in the intercalated discs of cardiac muscle [26,27]. Plakoglobin is required for the efficient binding of desmoplakins to the intermediate filaments to stabilize desmosomes at the membrane [28]. Furthermore, plakoglobin interacts with plakophilin-3 and recruits it to membrane to form desmosomes [29].

In spite of its well-validated role in the formation of desmosomes, the significance of plakoglobin in the assembly of adherens junctions remained controversial. Initial studies showed that plakoglobin only loosely interacted with E-cadherin suggesting that the E-cadherin/β-catenin/α-catenin complex was the primary complex involved in the formation of adherens junctions [15,30]. However, participation of plakoglobin in adherens junctions could not be dismissed since the co-immunoprecipitation studies also confirmed the association of plakoglobin with E-cadherin and α-catenin as an independent complex [29]. A subsequent study suggested that plakoglobin interaction with E-cadherin and formation of adherens junctions was an essential prerequisite for the assembly of desmosomes [31].

1.3.3. Plakoglobin and regulation of epithelial to mesenchymal transition

Previous studies have demonstrated that loss of E-cadherin leads to epithelial to mesenchymal transition (EMT) and acquisition of an invasive phenotype [32,33]. Furthermore, re-expression of E-cadherin and formation of adhesive complexes reverses the transformed phenotype and induces mesenchymal to epithelial transition (MET) [34,35]. These studies clearly suggested that malignant transformation of cells were regulated by the components of junctional complexes. However, the essential role of plakoglobin in triggering MET was long neglected until it was shown that exogenous expression of E- or P-cadherin in cadherin-null carcinoma cells with very low levels of plakoglobin failed to induce transition to an epithelial phenotype [36]. Although E-cadherin/β-catenin/α-catenin complexes were formed, plakoglobin was not detected at the adherens junctions and desmosomes were not assembled suggesting an essential role for plakoglobin in the regulation of cell-cell adhesion and MET [36]. Following this study, another group showed that the down-regulation of E-cadherin and plakoglobin led to the loss of adherens junctions and desmosomes and induction of EMT [31]. Expression of E-cadherin alone rescued the assembly of adherens junctions but not the epithelial phenotype [31]. Interestingly, expression of E-cadherin/plakoglobin chimeric protein not only led to the formation of stable adherens junctions and desmosomes but also induced MET [31].

To further investigate the importance of plakoglobin in the regulation of cell-cell adhesion and inhibition of a transformed phenotype, our laboratory used E-cadherin and plakoglobin deficient and N-cadherin expressing SCC9 cells, a human tongue

squamous cell carcinoma cell line. Ectopic expression of either E-cadherin or plakoglobin triggered MET concurrent with increased cell adhesion and decreased cell proliferation [37,38]. Furthermore, only plakoglobin expressing SCC9 cells formed desmosomes. More importantly, expression of plakoglobin also increased the stability of the mesenchymal marker N-cadherin and inhibited its oncogenic functions [37,38].

1.4. Catenin-mediated signal transduction

The importance of cadherin-catenin complexes is not limited to their role in maintaining cell-cell contact. Components of adherens junctions and desmosomes have been shown to participate in signaling pathways to regulate cell growth, differentiation and cell death [39,40]. In this context the cytoplasmic pool of catenin proteins have been shown to interact with various intracellular proteins from tumor suppressors to transcription factors in order to modulate signaling cascades [39].

With respect to the signaling functions of catenin proteins, β-catenin and p120-catenin have been studied extensively in the context of tumorigenesis and metastasis [41,42]. On the contrary, fewer studies have addressed the signaling functions of α-catenin and plakoglobin in the process of tumor development and cancer progression. Having said that, recent studies have suggested that both α-catenin and plakoglobin generally act as tumor and metastasis suppressor proteins through mechanisms that are beginning to be deciphered [22,43].

1.5. Plakoglobin and regulation of cell signaling

Plakoglobin and β-catenin have similar structural features and interacting protein partners [19,22] (Figure 1.2). However, they differ in their signaling functions in the context of tumorigenesis and metastasis. Unlike β-catenin, which has well-documented oncogenic functions via activating the Wnt pathway [41], plakoglobin mainly acts as a tumor and metastasis suppressor protein [21,22]. Not only does plakoglobin participate in the Wnt pathway but also it has been linked to other signaling cascades that are going to be discussed in detail.

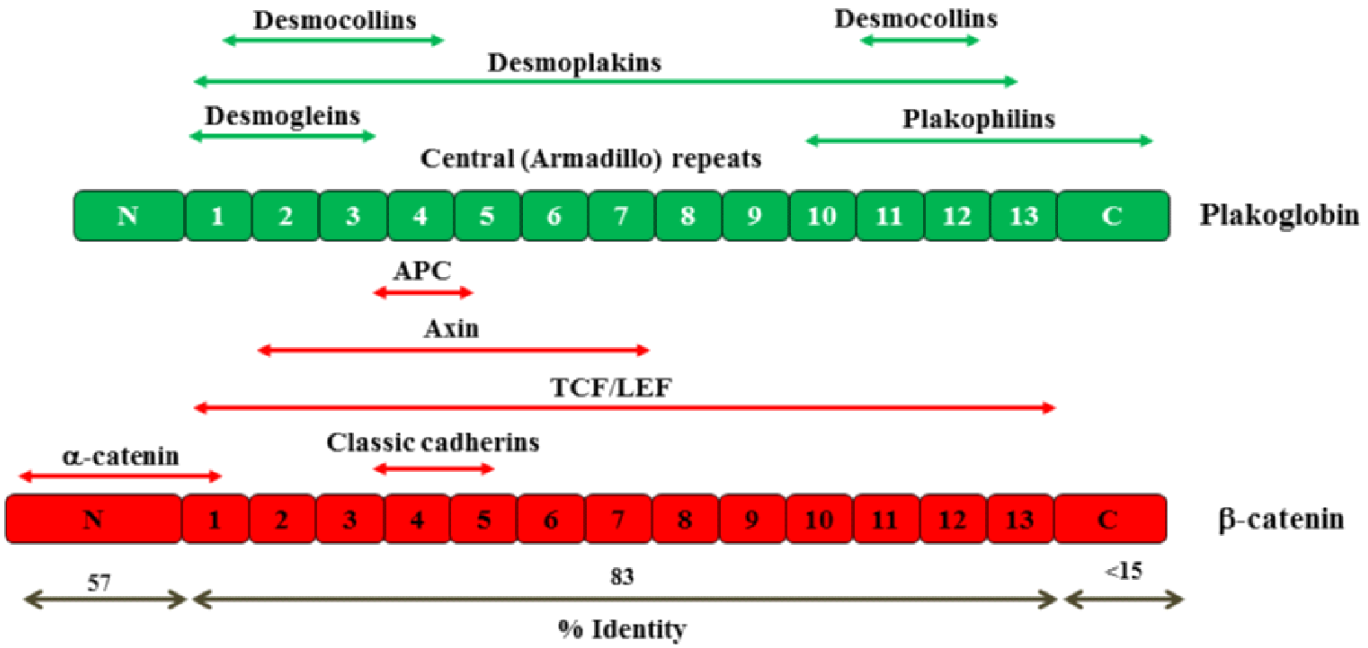

Figure 1.2. Schematic structure of plakoglobin and β-catenin. Plakoglobin and β-catenin are members of armadillo protein family and paralogues. They both have 13 armadillo repeats situated between their N- and C-terminal domains. The N-, central armadillo and C-terminal domains of the two protein are 57%, 83% and less than 15% identical, respectively. Red arrows indicate protein partners that interact with both β-catenin and plakoglobin and the domains involved in these interactions. Green arrows indicate proteins that interact with plakoglobin only and the domains involved in these interactions. APC, adenomatous polyposis coli; TCF/LEF, T-cell factor/Lymphoid enhancer factor

Modified from Pasdar M.

1.5.1. Plakoglobin and the Wnt signaling pathway

The Wnt pathway is one of the key regulators of normal development, cell proliferation and differentiation [44]. Disruption of the Wnt pathway has been linked to various diseases including cancer development and metastasis [45]. In the absence of Wnt signal, the destruction complex, axin/adenomatous polyposis coli (APC)/Glycogen Synthase Kinase 3 Beta (GSK3β)/casein kinase I (CKI), phosphorylates the excessive cytoplasmic pool of β-catenin, which is subsequently ubiquitinated and degraded via the proteasomal pathway [44] (Figure 1.3). When the Wnt ligand is present, it binds to the receptor Frizzled and the co-receptor low-density lipoprotein receptor-related protein (LRP) 5/6, and in turn they interact with Dishevelled via their intracellular domain [44]. Dishevelled recruits Axin and displaces GSK3β and CKI from tumor suppressor APC [44]. Upon disruption of the destruction complex function, β-catenin accumulates, translocates to the nucleus and binds to TCF/LEF transcription factors and activates the expression of Wnt targets that promotes proliferation, survival, migration, invasion and angiogenesis [44] (Figure 1.3).

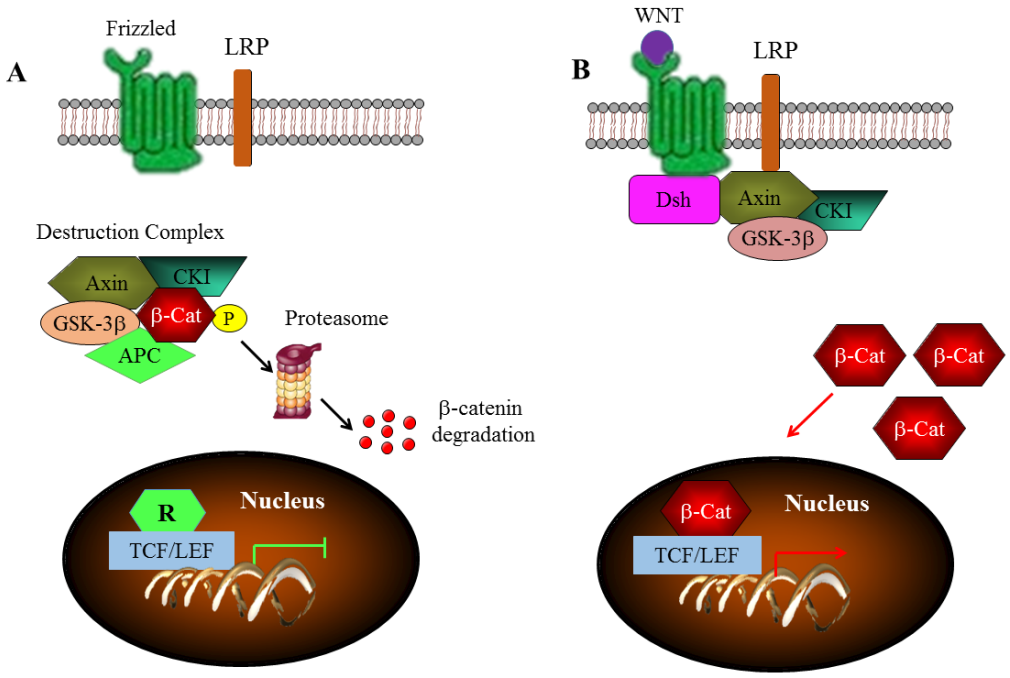

Figure 1.3. Regulation of β-catenin via the Wnt signaling pathway. **(A)** In the absence of Wnt signal, the destruction complex (Axin, APC, GSK3β, CKI) recruits and phosphorylates β-catenin. Phosphorylated β-catenin gets ubiquitinated and subsequently degraded by proteasomes. Consequently, TCF/LEF transcription factors in the nucleus are bound to the repressor and their respective target genes remain inactive. **(B)** In the presence of Wnt signal, Dishevelled recruits Axin and destruction complex dissociates. Thus β-catenin cannot get phosphorylated and degraded. Excessive cytoplasmic β-catenin translocates to the nucleus and binds to TCF/LEF that leads to the transactivation of their target genes involved in EMT, uncontrolled cell proliferation, migration and invasion. LRP, low-density lipoprotein receptor-related protein; CKI, casein kinase I; APC, adenomatous polyposis coli; GSK3β, Glycogen Synthase Kinase 3 β; Dsh, Dishevelled; TCF/LEF, T-cell factor/Lymphoid enhancer factor; R, repressor; β-cat, β-catenin

Modified from Pasdar M.

Early studies using PC12 pheochromocytoma cells showed that the exogenous expression of Wnt-1 increased plakoglobin levels and resulted in its membrane redistribution [46]. This was the first evidence suggesting that the Wnt pathway regulates the levels and localization of both β-catenin and plakoglobin [46]. Later on, Karnovsky *et al*., injected fertilized *Xenopus* embryos with mRNAs encoding plakoglobin and detected dorsalized gastrulation and anterior axis duplication, which was similar to Wnt/β-catenin induced segment polarization. In these embryos, plakoglobin localized in the cytoplasm, nucleus and membrane [47]. However, when mRNAs encoding plakoglobin and the cytoplasmic tail of desmoglein were co-injected into the embryos, plakoglobin primarily localized at the membrane and was excluded from the nucleus. In these embryos dorsalized gastrulation and anterior axis duplication did not occur, suggesting that the nuclear pool of plakoglobin participated in specification of dorsal mesoderm and had signaling functions similar to β-catenin [47].

While these initial studies suggested that both plakoglobin and β-catenin exerted similar signaling activities, many other studies provided contrary evidence. First evidence came from a study that showed both wild type and junction-dependent plakoglobin induced axis duplication, suggesting that presence or absence of nuclear plakoglobin had no effect on regulating the Wnt signaling [48]. To further investigate the signaling function of plakoglobin via the Wnt pathway, either β-catenin or plakoglobin were expressed in *Drosophila* embryos lacking functional Armadillo homologues [49]. This study showed that while expression of either β-catenin or plakoglobin resulted in the formation of cadherin-based cell adhesive

complexes, only the expression of β-catenin slightly induced the expression of the Wnt target, *engrailed* [49].

Several other lines of evidence have also suggested that plakoglobin has limited signaling activity in the context of the Wnt pathway. In MDCK cells, only β-catenin but not plakoglobin translocated to the nucleus in response to the over-expression of LEF-1 [50]. Moreover, HEK293T cells exhibited a significantly higher TOPFLASH reporter activity when β-catenin was expressed as compared to cells expressing plakoglobin [50]. The impact of plakoglobin on activating the Wnt pathway was also shown when HEK293T cells were transfected with the transcriptional active forms of β-catenin and plakoglobin, S37A and S28A mutants, respectively, and the expression of β-catenin mutant resulted in a much higher TOPFLASH reporter activity [51,52]. Subsequently, *in vitro* electrophoretic mobility shift assays using β-catenin, plakoglobin, TCF-4, LEF-1 proteins and radioactively labeled TCF/LEF DNA binding sequences showed that β-catenin formed a complex with TCF-4/LEF-1 and DNA, whereas, plakoglobin was unable to form similar complexes [53].

1.5.2. Plakoglobin and Src signaling

The proto-oncogene Src is a non-receptor tyrosine kinase that participates in various signaling pathways by phosphorylating specific tyrosine residues in other proteins [54]. Increased levels and activity of Src kinase has been shown to promote tumorigenesis and metastasis in different types of cancer [54]. A number of studies have demonstrated that signaling activities of plakoglobin and Src are inversely

correlated [55-58]. Based on the experimental findings, Src enhances migration by suppressing the expression and/or altering the phosphorylation of plakoglobin that leads to the inhibition of tumor and metastasis suppressor effects of plakoglobin [56-58]. Furthermore, treating MCF-7 breast carcinoma cells with human growth hormone reduced plakoglobin levels and promoted migration and invasion in a Src-dependent manner [59]. Interestingly, treatment with Src inhibitors increased the expression of plakoglobin and decreased the migratory and invasive potential of these cells [59]. A subsequent study confirmed that human growth hormone repressed plakoglobin expression and promoted cell migration via activating Src and JAK2 tyrosine kinases [58]. Upon activation of these kinases, they induced the expression of DNA methyltransferase-1, -3A and -3B, which resulted in the hypermethylation of plakoglobin promoter and the inhibition of its transcription [58].

Findings from the *in vitro* research were further validated by *in vivo* studies in breast tumors showing that growth hormone receptors were over-expressed in both epithelial and stromal components of axillary lymph node metastasis, which was concurrent with decreased expression of plakoglobin in nodal metastasis [60]. Also, in non-small cell lung cancer (NSCLC) cell lines, and mouse xenograft models combined inhibition of Src and MAPK upregulated the expression of E-cadherin and plakoglobin and downregulated the expression of Snail1, FAK and PAX, which led to the induction of MET [61].

Plakoglobin also inhibited cell motility and migration via regulating the extra-cellular matrix (ECM) dependent activation of Src. Increased levels of ECM

proteins including fibronectin and vitronectin leads to the activation of Src kinase. Plakoglobin modulates the deposition of ECM proteins, and therefore, reduces Src signaling activity and the migration of single keratinocyte cells [55,62,63].

1.5.3. Plakoglobin and Ras signaling

Ras proteins are a family of small GTPases that regulate different signaling pathways involved in cell growth, survival, differentiation, migration, and invasion [64]. They become active in response to an extracellular signal and in turn transmit the signal to intracellular effector proteins in order to modulate downstream signaling cascades [64]. Ras is a proto-oncogene and its mutations and/or aberrant activation have been reported in various types of cancers [64]. The first observation connecting plakoglobin and Ras was reported when the expression of dominant negative form of Ras (N17Ras) reduced the expression of plakoglobin and prevented the formation of 3-dimensional vascular structures in confluent cultures of endothelial cells [65]. Later studies validated this early observation by showing that inhibition of Ras farnesylation and disruption of the MAPK pathway increased the expression of catenin proteins including α-, β- and γ-catenin in breast, colon and liver cancer cells concurrent with decreased metastatic potential of these cells [66]. To further investigate the relationship between plakoglobin and Ras, Yim *et al*., expressed a mutant form of Ras (K-Ras12V) in Rat2 cells and showed that the mutant Ras decreased plakoglobin and histone deacetylase 4 (HDAC4), which subsequently led to increased metastatic potential of these cells [67]. Surprisingly, only the exogenous expression of plakoglobin but not β-catenin in these cells increased HDAC4 levels in a LEF-1 dependent manner [67]. However, whether

plakoglobin is directly involved in regulating the expression of HDAC4 and the possible mechanism(s) of this modulation have not yet been understood.

1.5.4. Plakoglobin and the Hippo signaling pathway

Imbalance between cell proliferation, apoptosis and differentiation is a critical step during tumor formation and cancer progression. Tissue homeostasis is regulated by multiple signaling cues coordinated by different molecules and signaling pathways. The Hippo signaling pathway and its downstream effectors, YAP and TAZ, have been identified as essential regulators of cell proliferation, organ size and cell-fate determination [68]. When the Hippo signaling pathway is active, the serine/threonine kinases MST1/2 activate LATS1/2 kinases, which in turn phosphorylate YAP at serine 127 which prevents its nuclear localization and transcriptional activities [69]. LATS1/2 also phosphorylate YAP at serine 397, which serves as a mark for ubiquitination and proteasomal degradation [70]. However, when the Hippo kinase cascade is off, YAP is active and translocates to the nucleus, binds to the transcription factors of the TEA domain (TEAD) family and activates transcription of downstream pro-proliferative and anti-apoptotic genes [70].

Although the core Hippo signaling pathway has been extensively studied, the upstream regulators of the kinase cascade are yet to be fully deciphered. Interestingly, cell-cell contact and adhesion complexes have been identified as essential regulators of the canonical Hippo signaling pathway [70]. Loss of cell adhesion proteins such as α–catenin and E-cadherin has been shown to enhance the

oncogenic potential of YAP in different carcinomas [71,72]. Plakoglobin has also been shown to interact with YAP, and this interaction sequesters YAP in the cytoplasm and inhibits its nuclear translocation [73]. Silencing the expression of YAP or preventing its nuclear localization has been shown to significantly reduce the growth and metastatic potential of cancer cells [74].

1.6. Tumor and metastasis suppressor activities of plakoglobin

Several lines of evidence suggest that plakoglobin interacts with an array of cellular proteins involved in regulating tumor cell growth and metastasis [21,22]. Our laboratory has proposed a model that suggests plakoglobin may act as a tumor and metastasis suppressor protein by at least three mechanisms. First, plakoglobin may sequester oncogenic functions of β-catenin via promoting proteasomal degradation of β-catenin and inhibiting its interaction with TCF, and therefore, repressing the expression of Wnt target genes. Second, by changing the levels, localization and/or function of growth and metastasis regulating molecules. Last but not least, by interacting with transcription factors and (in)directly regulating gene expression independent of the Wnt signaling pathway [20,21].

1.6.1. Plakoglobin and inhibition of the oncogenic signaling of β-catenin

One mechanism by which plakoglobin may act as a tumor and metastasis suppressor protein is the inhibition of β-catenin oncogenic activity. A previous study revealed that the expression of plakoglobin in β-catenin expressing and plakoglobin null cell lines resulted in the liberation of β-catenin from the adherens junctions and its subsequent proteasomal degradation [75]. Experimental evidence

from our laboratory further confirmed that in plakoglobin null SCC9 cells β-catenin protein levels decreased and cells underwent mesenchymal to epithelioid phenotypic transition upon the exogenous expression of plakoglobin [38].

A previous *in vivo* study using *Xenopus* embryos demonstrated that plakoglobin inhibited nuclear accumulation of exogenously expressed TCF by sequestering it in the cytoplasm and in turn repressing its transcriptional activity [52]. In addition, it was shown that plakoglobin and β-catenin interacted with two sequential and non-overlapping domains in the N-terminus of the TCF protein [56]. Comparison of the binding sites showed that β-catenin bound to amino acids 1-50 in the N-terminal domain of TCF and plakoglobin interacted with the region situated between amino acids 51-80. Furthermore, plakoglobin interacted with the β-catenin/TCF-4 complex and inhibited the binding of the complex to DNA [56].

Previous studies from our laboratory also showed that nuclear exclusive expression of plakoglobin in SCC9 cells decreased β-catenin/TCF interaction and β-catenin's oncogenic activity [76]. Additionally, it was recently shown that knockdown of desmoglein increased nuclear levels of plakoglobin, which in turn enhanced plakoglobin/TCF-4 interaction and decreased TOPFLASH reporter activity concurrent with reduced transactivation of Wnt/β-catenin target genes [77].

Another mechanism by which plakoglobin inhibits the oncogenic functions of β-catenin is through its interaction with SOX4, a transcription factor and an interacting partner of β-catenin that enhances β-catenin's transcriptional activity downstream of the Wnt signaling pathway [78]. SOX4 directly interacts with and

stabilizes β-catenin and enhances the expression of Wnt target genes via unknown mechanism(s). In this study, nuclear accumulation of plakoglobin in response to Wnt treatment led to the formation of plakoglobin/SOX4 complexes that facilitated the nuclear export of SOX4 and reduced transactivation of Wnt-responsive target genes [78].

Recent studies have also proposed another mechanism to explain how plakoglobin may inhibit the transcriptional activity of β-catenin/TCF-4. Cellular prion protein PrP(c) was shown to interact with both β-catenin and plakoglobin in the cytoplasm and nucleus [79,80]. Previously, it was shown that while in polarized epithelial cells, PrP(c) was primarily at the membrane, in rapidly proliferating cells it localized in the nucleus suggesting that it may participate in signaling pathways. Interestingly, further studies validated a signaling role for PrP(c) via Hippo and Wnt signaling pathways. PrP(c)'s interaction with β-catenin/TCF-4 complex induced transcriptional activation of Wnt target genes, whereas, its interaction with plakoglobin/TCF-4 repressed the Wnt-responsive transactivation [79,80].

1.6.2. Plakoglobin and modulation of intracellular growth and metastasis regulating molecules

As mentioned above, one mechanism by which plakoglobin may suppress tumor development and metastasis is via its interaction with various cellular partners and affecting their levels, localization, and/or activities. In agreement with this role of plakoglobin, work from our laboratory has shown that plakoglobin interacts with the nucleolar phosphoprotein, nucleophosmin (NPM) [81]. It is noteworthy that

NPM's functions during tumorigenesis is greatly dependent on its subcellular distribution [82,83]. Specifically, NPM is primarily localized in the nucleolus of untransformed cells, whereas, in cancer cells, NPM is distributed in the cytoplasm and nucleoplasm [84]. Our laboratory showed that in plakoglobin deficient breast carcinoma cell line, MDA-MB-231, exogenously expressed plakoglobin interacted with NPM and increased its protein levels and induced its redistribution from cytoplasm and nucleoplasm to the nucleolus concurrent with a significant decrease in *in vitro* growth, migration and invasion [81].

To further study the tumor and metastasis suppressor functions of plakoglobin via its interaction with cellular protein partners, our laboratory looked at the effect of plakoglobin expression on the protein levels of non-metastatic protein 23 (Nm23), the first metastasis suppressor protein to be discovered [85]. Expression of plakoglobin in SCC9 and MDA-MB-231 cells enhanced Nm23-H1 and Nm23-H2 protein levels [86] and resulted in the redistribution of Nm23 from cytoplasm to the membrane [86]. Work by other groups showed that Nm23 knockdown increased migration due to the loss of cell-cell adhesion [87]. In the absence of Nm23, adherens junction dissociated and nuclear localization of β-catenin increased, which in turn led to the transactivation of matrix metalloproteinases (MMPs) that are known to be β-catenin/TCF target genes [87]. Another study demonstrated that formation and stability of the endothelial junctions were dependent on the interaction between Nm23, plakoglobin and EMMPRIN (the extracellular matrix metalloproteinase inducer, also known as basigin or CD147) [88]. These findings

further supported the importance of the plakoglobin and Nm23 interaction in the inhibition of migration and metastasis of cancer cells.

1.6.3. Plakoglobin and regulation of gene expression

Several lines of experimental evidence have suggested that plakoglobin regulates gene expression independent of β-catenin and the Wnt signaling pathway. It was shown that in mouse keratinocytes, plakoglobin interacted with LEF-1 and they both associated with the promoter region of the *MYC* gene [89]. Plakoglobin/LEF-1 complex repressed the expression of *MYC* gene and suppressed cell growth independent of β-catenin [89]. In support of this observation, Tokonzaba *et al*., recently showed that plakoglobin induced the expression of desmocollins-2 (*DSC2*) gene in a LEF-1 dependent manner [90].

To assess the possible contribution of plakoglobin to gene regulation, our laboratory used plakoglobin deficient carcinoma cell lines and transfected them with wild type plakoglobin construct, as well as, plakoglobin cDNAs fused to nuclear localization sequence or nuclear export signal to generate plakoglobin-expressing transfectants with different subcellular localization of the protein in order to investigate its role at the membrane, in the cytoplasm and in the nucleus [76,86,91]. The results of microarray analyses using plakoglobin-expressing transfectants identified several p53 target genes that were differentially regulated upon plakoglobin expression [92,93]. These findings suggested that one way by which plakoglobin acts as a tumor suppressor may be via its interaction with p53 and regulation of p53 target gene expression [92,93]. Further characterization

validated the microarray results and confirmed that the expression of p53 targets including 14-3-3σ, SATB1 and Nm23-H1 were co-regulated by plakoglobin and p53 [92,93]. Chromatin immunoprecipitation (ChIP) experiments showed that both plakoglobin and wild type p53 associated with the 53 response element within the promoter region of *SFN* (14-3-3σ), *SATB1*, and *NME1* (Nm23-H1) genes and only in the presence of plakoglobin, mutant p53 interacted with the promoter of these genes [92,93]. Additionally, the luciferase reporter assays revealed that the transcriptional activity of both wild type and mutant p53 was enhanced upon plakoglobin expression [92,93]. Interestingly, based on our studies plakoglobin and p53 are co-regulating gene expression through both activating (*SFN* and *NME1*) and repressing (*SATB1*) mechanisms [92,93]. In agreement with these findings, another group showed that plakoglobin induced the expression of PML, a p53 target with tumor suppressor function [94]. Finally, it was recently shown that in non-small cell lung carcinoma cells plakoglobin reduced migration by regulating the expression of HAI-1, an upstream inhibitor of c-met, in a p53 dependent manner [95].

1.7. Plakoglobin and restoration of mutant p53 tumor suppressor function

As mentioned above, one of the interacting protein partners of plakoglobin that has been identified by our lab is the tumor suppressor protein p53 [92,93]. Plakoglobin interacts with both wild type and a number of mutant p53 proteins and they both associate with the promoter of several p53 target genes involved in the regulation of tumorigenesis and metastasis [92,93].

1.7.1. Tumor suppressor protein p53

p53 is a sequence specific DNA-binding transcription factor with tumor and metastasis suppressor activities [96]. Dubbed as the "guardian of the genome" [97], p53 maintains the integrity of the genome and normal functioning of cells in response to various stress signals including DNA damage, oxidative injury, hypoxia, improper cell adhesion, mitotic stress, oncogene activation and metabolic stress [98].

In the absence of stress signal, p53 levels are tightly regulated and are kept at low steady levels in order to prevent unnecessary cell death [99]. The primary regulator of p53 is Hdm-2, an E3 ubiquitin ligase and a p53 target gene, which interacts with and mono-ubiquitinates p53 in the nucleus [100,101]. Upon mono-ubiquitination, p53 translocates to the cytoplasm and gets poly-ubiquitinated by other E3 ubiquitin ligases including Pirh2, COP1, Arf-BP1, which leads to its subsequent degradation by the proteasomal pathway [101]. Upon exposure to cellular stress, p53 levels significantly increase and it becomes active in order to regulate the expression of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair and metabolism [98].

Various functions of p53 are mediated via its structural domains comprising of two N-terminal transactivation domains (TAD-1 and TAD-2), a proline rich domain, the core DNA binding domain and a carboxyl terminal domain [102] (Figure 1.4). TAD-1 and -2 interact with general transcription factors, chromatin modifiers and transcription cofactors to activate or repress p53 target genes [103]. Furthermore,

TAD-1 is essential for p53 stability via its interaction with Hdm-2 [104]. The proline rich domain plays a pivotal role in the regulation of p53-mediated apoptosis via its interaction with pro- and anti-apoptotic proteins in the cytoplasm [105]. Flanking between the N- and C-terminal domains, there is a DNA binding domain that interacts with the p53 response element in the promoter region of its target genes to regulate their transcription [102]. The carboxyl terminal of p53 contains three nuclear localization signals, an oligomerization domain and a non-specific DNA binding domain [102]. Oligomerization domain is essential for homo-dimerization and subsequent tetramerization of p53 protein, which is required for its transcriptional activities. While earlier *in vitro* studies suggested that the C-terminal DNA binding region negatively regulated binding to DNA [106], more recent studies demonstrated that this domain interacted with DNA in order to facilitate the binding of the core domain to the p53 response element [107,108].

As a transcription factor, wild type p53 activates or represses the expression of components of the cell cycle check points, DNA repair machinery and apoptotic pathway to inhibit propagation of damaged cells [99]. Depending on the cellular context and type of the damage a cell sustains, p53 either triggers cell cycle arrest to provide sufficient time for the injured cell(s) to undergo DNA repair or it induces senescence or apoptosis in case of irreparable damage [109].

Transient and/or permanent cell cycle arrest are two of the major anti-proliferative functions of p53. In response to DNA damage, p53 becomes active and in turn upregulates the expression of its target genes p21, 14-3-3σ and GADD45 (growth arrest and DNA damage-inducible). While p21 regulates both G1 and G2 arrest,

14-3-3σ and Gadd45 only induce G2 arrest and inhibit mitosis entry [110]. In addition to its ability to inhibit cell cycle progression, p53 also induces apoptosis by up-regulating the expression of pro-apoptotic genes including death receptor proteins (e.g. Fas, DR4, DR5 and PERP), NOXA, PUMA, BAX and BID [110] and down-regulating anti-apoptotic genes such as Bcl-X_L and Bcl-2 [110].

p53 also exerts its tumor suppressor functions independent of its transcriptional activity. p53 localizes to the mitochondria and directly binds to pro- and anti-apoptotic members of Bcl-2 family of proteins and promotes apoptosis by enhancing the mitochondrial outer membrane permeabilization (MOMP) [110,111].

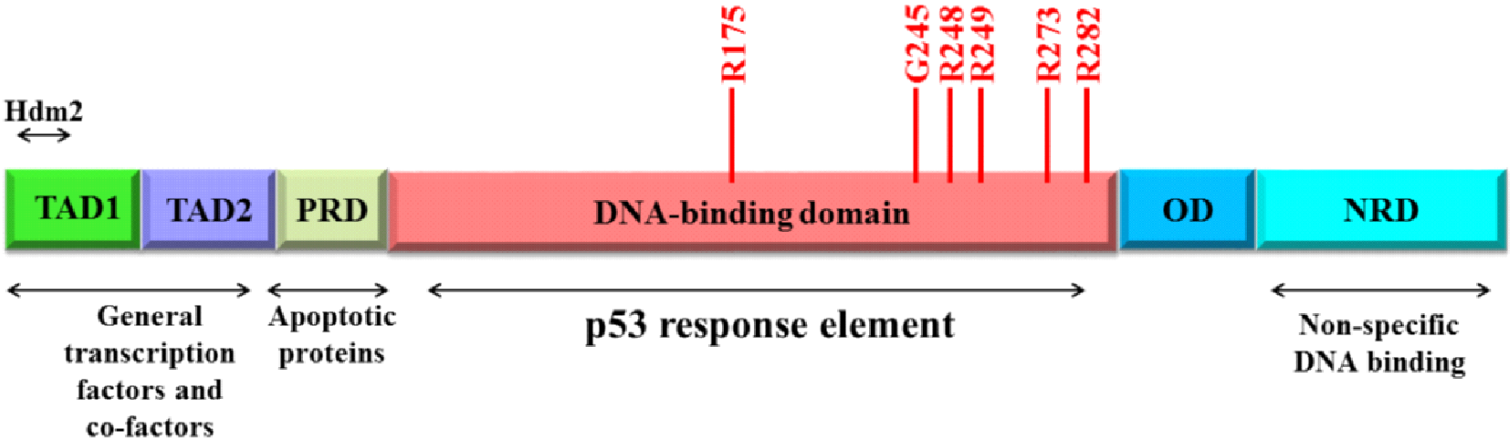

Figure 1.4. p53 domains and hotspot mutations. p53 has three different structural domains including; an intrinsically disordered N-terminal with two transactivation and a proline rich domains, a central DNA-binding domain and a C-terminal oligomerization domain which is required for tetramerization and is followed by a non-specific DNA binding domain. The majority of p53 mutations occur within the DNA-binding domain with six amino acid residues (R175, G245, R248, R249, R273 and R282) within the DNA-binding domain of p53 have been considered as mutation hotspots with the highest frequency of mutation. TAD, transactivation domain; PRD, proline rich domain; OD, oligomerization domain; NRD, negative regulatory domain; G, glycine; R, Arginine

1.7.2. Mutant p53 proteins

TP53 is the most commonly mutated tumor suppressor gene in cancers [113]. While the majority of tumor suppressor genes sustain deletions and truncations leading to their permanent inactivation and/or loss of expression [114], *TP53* gene mainly harbors missense mutations within the central DNA binding domain that interferes with its interaction with DNA and alters its transcriptional activity [115,116]. Six amino acid residues (175, 245, 248, 249, 273 and 282) within the DNA binding domain have been considered as mutation hotspots with the highest frequency of mutation [115,116] (Figure 1.4). In addition to the loss/partial loss of their tumor suppressor activities, many mutant p53 proteins exhibit dominant negative activities through their interaction with and inhibition of the functions of wild type p53 [117]. More importantly, some p53 mutants can gain oncogenic functions (GOF) that contribute to tumor cell growth, aggressiveness, metastasis and drug resistance [118]. GOF p53 mutations fall into two categories; structural mutations that alter the conformation of p53 and contact mutations that inhibit the direct interaction between p53 and DNA [118].

GOF p53 mutants exert their tumor promoting activities via four different mechanisms; 1- by interacting with transcription factors Sp1, ETS family, E2F1, and NF-1 and co-regulating the expression of target genes involved in cancer progression and metastasis [119-121], 2- by binding to different cellular protein partners including p53 family members, p63 and p73 and disrupting the recruitment of p63/p73 to the promoter region of their target genes [122-124], 3- by modulating

the expression of micro RNAs involved in tumorigenesis and metastasis [124], and, 4- by regulating epigenetic modifying enzymes [126,127] (Figure 1.5).

Experimental evidence from our laboratory suggested that in plakoglobin deficient carcinoma cell lines expressing mutant p53 proteins, exogenous expression of plakoglobin restored tumor suppressor activities of mutant p53s *in vitro*. These earlier studies are the basis for this thesis and will be discussed further.

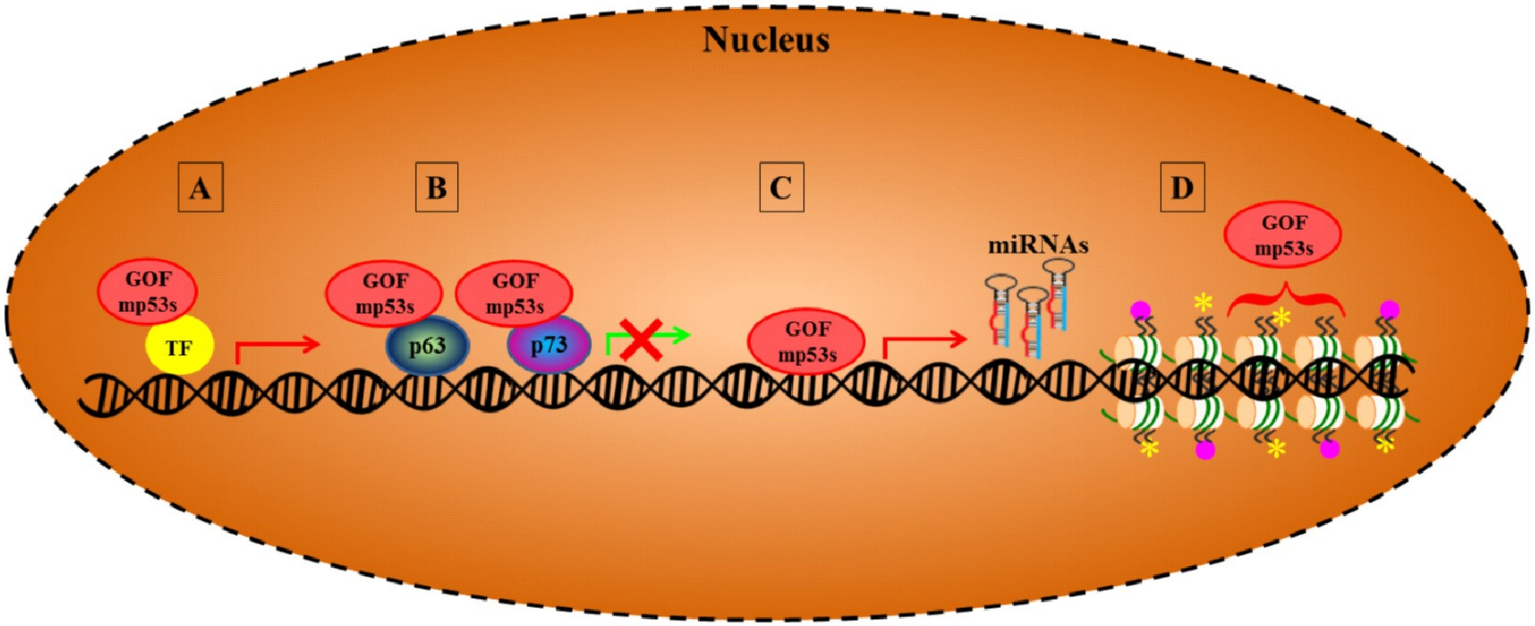

Figure 1.5. Mechanisms of oncogenic activities of gain of function (GOF) mutant p53 proteins. **(A)** GOF mutant p53s regulate the expression of genes by binding to the promoter of target genes via their interaction with other transcription factors. **(B)** GOF mutant p53 proteins interact with and sequester proteins with tumor and metastasis suppressor functions including other family members, p63 and p73 and impair their normal transcriptional activities. **(C)** GOF mutant p53s upregulate the expression of oncogenic micro RNAs and downregulate the expression of micro RNAs with tumor suppressor activities. **(D)** GOF mutant p53s modulate the expression of epigenetic enzymes and induce chromatin changes to silence or activate gene expression.

1.8. Earlier studies and research hypothesis

Previous studies from our laboratory revealed that exogenous expression of physiological levels of plakoglobin in plakoglobin-deficient SCC9 carcinoma cell line decreased growth and induced apoptosis [37] whereas its overexpression in SCC9 cells increased growth by inducing the expression of the anti-apoptotic protein Bcl-2 [91]. To further investigate the exact role of plakoglobin in regulating Bcl-2 expression, SCC9 cells were transfected with plakoglobin cDNAs fused to nuclear localization sequence or nuclear export signal. It was shown that plakoglobin induced Bcl-2 levels independent of its subcellular localization. Interestingly, expression of plakoglobin resulted in decreased interaction of β-catenin with N-cadherin and α-catenin leading to nuclear accumulation of β-catenin and its interaction with TCF [76]. Together these studies suggested that plakoglobin may regulate gene expression indirectly via regulation of β-catenin oncogenic activity.

Comparison of the mRNA profiles of plakoglobin-deficient human squamous and breast carcinoma cell lines and their plakoglobin-expressing transfectants showed increased expression of growth and metastasis suppressor genes and decreased expression of tumor promoting genes in plakoglobin expressing transfectants relative to parental cells. These studies identified a number of p53 targets among the differentially expressed genes, which are involved in tumor suppression, apoptosis and angiogenesis including *NME1*, *SFN* (14-3-3σ), *THBS1*, *SATB1*, *CDK1*, *CDC20*, *BCL2* and *BID* [92,93]. These observations raised the intriguing possibility of interaction between plakoglobin and p53.

To identify potential interaction between plakoglobin and p53 we performed co-immunoprecipitation experiments using a number of carcinoma cell lines with different plakoglobin and p53 expression profiles. Subsequent co-immunoprecipitation experiments showed that plakoglobin interacted with wild type as well as a number of mutant p53 proteins in both the cytoplasm and nucleus [92,93]. Additionally, ChIP assays showed that plakoglobin directly associated with the promoter regions of p53 target genes such as tumor and metastasis suppressors *NME1* and *SFN* [92,93] and the oncogenic genome organizer *SATB1* [93].

Based on the above studies, **we hypothesize that one mechanism underlying the tumor and metastasis suppressor functions of plakoglobin might be its interaction with mutant p53 proteins and restoration of their tumor suppressor function.**

The main focus of my PhD project was to gather experimental evidence for the following three specific aims:

1) To investigate the role of plakoglobin in high grade serous ovarian carcinoma cell lines and its functional interactions with wild type and mutant p53
2) To identify the interacting domains of plakoglobin and p53 and to assess the functional significance of their interaction
3) To determine the role of plakoglobin in down-modulation of the oncogenic activity of β-catenin induced by mutant p53 expression

1.9. References

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Chapter two

Plakoglobin reduces the *in vitro* growth, migration and invasion of ovarian cancer cells expressing N-cadherin and mutant p53

PREFACE

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Contributions:

I participated in conceiving and designing the experiments, performed the experiments and wrote the manuscript. GD performed replicates of experiment shown in figure 2.2A and migration and invasion assays for OV-90 cells.

2.1. Introduction

Ovarian cancer (OVCA), the fifth most prevalent cancer in women is the leading cause of all female reproductive cancer deaths worldwide, with an overall five-year survival rate of ~ 45% [1]. The major form of OVCA is the epithelial ovarian cancer (EOC), which accounts for ~80% of all ovarian neoplasms [2]. EOCs are classified into type I and type II [3]. Type I tumors are genetically stable, slow-growing, and have relatively good clinical outcome. However, the majority of OVCA are type II. Over 90% of these tumors harbor p53 mutations, are genetically unstable, highly aggressive and have poor clinical outcome [4-6]. *TP53* mutations are believed to be an early event during the development of type II tumors and contribute to both metastatic progression and chemoresistance [7-12]. p53 is a transcription factor and tumor suppressor that plays essential roles in regulating cell proliferation, survival, senescence, apoptosis and metabolism [13]. In response to stress, p53 activates DNA damage response, cell cycle arrest and cell death [14,15]. Different posttranslational modifications and protein-protein interactions regulate p53 stability and functions [16]. We have identified plakoglobin as a novel interacting partner of both wild type and mutant p53 (mp53) [17,18].

Plakoglobin is a member of the Armadilo family of proteins and a paralog of β-catenin [19,20]. Unlike, β-catenin, which only associates with adherens junctions and possesses well-known oncogenic functions, plakoglobin is a tumor/metastasis suppressor protein and participates in the formation of both adherens junctions and desmosomes [19,21]. Plakoglobin can confer growth/metastasis inhibitory effects via its interactions with cadherins and induction of contact inhibition of growth

[19]. In addition, it can interact with a number of intracellular partners including transcription factors [17-19,22-27]. We have shown that plakoglobin interacts with p53 and its tumor/metastasis suppressor function may, at least partially, be mediated by this interaction [17,18].

A number of studies have suggested that the loss of cadherin-catenin complex and activation of β-catenin oncogenic function play pivotal roles in the local invasion of ovarian tumor cells and subsequent metastasis [28-31]. Furthermore, the loss of heterozygosity of the plakoglobin gene (*JUP*) has been reported in sporadic OVCAs [32]. However, very little is known about the role of plakoglobin in OVCAs. In this study, we assessed the potential tumor/metastasis suppressor functions of plakoglobin in OVCAs, using the normal ovarian cell line IOSE-364 and OVCA cell lines OV-90 (plakoglobin and E-cadherin positive, mp53 expressing), ES-2 (plakoglobin and E-cadherin negative, N-cadherin positive and mp53 expressing), ES-2-PG (ES-2 tansfectants expressing plakoglobin), ES-2-E-cad (ES-2 tansfectants expressing E-cadherin) and ES-2-shN-cad (ES-2 cells in which N-cadherin has been knocked down). We examined plakoglobin levels, localization and interactions with E- and N-cadherin and p53 and assessed the growth, migratory and invasive properties of various cell lines. The results showed that plakoglobin interacted with both cadherins and p53. Exogenous expression of E-cadherin or plakoglobin or knockdown of N-cadherin significantly reduced the migration and invasion of ES-2 cells. Furthermore, plakoglobin expression and N-cadherin knockdown but not E-cadherin expression significantly reduced ES-2 cells growth.

2.2. Materials and methods

2.2.1. Cell lines and culture conditions

IOSE-364 (hereafter IOSE) were grown in a 1:1 M199 and MCDB M105 media plus 5% FBS and 1% PSK (Penicillin, Streptomycin, Kanamycin). OV90 cells were maintained in the same M199 and MCDB M105 media plus 15% FBS and 1% PSK. ES-2 cells were grown in McCoy's 5a media completed with 10% FBS and 1% PSK. ES-2-E-cad and ES-2-PG cells were grown in ES-2 media containing 400 μg/ml (selection) or 200 μg/ml (maintenance) G418. ES-2-shNcad transfectants were cultured in ES-2 media with 1μg/ml (selection) or 0.5 μg/ml (maintenance) puromycin.

2.2.2. Transfection

Plasmids encoding E-cadherin and plakoglobin have been described [33, 34]. Cultures of ES-2 cells in 60 mm or 100 mm dishes were transfected at 50-75% confluency with 10-25 μg of DNA using calcium phosphate. Twenty hours after transfection, cells were rinsed with PBS and allowed to recover for 24 hours in complete growth media. To select stable transfectants, 72 h after transfection, media containing 400 μg/ml G418 (ES-2- PG and ES-2- E-cad transfectants) were added to cells and resistant colonies selected for 3-4 weeks. Resistant clones were maintained in 200 μg/ml G418 and screened for plakoglobin and E-cadherin expression by immunofluorescence and immunoblotting assays.

2.2.3. N-cadherin knockdown

Human N-cadherin lentiviral shRNA plasmid [35] was used to transfect Phoenix-AMPHO cells using calcium phosphate. Lentiviral particles collected at 48 and 72

hours post transfection were combined and filtered using a 0.45μm low-protein binding filter. Lentiviral particles were used to transduce ES-2 cells in the presence of 8μg/ml polyberene (Santa Cruz). Puromycin-resistant stable cell lines expressing the N-cadherin shRNAs (ES-2-shN-cad) were isolated and the N-cadherin levels assessed by immunoblot and immunofluorescence.

2.2.4. Immunoblot Analysis

Confluent 100 mm culture plates were rinsed with cold PBS and solubilized in SDS sample buffer (10 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 50 mM dithiothreitol, 2 mM EDTA, 0.5 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4). Equal amounts of total cellular proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Biorad). The membranes were incubated in specific primary antibodies overnight at $4^{\circ C}$ followed by the appropriate secondary antibodies at room temperature (Table 2.1). Membranes were scanned using an Odyssey CLx infrared imaging system.

Table 2.1. Antibodies and their respective dilutions in specific assays.

Primary antibodies	Species	Assay			Source
		WB	IP	IF	
p53	Mouse	1:1000	1:100	-	Santa Cruz, sc-126
Plakoglobin	Mouse	1:1000	1:100	1:100	Translab, 610254
E-cadherin	Mouse	1:1000	-	1:100	Translab, 610404
N-cadherin	Mouse	1:1000	-	-	Santa Cruz, sc-59987
Cytokeratin (pan-keratin)	Mouse	1:1000	-	-	Sigma, C-2931
Vimentin	Mouse	1:1000	-	-	Sigma, V-6630
β-actin	Mouse	1:1000	-	-	Santa Cruz, sc-47778
Secondary antibodies					
Anti-mouse IgG, light chain	Goat	1:15000	-	-	Jackson Immuno Research, 115-625-174
Anti-rabbit IgG, light chain	Goat	1:15000	-	-	Jackson Immuno Research, 211-652-171
Alexa fluor 488	Mouse	-	-	1:2000	Molecular Probes, A11029
Alexa fluor 546	Rabbit	-	-	1:3000	Molecular Probes, A11035
Rhodamine	Rabbit	-	-	1:400	Boehringer Mannheim, 605107
Rhodamine	Rat	-	-	1:400	Sigma, T4280

2.2.5. Immunofluorescence

Confluent cell cultures were established on glass coverslips and rinsed with cold PBS containing 1 mM each of NaF, Na_3VO_4 and $CaCl_2$. Cells were fixed with 3.7% formaldehyde for 20 minutes and extracted with CSK buffer (50 mM NaCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 1.2 mM PMSF, and 1 mg/ml DNase and RNase; [17]) for 10 minutes. Coverslips were blocked with 4.0% goat serum and 50 mM NH_4Cl_4 in PBS containing 0.2% BSA for 1 hour. Coverslips were then incubated in the specific primary antibodies for 1 hour followed by the secondary antibodies for 30 minutes at concentrations indicated in Table 2.1. Nuclei were counterstained with DAPI (1:2000). Coverslips were mounted in elvanol containing 0.2% (w/v) paraphenylene diamine (PPD) and viewed using a 63x objective lens of a Zeiss confocal microscope.

2.2.6. Immunoprecipitation

Cultures were grown to confluency in 100 mm dishes and rinsed with cold PBS containing 1 mM NaF, Na_3VO_4 and $CaCl_2$. Cells were extracted in 1ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.7 μg/ml Pepstatin, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail) for 20 minutes on a rocker at 4^{oC}. Cells were scraped and centrifuged at 48000 xg for 10 minutes. Supernatants were processed for immunoprecipitation with p53, plakoglobin, E- and N-cadherin antibodies (Table 2.1) and 40 μl protein G agarose (Thermo Fisher Scientific) beads overnight on a rocker-rotator at 4^{oC}. Samples were then centrifuged at 14000 xg for 2 min, the beads were removed and the supernatants processed for a second

immunoprecipation for 3 hours. Beads from the two immunoprecipitations were combined and washed three times with the lysis buffer. Immune complexes were solubilized in 60 µl SDS sample buffer, separated by SDS-PAGE and processed for immunoblot as described above.

2.2.7. Growth, migration and invasion assay

For *in vitro* growth assay, $3x10^4$ cells from ES-2, ES-2-E-cad, ES-2-PG and ES-2-shN-cad cells were plated in a 24-well plate. At 1, 3, 5 and 7 days after plating, cultures were trypsinized and cells were counted. Each time point represents the average of three independent experiments.

For cell migration assays, 2×10^5 cells were resuspended in 0.5 ml serum-free media and plated in the upper chamber of transwell inserts (3 μ m pore, 6.5 mm diameter; BD Biosciences). Normal media containing 10% FBS was added to the lower chamber and cultures were incubated for 16 hours at 37°C. Inserts were then transferred into new dishes and rinsed with PBS to remove un-attached cells. Inserts were fixed with 3.7% formaldehyde (in PBS) for 2 minutes, permeabilized with 100% methanol for 20 minutes and stained with Giemsa stain for 15 minutes at room temperature. Following staining, membranes were viewed under an inverted microscope using a 20x objective lens and photographed.

For Matrigel invasion assays, cells were starved in serum-free media for 24 hours prior to the assay. For each cell line, 5×10^4 cells in 0.2 ml serum-free media were plated in the top compartment of Matrigel-coated invasion chambers (8 µm pore PETE membrane; BD Biosciences). Fibroblast conditioned media (0.8 ml) was added to the bottom chambers and plates were incubated overnight at 37°C. After

16 hours, membranes were recovered and processed as described for the migration assay. Mounted membranes were viewed under a 20x objective lens of an inverted microscope and photographed.

The migrated/invaded cells were counted in 5 random fields for each membrane using ImageJ Cell Counter program. Numbers for each cell line were averaged and normalized to those of the normal cell line or parental untransfected cells and histograms constructed. Histograms represent the average of at least 3 independent assays for each cell line.

2.2.8. Statistical analysis

Values are presented as means± SD. Statistical differences between groups were assessed by Student's t-tests. *P*-value <0.05 was considered significant.

2.3. Results

2.3.1. Protein expression of epithelial and mesenchymal markers and p53 in various OVCA cell lines

Protein expression of E-cadherin, N-cadherin, plakoglobin, cytokeratins, vimentin and p53 in IOSE, ES-2 and OV-90 cells were detected using immunoblot analysis (Figure 2.1). IOSE cells had very little, if any, E-cadherin and expressed N-cadherin and plakoglobin. These cells also expressed cytokeratins, vimentin and p53. These observations were consistent with previous findings indicating that normal OSE cells displayed both epithelial and mesenchymal markers [36]. In contrast, OV-90 cells that express mp53 [37] had no detectable N-cadherin, low levels of vimentin and high levels of epithelial markers including E-cadherin, plakoglobin and cytokeratins. ES-2 cells, which also express mp53 [38, 39], displayed a more

mesenchymal phenotype, lacked E-cadherin and plakoglobin and expressed N-cadherin, vimentin and very low levels of cytokeratins.

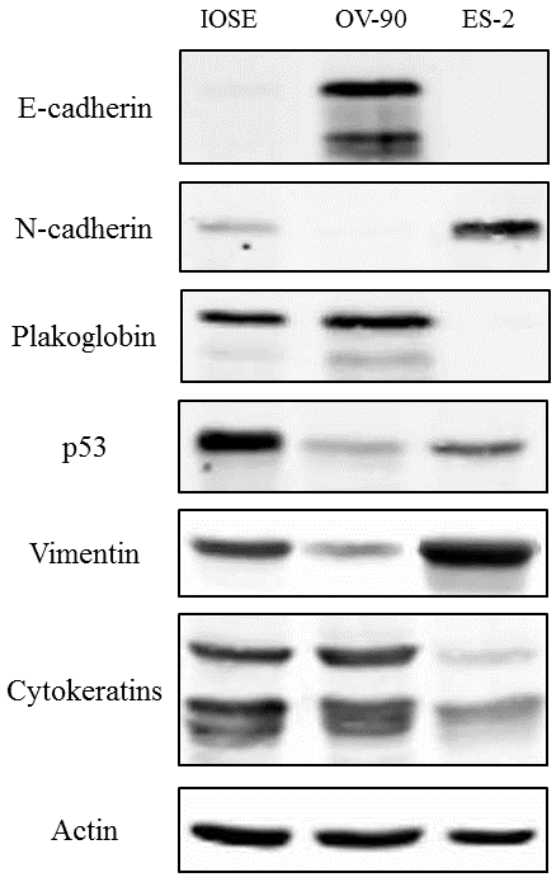


Figure 2.1. Protein expression of epithelial and mesenchymal markers and p53 in ovarian cancer cell lines. Total cell lysates from IOSE-364, ES-2 and OV-90 cells were processed for immunoblot analysis using **N-cadherin**, **E-cadherin**, **plakoglobin**, **vimentin**, **cytokeratins** and **p53 antibodies**. Equal loadings were confirmed by processing the same lysates with actin antibodies.

2.3.2. Levels, localization and interaction of E-cadherin, N-cadherin and plakoglobin in normal and carcinoma ovarian cell lines

Subcellular distribution and potential co-localization of E-/N-cadherin with plakoglobin were examined by double immunofluorescence staining (Figure 2.2A). In IOSE cells, consistent with the immunoblot results, E-cadherin levels were undetectable whereas N-cadherin and plakoglobin were expressed at high levels and were co-distributed at the membrane (Figure 2.2A, IOSE). In OV-90 cells, high levels of E-cadherin and plakoglobin were present and were colocalized at the membrane. We also detected scarcely distributed small patches of N-cadherin positive cells in OV-90 cultures. In these patches, N-cadherin was colocalized with plakoglobin (Figure 2.2A, OV-90). In ES-2 cells, there was no detectable E-cadherin or plakoglobin, whereas they expressed high levels of N-cadherin, which was distributed throughout the cytoplasm (Figure 2.2A, ES-2). Consistent with the absence of plakoglobin and adhesive junctions, ES-2 cells exhibited significantly less cell-to-cell contact and their morphology was distinctly different than IOSE and OV-90 cells. Co-immunoprecipitation studies showed that plakoglobin interacted with N-cadherin in IOSE cells and with E-cadherin in OV-90 cells (Figure 2.2B).

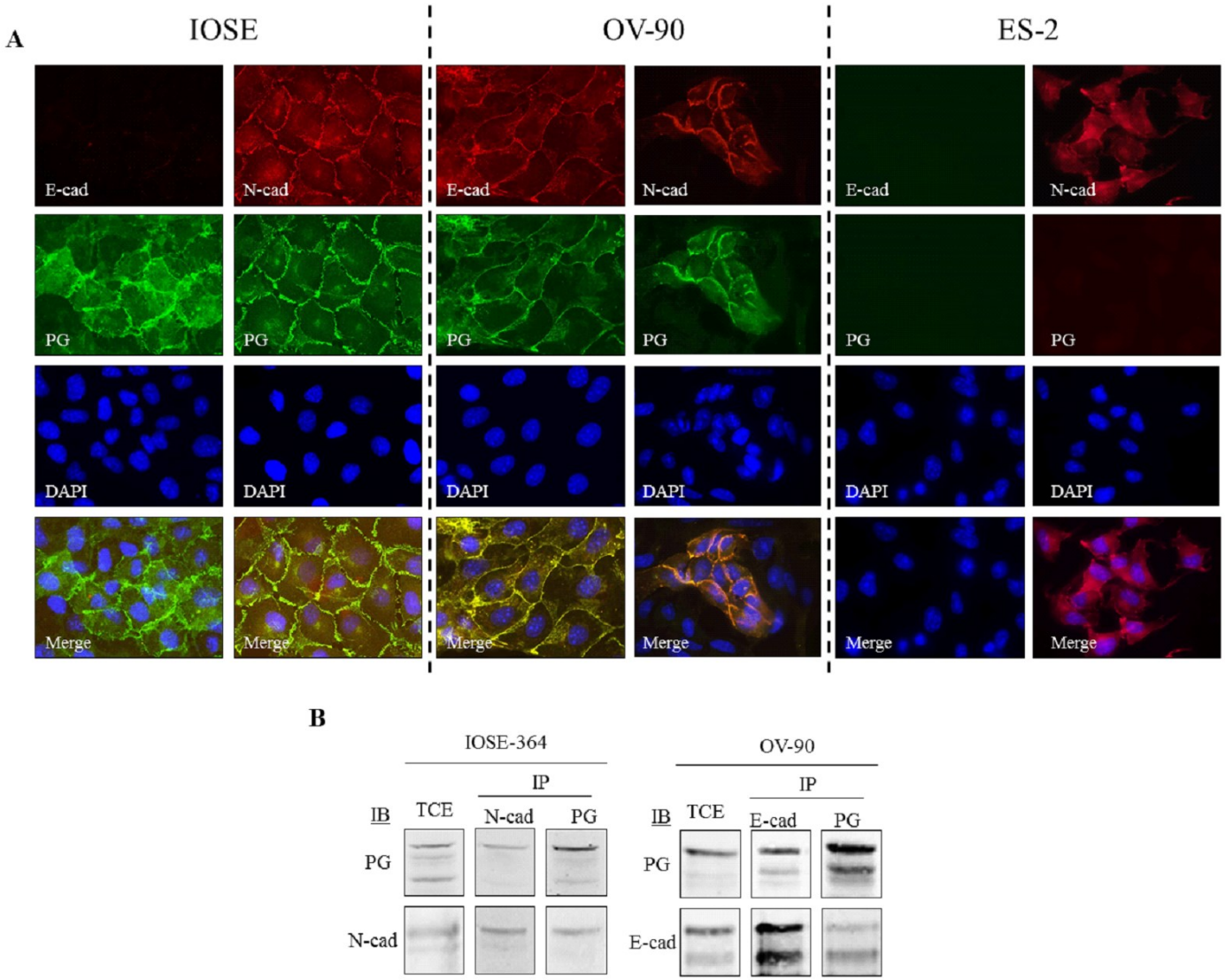


Figure 2.2. (A) Levels and localization of E-cadherin, N-cadherin and plakoglobin in normal and carcinoma ovarian cell lines. IOSE-364, ES-2 and OV-90 cells were grown on coverslips and processed for double immunofluorescence staining. E-cadherin (**E-cad**, red) or N-cadherin (**N-cad**, red) and plakoglobin (**PG**, green) antibodies were used at concentrations indicated in Table 2.1. Nuclei were stained with DAPI (blue). Bar, 25 mm. **(B) Interaction of plakoglobin and E-/N-cadherin in normal and ovarian carcinoma cell lines.** Equal amounts of total cell extracts from IOSE-364 and OV90 cells were processed for reciprocal and sequential immunoprecipitation and immunoblotting using N-cadherin, E-cadherin and plakoglobin antibodies. OV-90 cells expressing endogenous E-cadherin and plakoglobin were used to show the interaction between E-cadherin and plakoglobin. IOSE-364 cells expressing endogenous N-cadherin and plakoglobin were used to confirm the interaction of N-cadherin and plakoglobin. E-cad, E-cadherin; N-cad, N-cadherin; PG, plakoglobin.

2.3.3. The absence of E-cadherin and plakoglobin expression and the presence of N-cadherin contribute to the migratory and invasive properties of ES-2 cells.

Previously, we have shown that the expression of plakoglobin in plakoglobin - deficient carcinoma cells that lack E-cadherin and express N-cadherin decreases their *in vitro* growth, migration and invasion [33, 34]. To examine whether plakoglobin had similar effects in OVCA cells, we first examined the migration and invasion properties of IOSE, OV-90 and ES-2 cells. Then, we exogenously expressed E-cadherin or plakoglobin or knocked down N-cadherin in these cells and assessed changes in their growth, migration and invasion. As depicted in Figure (Figure 2.3A), OV-90 cells showed significantly lower migration and invasion relative to IOSE cells (8.4% and 0.4 %, respectively). In contrast ES-2 cells were significantly more migratory and invasive compare to IOSE cells (138% and 196.4%, respectively).

Exogenous expression of E-cadherin and plakoglobin and stable knockdown of N-cadherin in ES-2 transfectants was confirmed using immunoblot (Figure 2.3B) and immunofluorescence analyses (Figure 2.3C). In ES-2-E-cad cells (Figure 2.3B, C, ES-2-Ecad), E-cadherin was expressed and mainly localized at the membrane although it was also detected in the cytoplasm of the transfectants. Interestingly, plakoglobin expression in ES-2-PG cells (Figure 2.3B, C, ES2-PG) led to the upregulation of endogenous E-cadherin. In these cells, the exogenously expressed plakoglobin colocalized with both N-cadherin and E-cadherin (Figure 3B, C, ES2-PG). N-cadherin knockdown reduced the levels of the endogenous N-cadherin

(>90%). Staining of these cultures with N-cadherin antibodies detected occasional cells that were barely stained (Figure 2.3B, C, ES2-shN-cad). Furthermore, co-immunoprecipitation studies showed that in ES-2-PG cells, N-cadherin interacted with exogenously expressed plakoglobin (Figure 2.3D).

Assessment of the migration and invasion of ES-2 transfectants showed a significant reduction in both migration and invasion of ES-2-Ecad and ES-2-PG cells relative to parental ES-2 cells (Figure 2.4A, B and D). E-cadherin expression in ES-2 cells reduced migration and invasion of these cells by 39% and 42%, respectively. Plakoglobin expression in ES-2 cells decreased migration and invasion by 58% and 44%, respectively. The effect of N-cadherin knockdown on migration was similar to that of plakoglobin expression, i.e. a reduction of 65% whereas the invasion of ES-2-shN-cad cells was significantly less than that of ES-2-E-cad and ES-2-PG cells (68% reduction) (Figure 2.4A, B and D). We also compared the growth of ES-2 cells with those of ES-2-E-cad, ES-2-PG and ES-2-shN-cad transfectants (Figure 2.4C, D). At day 7, ES-2-E-cad cells showed similar growth rate to ES-2 cells while ES-2-PG and ES-2-shN-cad cells showed significantly lower growth than ES-2 cells (21% and 25% reduction, respectively, (Figure 2.4C, D). However, while ES-2-shN-cad cells showed decreased growth throughout the 7 days, ES-2-PG cultures showed decreased cell number after day 5, likely due to the induction of contact inhibition upon culture confluency (Figure 2.4C).

Taken together, these results suggested that expression of E-cadherin or plakoglobin or knockdown of N-cadherin effectively reduced migration and

invasion of ES-2 cells. However, only plakoglobin expression or N-cadherin knockdown significantly decreased the growth of these cells.

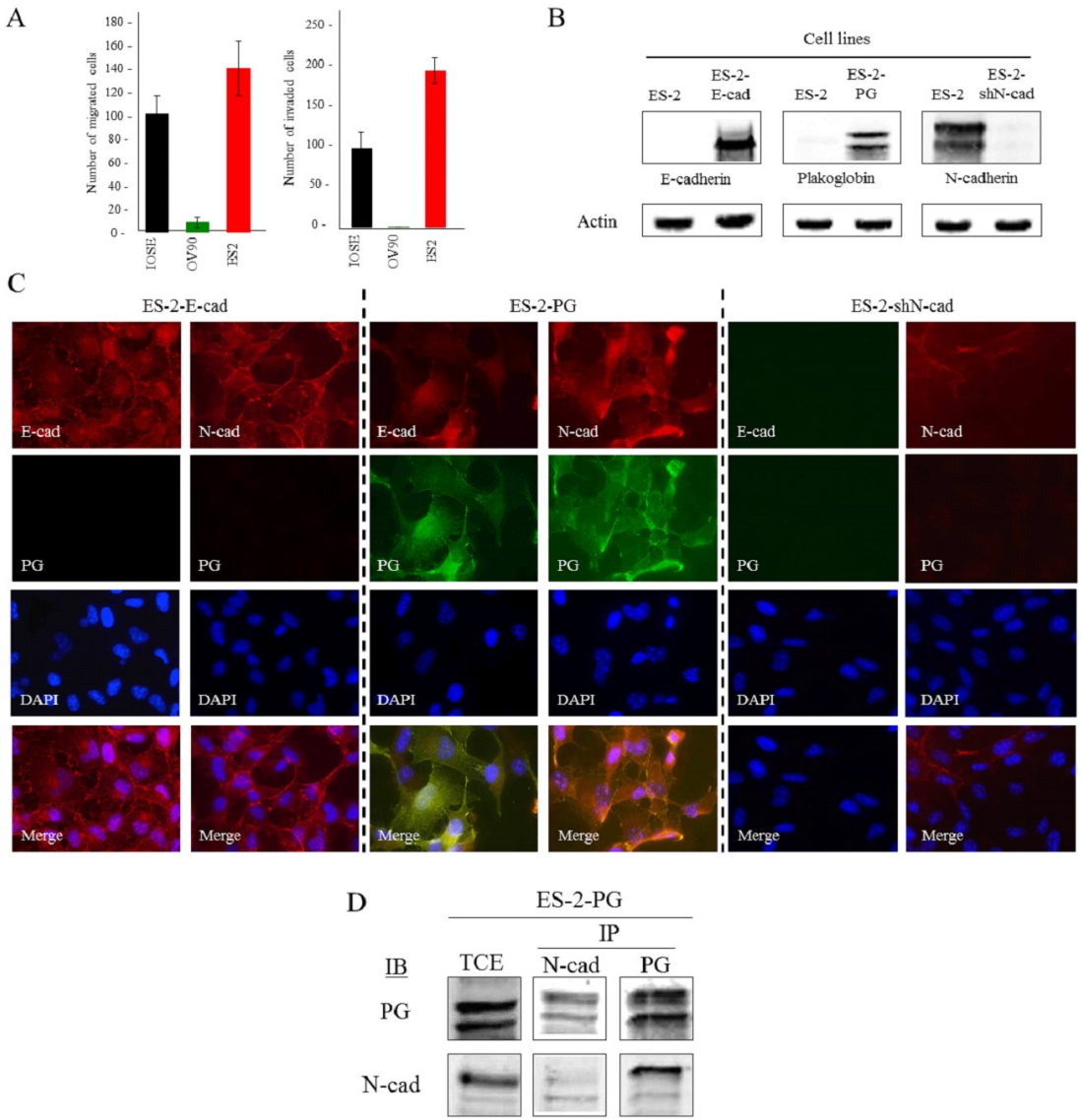
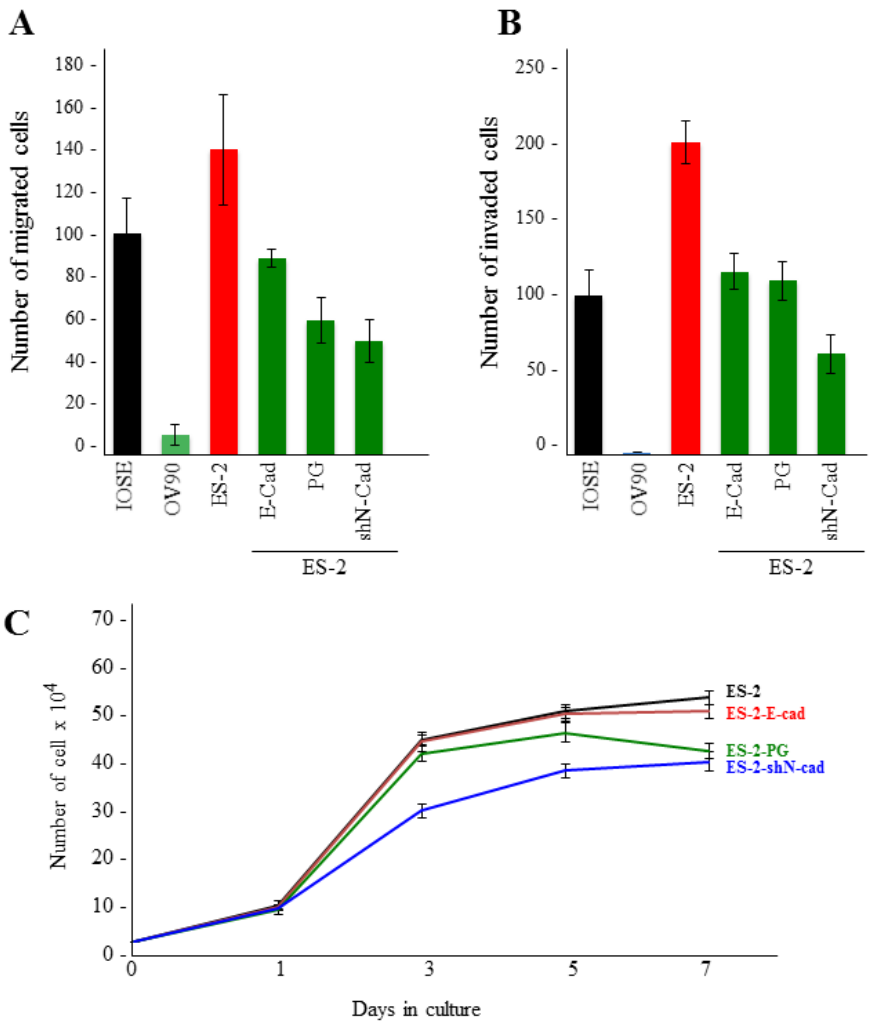


Figure 2.3. (A) migration (Left) and invasion (Right) of IOSE-364, OV-90, ES-2 cells. Cultures were processed for in vitro migration and invasion assays as described in Materials and Methods. The number of migrated/invaded cells were normalized to those of the IOSE-364 cells. **(B) Expression of E-cadherin, N-cadherin and plakoglobin in ES-2 transfectants expressing E-cadherin (ES-2-E-cad) or plakoglobin (ES-2-PG) or N-cadherin shRNAs (ES-2-shN-cad).** Stable transfectants were processed for immunoblotting using E-cadherin, plakoglobin and N-cadherin antibodies. To confirm equal loadings, the same cell lysates were processed with actin antibodies. **(C) Subcellular distribution and colocalization of E-cadherin, N-cadherin and plakoglobin in ES-2- E-cad, ES-2-PG and ES-2-shN-cad transfectants.** Stable transfectants were established on coverslips and processed for double immunofluorescence with E-cadherin **(E-cad, red)** or N-cadherin (**N-cad**, red) and plakoglobin (**PG**, green) antibodies. Nuclei were stained with **DAPI** (blue). Bar, 25 mm. **(D) Interaction of plakoglobin and N-cadherin in ES-2-PG cells.** Total cell extracts from ES-2-PG cells were processed for reciprocal and sequential immunoprecipitation and immunoblotting using N-cadherin and plakoglobin antibodies to confirm the interaction of N-cadherin and plakoglobin. E-cad, E-cadherin; N-cad, N-cadherin; PG, plakoglobin.



D

Cell lines	Decreased migration relative to ES-2	Decreased invasion relative to ES-2	Decreased growth relative to ES-2
ES-2-Ecad	39% **	42% *	5%
ES-2-PG	58% **	44% *	21% **
ES-2-shNcad	65% **	68% *	25% *

Figure 2.4. Migration, invasion and growth properties of normal and carcinoma ovarian cell lines. IOSE-364, OV-90, ES-2 and ES-2 transfectants (ES-2-E-cad, ES-2-PG, ES-2-shN-cad) were processed for **migration** (**A**) and **invasion (B)** assays as described in Materials and Methods. The number of migrated/invaded cells were normalized to those of the IOSE-364 cells. **(C)** Replicate cultures of ES-2 cells and ES-2 transfectants (E-cad, PG and shN-cad) were plated at single cell ($3x10^4$) density. Cultures were counted at day 1, 3, 5 and 7. Each time point is the average of three independent experiments. **(D)** Summary of changes in growth, migration and invasion of ES-2 transfectants. Transfectants values were normalized to ES-2 cells. *p* values, * <0.05, ** < 0.001.

2.3.4. Interaction of plakoglobin and p53 in normal and ovarian carcinoma cell lines

We have shown that plakoglobin interacted with both wild type and mp53 in various carcinoma cell lines and they both associated with promoters of a number of p53 target genes [17, 18, 40]. Plakoglobin's interactions with mp53 expressing carcinoma cells led to decreased growth, migration and invasion of these cells. To this end, we examined whether plakoglobin associated with p53 in OVCA cells. Total cell extract of IOSE, ES-2 and ES-2-PG cells were processed for reciprocal co-immunoprecipitation (co-IP) and immunoblotting with plakoglobin and p53 antibodies (Table 2.1). In IOSE cells plakoglobin antibodies co-precipitated p53 and plakoglobin (Figure 2.5). The reciprocal co-IP using p53 antibodies co-precipitated plakoglobin, further validating the interaction between plakoglobin and p53 in these cells. In ES-2 cells expressing exogenous plakoglobin and endogenous mp53, plakoglobin antibodies co-precipitated p53 and plakoglobin. In the reciprocal co-IP of ES-2-PG cells, p53 antibodies brought down both plakoglobin and p53 (Figure 2.5). In contrast, in ES-2 cells with no plakoglobin expression, p53 antibodies precipitated p53 only (Figure 2.5). Control immunoprecipitations with p53 and plakoglobin pre-immune antibodies did not detect either protein in the total cell lysates (Figure 2.5B).

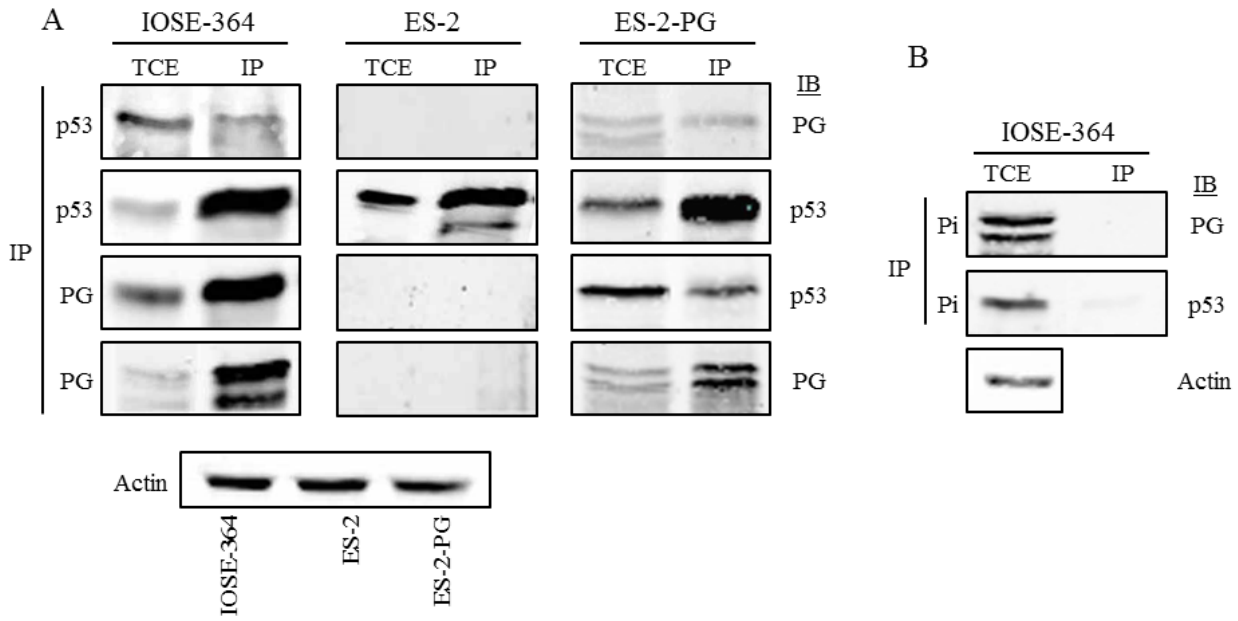


Figure 2.5. Interaction of plakoglobin and p53 in normal and ovarian carcinoma cell lines. Equal amounts of total cell extracts (TCE) from IOSE-364, ES-2 and ES-2-PG cells were processed for receiprocal and sequential immunoprecipitation (IP) and immunoblotting (IB) using p53 and plakoglobin antibodies **(A)** or preimmune antibodies **(B)** as described in Materials and Methods. The same lysates were processed with actin antibodies to confirm equal loadings. PG, plakoglobin; Pi, pre-immune.

2.4. Discussion

In the current study, for the first time, we investigated the *in vitro* tumor/metastasis suppressor effects of plakoglobin in EOC cell lines with mp53 expression and different cadherin profiles. We showed that ES-2 cells that express N-cadherin and are deficient in E-cadherin and plakoglobin were highly migratory and invasive. In contrast, OV-90 cells that express both E-cadherin and plakoglobin and very little N-cadherin were not migratory or invasive. The exogenous expression of plakoglobin or E-cadherin or knockdown of N-cadherin in ES-2 cells significantly reduced their migration and invasion. Our data showed that plakoglobin colocalized with both E-cadherin and N-cadherin in adhesion complexes. Consistent with these observations, we detected significant reduction in ES-2-PG and ES-2-shN-cad growth relative to ES-2 and ES-2-E-cad cells. Furthermore, plakoglobin interacted with wild type p53 in IOSE cells and mp53 in ES-2-PG transfectants.

Cadherin switching from E- to N-cadherin is a critical step in the epithelial to mesenchymal transition (EMT)-mediated malignancies [41, 42]. EMT leads to the cell-cell junction disassembly, loss of cell polarity and gain of migratory and invasive properties [30, 44, 44]. While E-cadherin is an epithelial marker and a known tumor suppressor, N-cadherin is a mesenchymal marker and its expression is associated with a more migratory and invasive phenotype [44, 45]. Normal ovarian surface epithelial (OSE) cells express a combination of epithelial and mesenchymal markers. These cells do not have E-cadherin but express N-cadherin, catenins, vimentin and cytokeratins [36, 46-50]. In agreement with these reports, IOSE cells expressed N-cadherin and vimentin as well as catenins including

plakoglobin, and cytokeratins. The exact role of E-/N-cadherin switch in the initiation and progression of ovarian carcinomas is not very clear since both cadherins can be expressed in ovarian tumors of different origins and at different stages [51, 52]. However, while a few studies suggest that E-cadherin is upregulated in OVCA effusions [52, 53], the great majority suggest that the loss or reduced levels of E-cadherin contribute to the transition from benign to borderline ovarian lesions, to poorly differentiated ovarian tumors, and to the local invasion and metastasis [28, 47, 55-58]. Consistent with the tumor suppressor activities of E-cadherin, downregulation/lack of E-cadherin expression due to the high levels of its transcriptional repressors Snail, Twist and ZEB-2 has been associated with the migratory and invasive properties of ES-2 and other OVCA cells [59-71]. In addition, E-cadherin suppresses growth and metastasis via inhibiting receptor tyrosine kinase signaling and PI3k/Akt pathways [72, 73]. In agreement with these studies, we showed that ES-2-E-cad cells had significantly lower migration and invasion (39% and 42%, respectively) compared to ES-2 cells.

Although N-cadherin is expressed in normal OSE, its expression is generally associated with increased migration and invasion of OVCA [74-76]. N-cadherin levels have been shown to be elevated in cell lines expressing Snail and ZEB-1, as well as, in patients with higher FIGO tumor grade and metastasis [51, 64, 77]. Exogenous expression of MUC4 in SKOV3 cells led to the downregulation of E-cadherin, upregulation of N-cadherin and increased motility. N-cadherin knockdown in these cells reduced MUC4 induced motility, concurrent with decreased activity of ERK1/2, AKT and MMP9 [78]. Supporting these studies, a

selective anti-N-cadherin antibody (Exherin, ADH-1) was recently shown to be effective in stabilizing disease progression in two OVCA patients in a small phase I clinical study, which assessed patients with various solid tumors [79]. Here, we showed that relative to ES-2 cells, the migration and invasion of ES-2-shN-cad transfectants were reduced by 65% and 68%, respectively. Furthermore, knocking down N-cadherin was much more effective in reducing migration and invasion than expressing E-cadherin in ES-2 cells. Similarly, while E-cadherin expression had very little effect (5%) in decreasing growth, plakoglobin expression or N-cadherin knockdown significantly reduced ES-2 cells growth (20%, 25%, respectively).

Unlike cadherins, very little is known about the role of plakoglobin in OVCA. Plakoglobin has been shown to have growth/metastasis inhibitory function, both *in vitro* and *in vivo* [19]. This function of plakoglobin can be mediated by stabilizing /sequestering N-cadherin and induction of contact inhibition of growth and/or interacting with different cellular proteins including transcription factors [17-19, 27,34,40,80-82]. Here, the exogenous expression of plakoglobin significantly reduced migration and invasion of ES-2 cells (58% and 44% respectively). The effect of plakoglobin on inhibiting migration was significantly higher than that of E-cadherin. Since plakoglobin expression is necessary for the formation of both adherens junctions and desmosomes [19,33], this may suggest that plakoglobin reduced migration via association with N-cadherin and formation of junctions as well as interaction with transcription factors and regulation of gene expression. Interaction of plakoglobin with several transcription factors such as TCF/LEF, CBP, SOX4 and p53 has been reported previously [17, 22-26, 81]. We have shown

that plakoglobin interacted with mp53 in several carcinoma cell lines and they both associated with promoters of a number of p53 target genes including tumor suppressors *SFN* (14-3-3s) and *NME1*and the oncogenic genome organizer *SATB1*. Furthermore, these associations were concurrent with reduced growth, migration and invasion [17,18]. Plakoglobin also regulated the expression of HAI-1 and reduced migration in a p53 dependent manner in NSCLC cells [27]. Here, we showed that plakoglobin interacted with wild type p53 in IOSE cells and with mp53 in ES-2-PG cells. p53 regulates the expression of EMT markers such as Twist, Snail and Slug [82-85]. We detected low levels of E-cadherin in ES-2-PG cells upon plakoglobin expression. Whether this E-cadherin expression is due to the downregulation of E-cadherin transcriptional repressors via plakoglobin/p53 interaction or stabilization of E-cadherin protein via its interaction with plakoglobin warrants further studies.

In summary, this is the first demonstration of the role of plakoglobin in OVCA cells. Our data showed that exogenous expression of plakoglobin or knockdown of N-cadherin were more effective than expression of E-cadherin in inhibiting the growth, migratory and invasive properties of ES-2 cells. These results suggest that Plakoglobin expression sequestered tumor/metastasis promoting activities of N-cadherin. Induction of E-cadherin expression in ES-2 cells expressing exogenous plakoglobin, which interacted with the endogenous mp53 raises the possibility that plakoglobin may also be involved in the regulation of p53 target genes involved in migration and invasion. Collectively, the results suggest that plakoglobin may act as a tumor/metastasis suppressor in OVCA, as has been shown for other cancers.

The larger implication of our studies is the potential of plakoglobin as a therapeutic target for the majority of OVCAs with mp53 and N-cadherin expression.

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Chapter three

The Physical Interaction of p53 and Plakoglobin is necessary for Their Synergistic Inhibition of Migration and Invasion

PREFACE

This chapter has been published as: Alaee M*, Padda A*, Mehrabani V, Churchill L, Pasdar M. The physical interaction of p53 and plakoglobin is necessary for their synergistic inhibition of migration and invasion. Oncotarget. 2016;7(18):26898-915.

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Contributions:

I participated in designing and performing several of the experiments and wrote the first draft of the discussion. I generated H1299 stable transfectants expressing plakoglobin-ΔN, -ΔArm, and -ΔC as well as H1299 cells co-expressing plakoglobin and different fragments of p53. I performed western blot and immunofluorescence assays to assess the expression levels and localization of the proteins and co-immunoprecipitation studies to identify the interacting domains. I also performed many of the growth, migration and invasion assays.

3.1. Introduction

The p53 transcription factor is a tumor suppressor that is absent or mutated in over half of all tumors [1-3]. p53 can be activated by various stress signals, including DNA damage, oncogenic insults, hypoxia, loss of cell-cell contact and changes in metabolic behavior. In response to stress, p53 activates physiological pathways that regulate cell cycle arrest, DNA repair, apoptosis, autophagy and metabolism [2, 3]. In addition to being a transcriptional regulator, p53 interacts with various cytoplasmic proteins, which mediate its growth regulating activity [4, 5].

The three structural domains [N-terminus (NT), DNA binding (DBD) and C-terminus (CT)] of p53 regulate its cellular functions. The NT contains two transactivation domains (TAD1 and 2). In addition to binding to coactivators, the NT is also the binding site for Hdm-2, which is an E3-ubiquitin ligase mediating p53 degradation, thus serving as the primary regulator of p53 levels [6, 7]. The CT contains an oligomerization domain, which allows p53 tetramerization, and a short regulatory domain, which may function as a non-specific DNA binding domain necessary for growth arrest and apoptosis [8, 9]. Flanked by the NT and CT, the DBD confers transcriptional activity on p53 and harbors the majority of p53 mutations [1, 10, 11]. p53 functions are regulated by posttranslational modifications and protein-protein interactions [5, 12, 13]. We have identified plakoglobin as an endogenous interacting partner of both wild type and a number of mutant p53s, and have shown that plakoglobin's interaction with these mutants can restore their wild type functions [14, 15].

Plakoglobin is an Armadillo protein family member and a paralog of β-catenin with dual adhesive and signalling functions [16, 17, 18]. Structurally, these proteins consist of a N-terminal α-catenin binding domain, a core of Armadillo (Arm) repeats, which bind adhesive and signalling partners, and a TA domain [18]. In adherens junctions, both β-catenin and plakoglobin mediate cell-cell adhesion by interacting with classic cadherins and α-catenin, which link the complex to the cytoskeleton [18]. Plakoglobin is also an essential desmosomal junction component and as such plays an integral role in cell-cell adhesion [18, 19]. Both β-catenin and plakoglobin affect cell signalling through interactions with intracellular partners involved in cell proliferation, differentiation, survival and apoptosis [18, 19]. Although β-catenin has a well-documented oncogenic function [18], plakoglobin is known to generally act as a tumor/metastasis suppressor by mechanisms that are beginning to be deciphered [19-22]. Our laboratory has shown that the tumor supressor activity of plakoglobin, is, at least in part, mediated by its interaction with p53. We have shown that plakoglobin interacted with p53, and both were associated with the promoters of p53 target genes [e.g. *NME1*, *SFN* (14-3-3σ), *SATB1*, *THBS1*] [14, 15, 20]. Together, these results suggest that the tumor/metastasis suppressor activity of plakoglobin may be mediated by its interaction with p53 and regulation of p53 target genes.

In this study, we assessed the roles of p53 and plakoglobin, individually and together, in cell growth, migration and invasion, and identified the domains of p53 and plakoglobin that mediated their interaction. H1299 and SCC9 cells were co-transfected with expression constructs encoding HA-p53- (wild type, NT, DBD and

CT) and FLAG-plakoglobin- (wild type, ΔN, ΔArm and ΔC). Transfectants were characterized for their growth, migration and invasion. p53/plakoglobin interaction and localization were determined by co-immunoprecipitation and confocal immunofluorescence microscopy. Our results suggested that 1) p53 and plakoglobin cooperated to decrease growth whereas they acted synergistically to significantly reduce migration and invasion of H1299 cells, 2) p53/ plakoglobin interaction was mediated by the DBD of p53 and the C-terminus of plakoglobin, and 3) the C-terminal domain of plakoglobin was necessary for its maximum invasion inhibitory function via interaction with p53.

3.2. Material and methods

3.2.1. Reagents, cells and culture conditions

Chemical reagents were purchased from Sigma-Aldrich and tissue culture reagents from Invitrogen, unless stated otherwise. Dr. Roger Leng, University of Alberta, provided the p53 and plakoglobin null non-small cell lung carcinoma cell line H1299 [21]. The p53 mutant and plakoglobin deficient human tongue squamous cell carcinoma cell line SCC9 has been described [24, 25]. All cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin-kanamycin (PSK) antibiotics.

3.2.2. Plasmid construction and transfection

The FLAG-tagged-plakoglobin (PG) (-wild type (WT), -ΔN, -ΔArm, -ΔC) constructs and their SCC9 transfectants have been described [24]. A plasmid encoding WT-p53 (PGEX2TK-WT-p53, gift from Dr. Roger Leng) served as the

template for constructing HA-tagged p53 WT, and p53 fragments, NT, DBD, and CT.

Various primers (Table 3.1) were used to generate the four p53 inserts by PCR. For all PCR reactions, pre-denaturation was done at 95°C for 2 minutes followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C (first 7 cycles) and 55°C (last 25 cycles) for 30 seconds, and extension at 72°C for 90 seconds. The PCR products were then subcloned into pcDNA 3.1 containing an HA tag at the C-terminus. The pcDNA 3.1 vector was modified with the HA epitope tag sequence (TAC CCA TAC GAT GTT CCA GAT TAC GCT), which contained restriction sites to facilitate the subcloning of the p53 inserts and a stop codon. The constructs encoding HA-tagged p53-WT, NT, DBD, or CT were verified by sequencing.

H1299 or SCC9 cells cultured in 60 mm dishes or on glass coverslips were transfected at 60-80% confluency with 2-10 μg of DNA. Twenty hours later, cells were rinsed and allowed to recover for 24 hour in complete MEM. For transient transfections, transfected cells were processed for different assays 48 hour after transfection. For stable transfectants, 48 hours after transfection, media were replaced with media containing 500 μg/ml hygromycin B (p53) or 400 μg/ml G418 (plakoglobin) and the resistant colonies selected for 2-3 weeks and verified for HA-p53 and FLAG-plakoglobin expression. Positive clones were subcultured by limiting dilution and maintained in media containing 350 μg/ml hygromycin B and 200 μg/ml G418.

Table 3.1. Oligos/primers sequences used to create p53 constructs

Construct	Primers		Size (nucleotide)
p53-WT	Forward	5’ ttt taa gct tat gga gga gcc gca gtc ag 3’	29
	Reverse	5’ ttt tgc ggc cgc gtc tga gtc agg ccc ttc tgt c 3’	34
P53-NT	Forward	5’ ttt taa gct tat gga gga gcc gca gtc ag 3’	29
	Reverse	5’ ttt tgc ggc cgc agg agc tgc tgg tgc agg 3’	30
P53-DBD	Forward	5’ ttt taa gct tat gtc cca agc aat gga tga ttt g 3’	34
	Reverse	5’ ttt tgc ggc cgc ccc ttt ctt gcg gag att ctc 3’	33
P53-CT	Forward	5’ ttt taa gct tat gac cag ctc ctc tcc cca gc 3’	32
	Reverse	5’ ttt tgc ggc cgc gtc tga gtc agg ccc ttc tgt c 3’	34
HA tag	Forward	5’ ttt gct agc atg gcg gcc gca tac cca tac gat gtt cca gat 3’	42
	Reverse	5’ aaa tct aga cta aag ctt agc gta atc tgg aac atc gta 3’	39

3.2.3. Preparation of total cell extracts and immunoblotting

Confluent 100 mm culture dishes were rinsed with cold PBS, solubilized in hot SDS sample buffer (10 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 50 mM dithiothreitol (DTT), 2 mM EDTA, 0.5 mM PMSF) and boiled for 10 minutes. Twenty-five - 50 μg of total cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes and processed for immunoblotting using HA, FLAG and actin primary antibodies followed by the appropriate secondary antibodies (Table 3.2). Membranes were developed by either ECL (Perkin Elmer LAS) or LI-COR IR fluorescence dyes.

Table 3.2. Antibodies and their respective dilutions in specific assays

Primary antibodies	Species	Assay			Company/Catalog number
		WB	IP	IF	
Anti-HA	Rat	1:500	1:150	1:100	Roche/11867423001
Anti-β-Actin	Mouse	1:2000	--	--	Santa Cruz/sc-47778
Anti-FLAG	Mouse	1:500	1:150	1:100	Sigma/F-3165
Secondary antibodies					
HRP anti-mouse, IgG light chain	Goat	1:2000	--	--	Jackson/115-005-174
HRP anti-rat, IgG light chain	Goat	1:2000	--	--	Jackson/112-005-175
Alexa Fluor anti-mouse IgG, light chain	Goat	1:25000	--	--	Jackson/112-625-175
Alexa Fluor anti-rat IgG, light chain	Goat	1:25000	--	--	Jackson/115-625-174
Alexa 488 anti-mouse IgG	Goat	--	--	1:1000	Molecular probes/ A11029
Rhodamine/TRITC anti-Rat IgG	Rabbit	--	--	1:1000	Sigma/T4280

3.2.4. Immunoprecipitation

Confluent cultures in 100 mm plates were rinsed with cold PBS containing 1mM NaF, Na_3VO_4 and $CaCl_2$ and extracted in 2 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.7 ug/ml Pepstatin, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail) for 30 minutes at 4ºC on a rocker. Cells were scraped and centrifuged at 48000 xg for 10 minutes. Supernatants were divided into equal aliquots and processed for immunoprecipitation with FLAG and HA antibodies **(**Table 3.2) and 40 µl protein G agarose (for monoclonal antibodies) or protein A sepharose beads (Pierce Biotechnology) for polyclonal antibodies) beads (Pierce Biotechnology) overnight at 4°C on a rocker-rotator. Samples were then centrifuges at 14000xg for 2 minutes

to separate the beads from the supernatants and the supernatants were processed for a second immunoprecipitation for 2-3 hours. Beads from the two immunoprecipitations were combined and washed three times with the lysis buffer. Immune complexes were solubilized in 40 μl SDS sample buffer, separated by PAGE and processed for immunoblot using HA, FLAG and actin primary antibodies followed by the appropriate secondary antibodies (Table 3.2) as described above.

3.2.5. Immunofluorescence

Cells were grown to confluency on glass coverslips and rinsed twice with cold PBS containing 1 mM NaF, Na_3VO_4 and $CaCl_2$. Cells were then fixed with 3.7% formaldehyde for 20 minutes and extracted with CSK buffer (50 mM NaCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 1.2 mM PMSF, and 1 mg/ml DNase and RNase) for 7 minutes. Coverslips were blocked with 4.0% goat serum and 50 mM NH_4Cl_4 in PBS containing 0.2% BSA (PBS–BSA) for 1 hour and processed for indirect immunofluorescence. Coverslips were incubated in the primary antibodies followed by the species-specific secondary antibodies at concentrations indicated in Table 3.2 for 1 hour and 20 minutes, respectively. All antibodies were diluted in PBS–BSA. Nuclei were counterstained with DAPI (1:2,000) in PBS. Coverslips were mounted in elvanol containing 0.2% (w/v) paraphenylene diamine (PPD) and viewed using a Zeiss confocal microscope.

3.2.6. *In vitro* growth, migration and invasion assays

For growth assays, triplicate cultures of various cell lines were plated in 24-well plates at single cell density (2.5 x $10^4/cm^2$). At 1, 3, 5 and 7 days after plating, cultures were trypsinized and cells counted. Each time point represents the average of three independent experiments.

For cell migration assays, 2×10^5 cells were resuspended in 500 µl serum-free media and plated in the upper chamber of transwell inserts (3 µm pore, 6.5 mm diameter; BD Biosciences). Normal media containing 10% FBS was added to the lower chamber. Cultures were incubated at 37°C in 5% CO_2 for 24 hours to allow cell migration. Inserts were transferred into new dishes and rinsed with PBS to remove un-attached cells. Inserts were fixed with 3.7% formaldehyde (in PBS) for 2 minutes, permeabilized with 100% methanol for 20 minutes and stained with Giemsa stain for 15 minutes at room temperature. Following staining, membranes were cut, mounted using permount (Fisher Scientific), viewed under an inverted microscope using a 20x objective lens and photographed. The migrated cells on the underside of the membranes were counted in 5 random fields from the photographs.

Matrigel invasion assays were performed according to the manufacturer's protocol (BD Biosciences). Cells were starved in serum free media 24 hour prior to plating. For each cell line, 5×10^4 cells in 0.2 ml serum-free media were plated in the top compartment of Matrigel-coated invasion chambers (8 µm pore PETE membrane). Fibroblast conditioned media (0.8 ml) was added to the bottom chambers and plates were incubated overnight at 37°C in 5% CO_2. After 24 hours, membranes were recovered and processed as described for the migration assay. Mounted membranes were viewed under a 20x objective lens of an inverted microscope and

photographed. The invaded cells were counted in 5 random fields for each membrane.

ImageJ Cell Counter program was used to calculate the numbers of migrated/invaded cells. Counted cell numbers were averaged and histograms were constructed after normalizing the average numbers of migrated/invaded cells in each transfected cell line to those of their parental untransfected cells. Each assay was repeated 2-5 independent times.

3.2.7. Statistical analysis

Values are presented as means ± SD. Statistical differences between groups were assessed by Student's t-tests. All experiments were performed at least three times. P-values <0.05 were considered significant.

3.3. Results

3.3.1. Reduced growth, migration and invasion of transfectants expressing p53, plakoglobin or p53 and plakoglobin

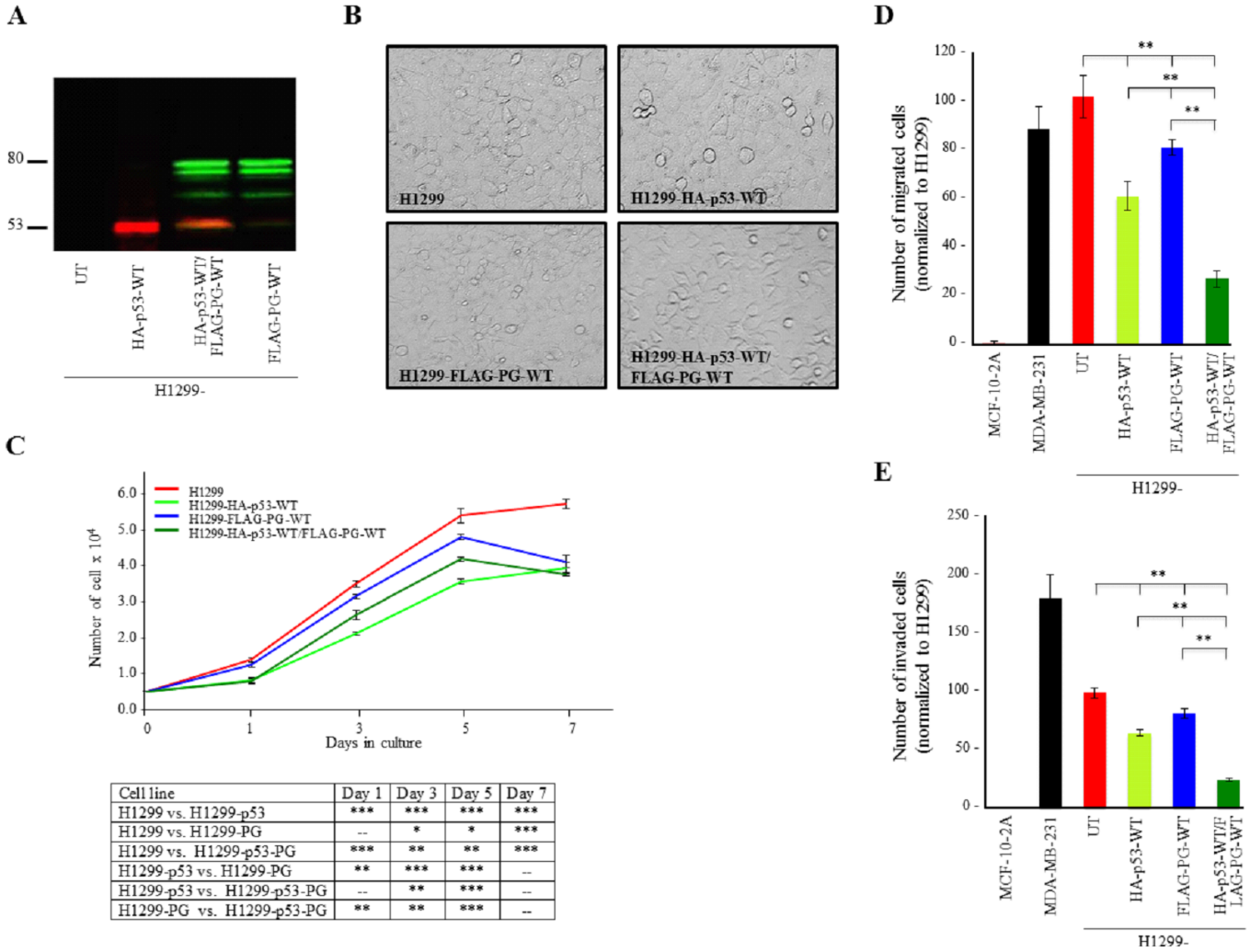
The expression of HA-p53-WT, FLAG-PG-WT and HA-p53-WT/FLAG-PG-WT in single and double transfectant**s** was validated by western blot using anti-HA and anti-FLAG antibodies (Figure 3.1A) or p53 and plakoglobin antibodies (Figure 3.2). Figure 3.1B is a phase micrograph of confluent cultures of H1299 cells and its transfectants expressing HA-p53-WT, FLAG-PG-WT and HA-p53-WT/FLAG-PG-WT. Relative to H1299 cells, HA-p53-WT expressing transfectants were slightly larger and flatter. There were also some rounded, detached and presumably apoptotic cells in these cultures (H1299- HA-p53-WT). In contrast, FLAG-PG-WT

cells appeared to form a tighter monolayer, consistent with the formation of adhesive junctions upon plakoglobin expression in these cells (H1299- FLAG-PG-WT). Interestingly, the double transfectants formed monolayers that were tighter than HA-p53-WT cells but not as tight as FLAG-PG-WT cells and furthermore showed some apoptotic cells (H1299- HA-p53-WT /FLAG-PG-WT) (Figure 3.1B).

The functional consequence of WT p53 and plakoglobin expression were assessed by examining the *in vitro* growth, migration and invasion of H1299 and H1299 transfectants (Figure 3.1C, D, E). Although the H1299-HA-p53 cells showed consistently and significantly less growth than H1299 cells (Figure 3.1C, H1299-HA-p53), the growth of H1299-FLAG-PG and H1299-HA-p53/FLAG-PG transfectants was similar to that of H1299 cells until day 5, when cultures became confluent and cell numbers sharply declined (Figure 3.1C, H1299-FLAG-PG, H1299-HA-p53/FLAG-PG). At day 7, H1299-HA-p53/FLAG-PG cells showed ~40% less growth than H1299 cells, whereas cells expressing either p53 or plakoglobin showed ~30% less growth (Figure 3.1C, Table 3.3).

Individual expression of either p53 or plakoglobin decreased migration by 40% and 21% relative to H1299 cells, respectively, whereas the co-expression of p53 and plakoglobin reduced migration by 73%. (Figure 3.1D, Table 3.3). Similarly, the invasiveness of H1299-HA-p53 and H1299-FLAG-PG cells was decreased by 35% and 21%, respectively, while the invasiveness of H1299-HA-p53/FLAG-PG cells was decreased by ~75% relative to H1299 cells (Figure 3.1D, Table 3.3). These results indicated that co-expression of p53 and plakoglobin synergistically and significantly decreased the migration and invasion of H1299 cells, and were also

consistent with the reduced growth, migration and invasion of SCC9 cells upon the exogenous expression of plakoglobin [15, 24].



Cell line	Day 1	Day 3	Day 5	Day 7
H1299 vs. H1299-p53	***	***	***	***
H1299 vs. H1299-PG	--	*	*	***
H1299 vs. H1299-p53-PG	***	**	**	***
H1299-p53 vs. H1299-PG	**	***	***	--
H1299-p53 vs. H1299-p53-PG	--	**	***	--
H1299-PG vs. H1299-p53-PG	**	**	***	--

Figure 3.1. Growth, migration and invasion of H1299 cells expressing HA-p53, FLAG-PG or HA-p53 and FLAG-PG.
(A) Protein expression of HA-p53 and FLAG-PG in H1299 cells. Total cell lysates from H1299 cells and H1299 cells transfected with HA-p53, FLAG-PG or both were processed for immunoblot with HA and FLAG antibodies at dilutions indicated in Table 3.2. **(B)** Phase contrast micrograph (20x) of confluent cultures of H1299 and H1299-HA-p53, FLAG-PG or HA-p53/FLAG-PG. **(C)** Untransfected (UT), HA-p53 or FLAG-PG or HA-p53/FLAG-PG expressing H1299 cell were plated at single cell density (5×10^4) in replicate cultures and allowed to grow for 7 days. At days 1, 3, 5, and 7 cultures were trypsinized and cells counted. Each time point represents the average of three independent experiments. The absence of error bars at some time points is due to the small differences among the experiments. **(D)** Twenty-four-hour Transwell migration assays were performed in triplicate for the untransfected H1299 cells (UT) and H1299 transfectants expressing HA-p53, FLAG-PG or HA-p53 and FLAG-PG. The membranes were fixed, stained, cut and mounted on slides and viewed under an inverted microscope. MCF-10-2A, a normal and MDA-MB-231, a highly invasive mammary epithelial cell lines were included in the assays as negative and positive controls, respectively. The number of migrated cells in five random fields for each membrane was calculated using the ImageJ Cell Counter program and averaged. Histograms represent the average ± SD of the number of migrated/invaded cells for each cell line. *p* values, * <0.05, ** < 0.001. **(E)** Twenty-four-hour Matrigel invasion assays were performed as described in B using matrigel coated transwell membranes. The number of invaded cells was calculated as described in **B**. PG, plakoglobin.

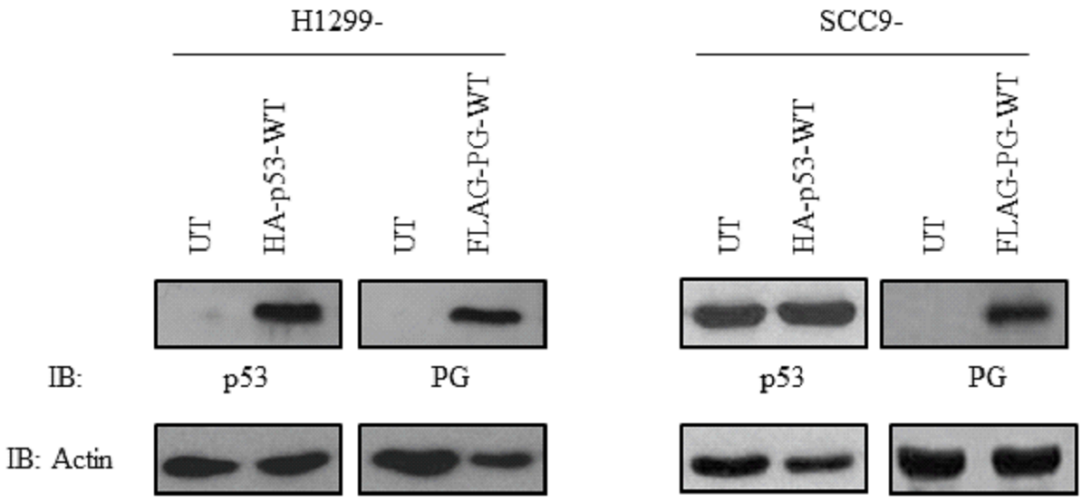


Figure 3.2. Expression of p53 and plakoglobin proteins in H1299 and SCC9 cells. Total cell lysates from untransfected (UT) H1299 and SCC9 cells and their transfectants expressing HA-p53-WT or FLAG-PG-WT were processed for immunoblotting with anti-p53, anti-plakoglobin or anti-actin antibodies. PG, plakoglobin.

3.3.2. Generation and characterization of cell lines expressing wild-type p53 and plakoglobin, various p53 fragments and plakoglobin deletion mutants

To identify the domains of p53 and plakoglobin mediating their interactions, we created constructs encoding various deletions of FLAG-tagged PG, and constructs encoding different fragments of HA-tagged p53 (Figure 3.3). The plakoglobin constructs have been described previously [24,25] and include PG-WT (a.a. 1-745), -ΔN (a.a. 123-745; lacking the α-catenin binding domain), -ΔArm [a.a. 1-216 and 464-745; lacking Armadillo domains 3-7, involved in binding to classic cadherins and adenomatous polyposis coli)] and -ΔC (a.a. 687-745; lacking the TA). All plakoglobin constructs contained a C-terminal FLAG tag (Figure 3.3A, left), and were previously characterized in SCC9 cells [25]. These constructs were transfected into H1299 cells and their expression was verified by immunoblotting with FLAG antibodies (Figure 3.3A, right).

Constructs encoding C-terminally HA-tagged WT and fragments of p53 were generated, including p53-WT (a.a. 1-393), -NT [a.a. 1- 96; containing both TAs (a.a. 1-42; 43-92), the nuclear export signal (a.a. 11-27) and the proline-rich domain (a.a. 64-92)], -DBD [a.a. 51-309; including the second TAD, proline-rich domain, and entire DBD (a.a. 101-300)], and -CT [a.a. 312-393; containing the 3 nuclear localization sequences (a.a. 305-322; 369-375; 379-384), tetramerization domain (a.a. 326-356), and regulatory domain (a.a. 364-393)] (Figure 3.3B, left). The HA-p53 constructs were transfected into H1299 cells and protein expression was confirmed by immunoblotting with HA antibodies (Figure 3.3B, right).

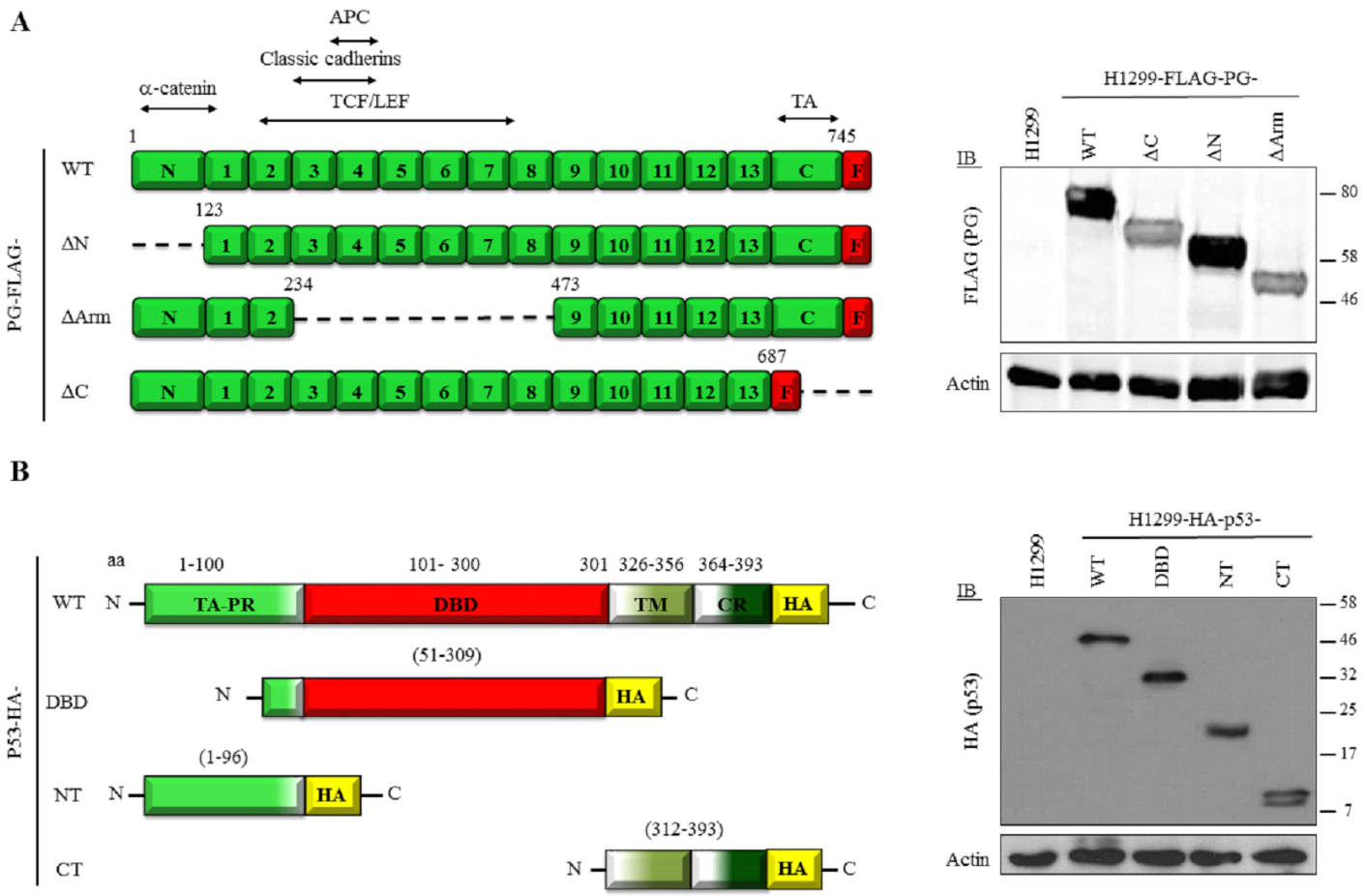


Figure 3.3. (A) (Left) Domain structure of FLAG-tagged plakoglobin and plakoglobin deletion proteins. (Right) Expression of FLAG-tagged PG proteins in H1299 cells. H1299 cells were transfected with expression constructs encoding FLAG-tagged **PG-WT**, **?N, ?Arm** or **?C**. FLAG-PG-expressing stable cell lines were processed for immunoblot using **FLAG** and **Actin** (loading control) antibodies. APC, Adenomatous polyposis coli; TCF/LEF, T-cell factor/lymphoid enhancer factor; TA, Transactivation domain; F, FLAG tag.

(B) (Left) Domain structure HA-tagged p53 WT and deletion proteins. (Right) Expression of HA-tagged p53 proteins in H1299 cells. H1299 cells were transfected with constructs encoding **HA-tagged p53-full length (WT)**, DNA binding domain **(DBD)**, N-terminus **(NT)** and C-terminus **(CT)**. p53-expressing stable cell lines were processed for immunoblot using **HA** and **Actin** (loading control) antibodies. TA, Transactivation; PR, proline-rich; TM, Tetramerization; CR, C-terminal regulatory domain; HA, HA tag.

3.3.4. Expression of HA-p53 and FLAG-plakoglobin proteins in H1299 double transfectants

To study p53 and plakoglobin interaction, we generated H1299 double transfectants coexpressing HA-p53-WT with FLAG-PG-WT, -ΔN, -ΔArm or -ΔC or -FLAG-PG-WT with HA-p53-WT, -NT, -DBD or -CT. Protein expression in H1299 (Figure 3.4 A, B) double transfectants was confirmed by immunoblotting with HA and FLAG antibodies (Figure 3.4 A, B).

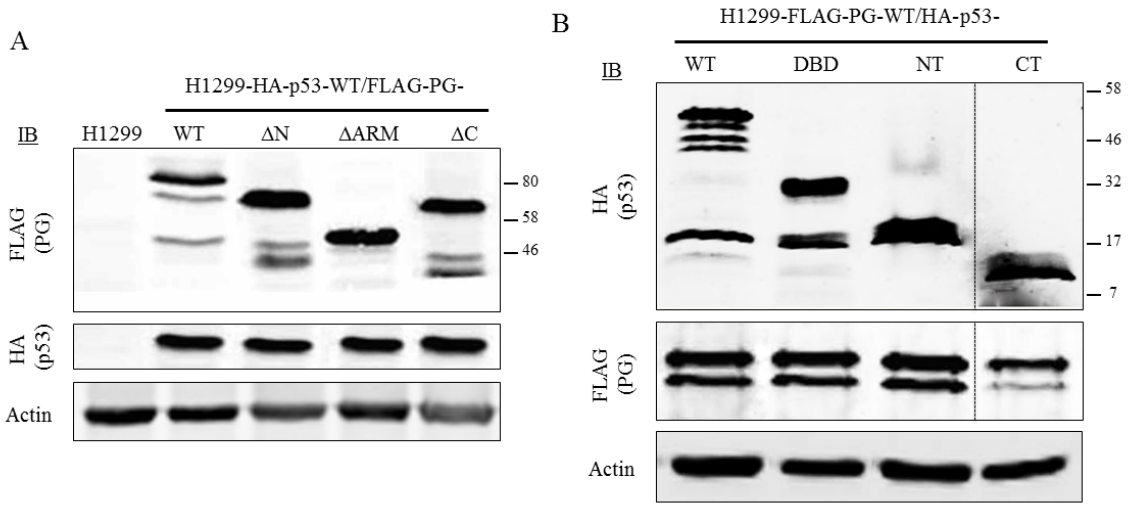


Figure 3.4. Protein expression of WT and fragments of p53 and plakoglobin in double transfectants. Equal amounts of total cellular proteins from stable H1299-HA-p53-WT transfectants co-expressing FLAG-PG-WT, - or ΔN, -ΔArm or -ΔC **(A)** or H1299-FLAG-PG-WT co-expressing HA-p53 WT, -NT, -DBD or -CT **(B)** were processed for immunoblots with HA or FLAG antibodies as described in Materials and Methods. PG, plakoglobin; WT, wild type; N, N-terminus; C, C-terminus; Arm, armadillo; DBD, DNA binding domain.

3.3.5. DNA binding domain of p53 and the C-terminal domain of plakoglobin mediate p53/plakoglobin interactions

H1299 double transfectants co-expressing various pairs of HA-p53 and FLAG-PG proteins/fragments were processed for reciprocal co-immunoprecipitation and immunoblotting with HA and FLAG antibodies. Figure 3.5A shows the co-immunoprecipitation results with H1299 cells expressing HA-p53-WT together with FLAG-PG-WT, -ΔN, -ΔArm or -ΔC. In lysates from these transfectants, FLAG antibodies co-precipitated HA-p53-WT with FLAG-PG-WT, -ΔN and -ΔArm, but not with FLAG-PG-ΔC. The reciprocal co-immunoprecipitation using HA antibodies confirmed these findings, as FLAG-PG-ΔC was the only FLAG-PG fragment that was not co-precipitated with HA-p53-WT. These results suggested that the C-terminus domain of plakoglobin is necessary for p53/plakoglobin interactions (Figure 3.5A). When H1299 cells expressing FLAG-PG-WT with HA-p53-WT, -NT, -DBD or -CT were subjected to reciprocal co-immunoprecipitation, FLAG antibodies co-precipitated HA-p53-WT and -DBD, but not HA-p53-NT or -CT (Figure 3.5B). These results were confirmed when HA antibodies co-precipitated FLAG-PG-WT with HA-p53-DBD, but not HA-p53-NT or -CT (Figure 3.5B). Taken together, these results suggest that the C-terminus of plakoglobin, and the DBD of p53 mediate p53/plakoglobin interaction.

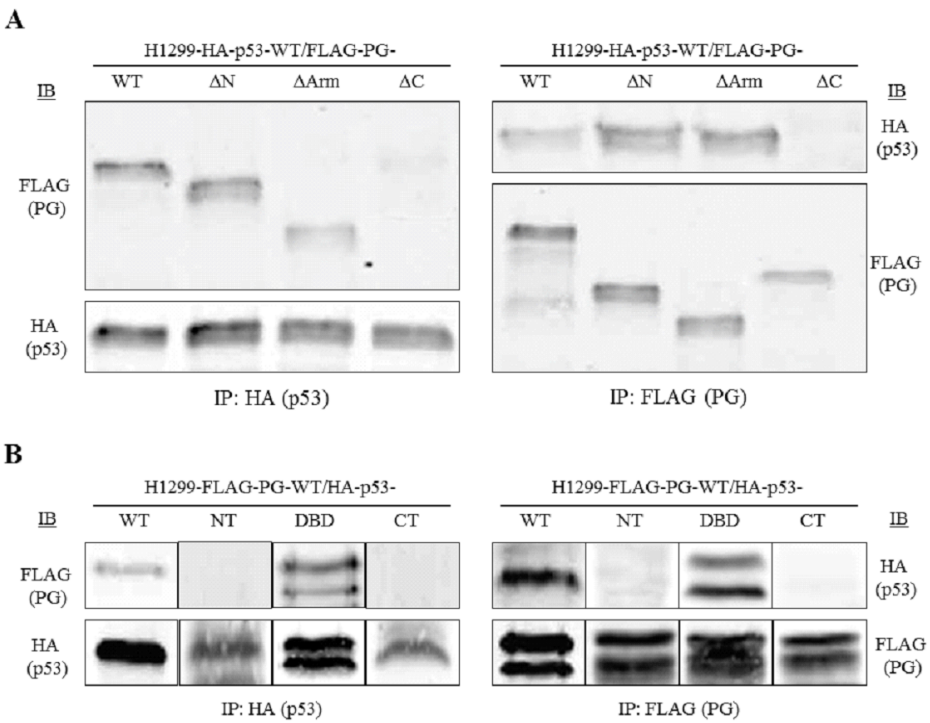


Figure 3.5. DNA binding domain of p53 interacts with the C-terminal domain of plakoglobin. Equal amounts of total cell extracts from double transfectants co-expressing HA-p53-WT and various FLAG-tagged PG proteins **(A)** or FLAG-PG-WT and various HA-tagged p53 proteins **(B)** were processed for reciprocal and sequential immunoprecipitation and immunoblotting using HA and FLAG antibodies as described in Materials and Methods. The immune complexes in **A** were separated on 7.5%, and in **B** on 5-20% SDS gradient gels. PG, plakoglobin; WT, wild type; N, N-terminus; C, C-terminus; Arm, Armadillo; DBD, DNA binding domain.

3.3.6. Subcellular location of p53 and plakoglobin in H1299-HA-p53 and H1299-FLAG-PG transfectants

We previously demonstrated that p53 and plakoglobin interacted in both the cytoplasm and nucleus [14]. Here, HA-p53 and FLAG-PG transfectants were processed for immunofluorescence using HA and FLAG antibodies. Figure 3.6A shows the subcellular localization of HA-p53 in various H1299-HA-p53 transfectants. In HA-p53-WT transfectants, p53 was primarily nuclear, with a faint cytoplasmic distribution (Figure 3.6A, H1299-HA-p53-WT). In contrast, p53 was distributed mainly in the cytoplasm of H1299-HA-p53-DBD transfectants with very little nuclear staining (Figure 3.6A, H1299-HA-p53-DBD). In H1299-HA-p53-NT transfectants, p53 was mainly cytoplasmic, with a distinct peri-nuclear distribution (Figure 3.6A, H1299-HA-p53-NT). Finally, in HA-p53-CT transfectants, p53 was detected exclusively in the nucleus, resembling the HA-p53-WT transfectants (Figure 3.6A, H1299-HA-p53-CT). Collectively, these results are consistent with the presence of the nuclear localization sequence in p53-WT and -CT, and its absence in p53-DBD and -NT.

H1299 cells expressing FLAG-PG-WT or its three deletions showed different PG staining and cell morphology (Figure 3.6B). H1299-FLAG-PG-WT transfectants exhibited typical epithelial morphology and extensive cell-cell contact, with plakoglobin localized primarily to the areas of cell-cell contact (Figure 3.6B, H1299-FLAG-PG-WT). H1299-FLAG-PG-ΔN and H1299-FLAG-PG-ΔArm transfectants had numerous processes and little cell-cell contact, consistent with these fragments lacking the ability to interact with α-catenin and cadherins and

localize to adhesive junctions. In these transfectants, FLAG-PG-ΔN and FLAG-PG-ΔArm were mainly detected throughout the cytoplasm, without any distinct membrane staining (Figure 3.6B, H1299-FLAG-PG-ΔN, -FLAG-PG-ΔArm). In contrast, FLAG-PG-ΔC transfectants showed epithelial morphology, but were flatter than H1299-FLAG-PG-WT cells. In these cells, PG-ΔC was localized to the areas of cell-cell contact and cytoplasm, but was clearly excluded from the nucleus (Figure 3.6B, H1299-FLAG-PG-ΔC). Together, these results suggest that the C-terminus of plakoglobin may be necessary for its nuclear localization.

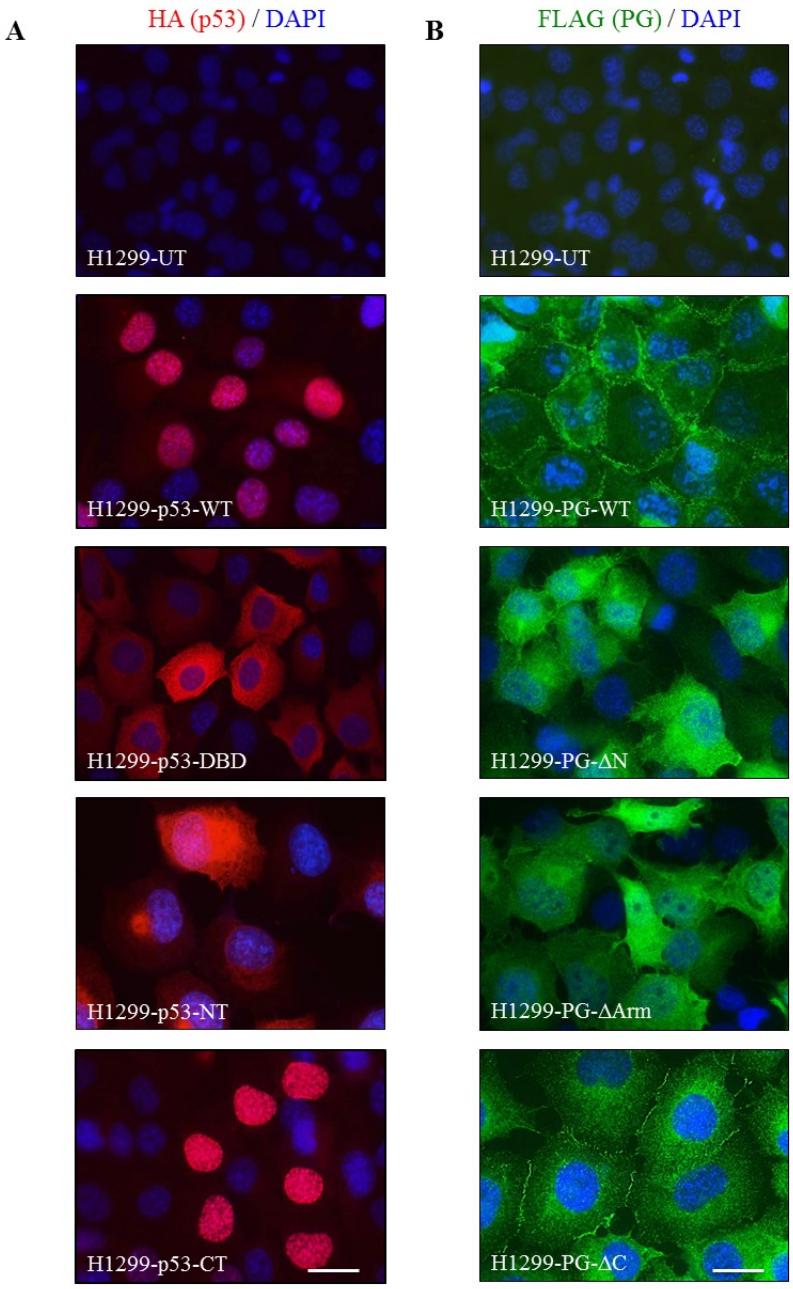


Figure 3.6. Subcellular localization of HA-tagged p53 (A) and FLAG-tagged plakoglobin (B) proteins in H1299 cells. H1299 cells expressing various FLAG-PG and HA-p53 proteins were grown to confluency on coverslips, fixed with formaldehyde and permeabilized with CSK buffer. Coverslips were processed for confocal immunofluorescence microscopy using FLAG (green) and HA (red) antibodies. Nuclei were counterstained with DAPI (blue) and coverslips mounted and viewed using a Zeiss confocal microscope. PG, plakoglobin; WT, wild type; N, N-terminus; C, C-terminus; Arm, Armadillo; DBD, DNA binding domain. Bar, 40 μm.

3.3.7. Subcellular distribution of plakoglobin and p53 in H1299 double transfectants expressing FLAG-PG-WT and HA-p53-WT, -NT, -DBD or -CT

In HA-p53-WT and FLAG-PG-WT co-transfectants, p53-WT was primarily nuclear with faint cytoplasmic staining, whereas plakoglobin was localized to the areas of cell-cell contact as well as in the cytoplasm and nucleus. There was an overlap between the nuclear p53 and the nuclear plakoglobin staining in these cells (Figure 3.7, H1299-FLAG-PG-WT/HA-p53-WT). Membrane and cytoplasmic distribution of plakoglobin was also detected in H1299-FLAG-PG-WT/HA-p53-NT transfectants, in which p53-NT distribution was almost exclusively cytoplasmic/perinuclear, overlapping with the cytoplasmic plakoglobin staining. Nuclear plakoglobin was not detected in these cells (Figure 3.7, H1299-FLAG-PG-WT/HA-p53-NT). In H1299-FLAG-PG-WT/HA-p53-DBD cells, plakoglobin was primarily membrane localized, whereas p53-DBD was primarily cytoplasmic and overlapped with a pool of cytoplasmic plakoglobin (Figure 3.7, H1299-FLAG-PG-WT/HA-p53-DBD). FLAG-PG-WT/HA-p53-CT transfectants showed membrane localization of plakoglobin with some homogeneous cytoplasmic staining, whereas p53-CT was almost exclusively nuclear. No overlap was detectable in the distribution of the two proteins (Figure 3.7, H1299-FLAG-PG-WT/HA-p53-CT). These observations are consistent with the presence of nuclear localization signals in p53-CT and suggest that plakoglobin was co-distributed only with the p53-WT and p53-DBD, albeit in the cytoplasm.

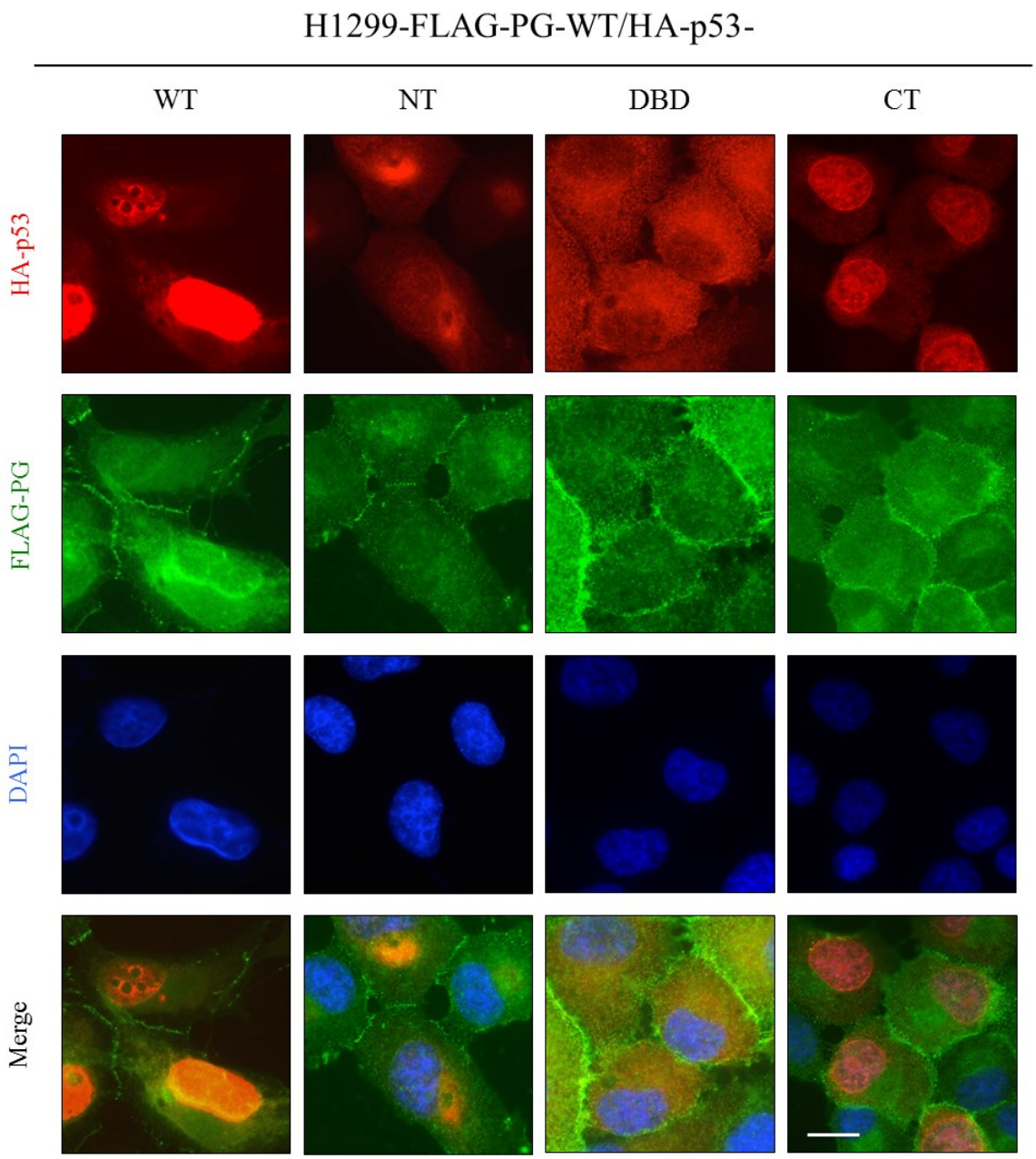


Figure 3.7. Subcellular localization of plakoglobin and p53 in H1299 double transfectants co-expressing FLAG-PG-WT and HA-p53-WT, -NT, -DBD or -CT. Cultures were processed for double immunofluorescence with FLAG and HA antibodies. Cells were grown to confluency on coverslips, fixed with formaldehyde and permeabilized with CSK buffer. Coverslips were processed for confocal immunofluorescence microscopy using FLAG (green) and HA (red) antibodies. Nuclei were counterstained with DAPI (blue) and coverslips mounted and viewed using a Zeiss confocal microscope. WT, wild type; PG, plakoglobin; NT, N-terminus; CT, C-terminus; DBD, DNA binding domain. Bar, 25 μm.

3.3.8. Subcellular distribution of plakoglobin and p53 in SCC9 double transfectants expressing HA-p53-WT and FLAG-PG-WT, -ΔN, -ΔArm or -ΔC

In SCC9 cells expressing HA-p53-WT and FLAG-PG-WT, the distribution of p53 and plakoglobin was similar to that of H1299-FLAG-PG-WT/HA-p53-WT cells. plakoglobin was detected at the membrane, and in the cytoplasm and nucleus. Nuclear plakoglobin was co-distributed with p53, which was almost exclusively nuclear (Figure 3.8A, SCC9-HA-p53-WT/FLAG-PG-WT). In the HA-p53-WT/FLAG-PG-ΔN transfectants, PG-ΔN was detected throughout the cells, overlapping in distribution with p53, which was detected in both the cytoplasm and nucleus (Figure 3.8A, SCC9-HA-p53-WT/FLAG-PG-ΔN). In HA-p53-WT/FLAG-PG-ΔArm transfectants, PG-ΔArm was detected throughout the cell, while p53 was primarily nuclear with some cytoplasmic distribution. In these cells, p53 was co-distributed with PG-ΔArm in both the cytoplasm and nucleus (Figure 3.8A, SCC9-HA-p53-WT/FLAG-PG-ΔArm). In contrast to the FLAG-PG-WT, -ΔN or -ΔArm transfectants in which plakoglobin was detected in the nucleus, FLAG-PG-ΔC transfectants had no detectable nuclear PG-ΔC. Due to the exclusively nuclear distribution of p53 in these cells, no overlap of p53 and PG-ΔC was detected (Figure 3.8A, SCC9-HA-p53-WT/FLAG-PG-ΔC). Collectively, these results suggested that the C-terminus of plakoglobin is necessary for its localization to the nucleus and its colocalization with p53.

Protein expression in SCC9 (Figure 3.8B) double transfectants was confirmed by immunoblotting with HA and FLAG antibodies (Figure 3.8B).

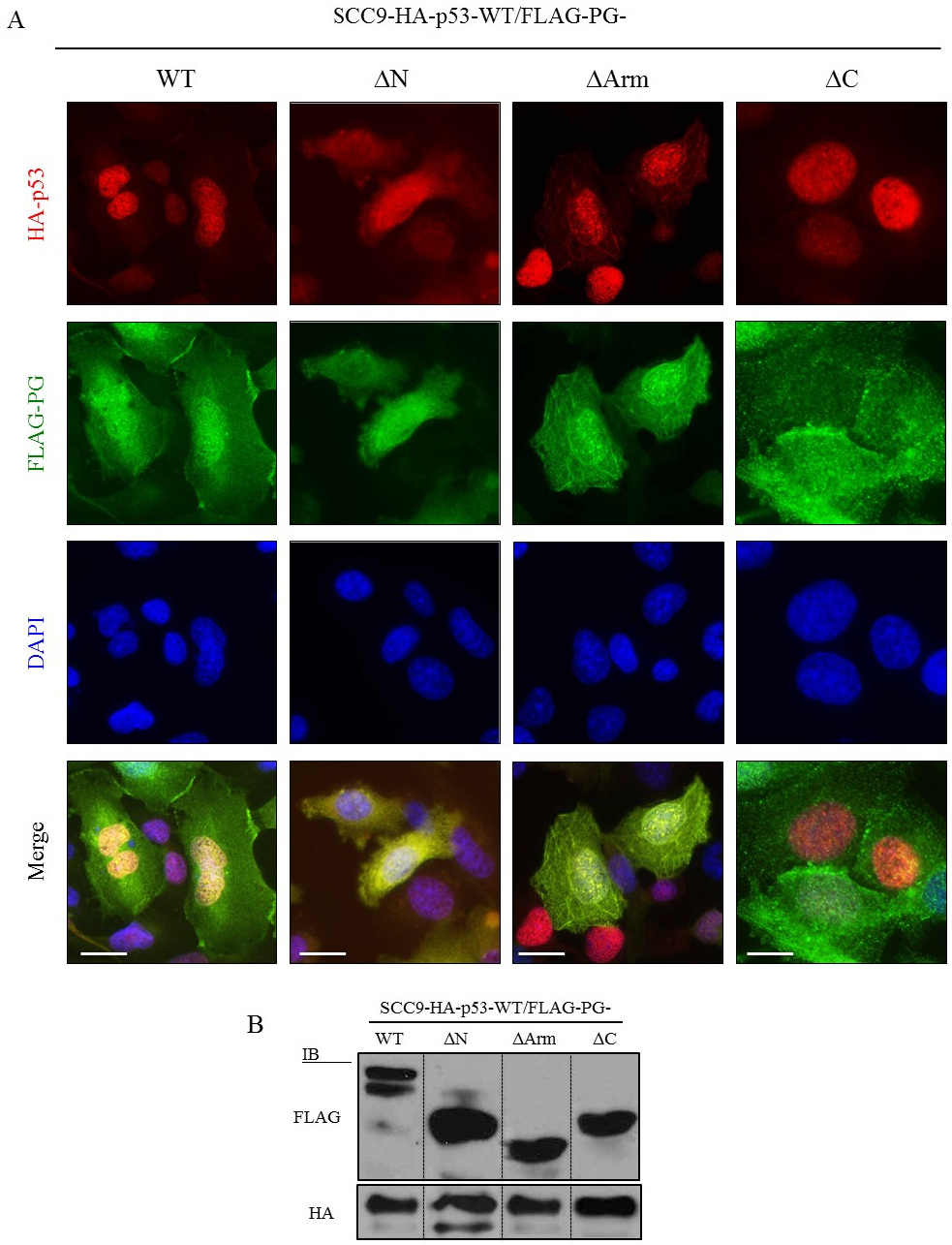


Figure 3.8. Subcellular localization of plakoglobin and p53 in SCC9 double transfectants co-expressing HA-p53-WT and FLAG-PG-WT, -ΔN, -ΔArm or -ΔC. (A) Cultures were processed for double immunofluorescence with FLAG (green) and HA (red) antibodies. Nuclei were counterstained with DAPI (blue). WT, wild type; PG, plakoglobin; N, N-terminus; C, C-terminus; Arm, Armadillo. Bar, 25 μm (HA-p53-WT and FLAG-PG-WT, -ΔN, -ΔArm) and 15 μm (HA-p53-WT and FLAG-PG -ΔC). **(B)** Total cell lysates from SCC9 cells expressing HA-p53-WT and FLAG-PG-WT, -ΔN, ΔArm or -ΔC were processed for immunoblots with FLAG and HA antibodies.

3.3.9. Cooperation of p53 and plakoglobin in regulating growth, migration and invasion of H1299 cells

We also investigated the role of various structural domains of p53 and plakoglobin in their combined inhibition of the growth, migration and invasion of H1299 cells. *In vitro* growth assays showed a small reduction (~10%) in the growth of transfectants expressing FLAG-PG-WT and p53-NT, -DBD or -CT compared to H1299 cells. In comparison, the growth of H1299-HA-p53-WT/FLAG-PG-WT cells was reduced by ~40% (Figure 3.9; Table 3.3). In contrast, the growth of H1299 cells expressing HA-p53-WT and FLAG-PG-ΔN, -ΔArm or -ΔC was the same or slightly less than H1299-HA-p53-WT/FLAG-PG-WT cells (Figure 3.9; Table 3.3).

Figure 3.10A shows the effect of various p53 domains on cell migration in a FLAG-PG-WT background. The co-expression of HA-p53-WT and FLAG-PG-WT reduced the migration of H1299 cells by >70% compared to parental H1299 cells (Figures 3.1B, 3.10A, Table 3.3). Cells co-expressing FLAG-PG-WT and various HA-p53 fragments (H1299-FLAG-PG-WT/p53-NT, -DBD, -CT) were more migratory than H1299-FLAG-PG-WT/p53-WT cells, but significantly less than H1299 cells (Figure 3.10A, Table 3.3). Among the fragments, HA-p53-DBD transfectants were less migratory than HA-p53-NT or CT transfectants, which had similar migration levels (Figure 3.10A, Table 3.3).

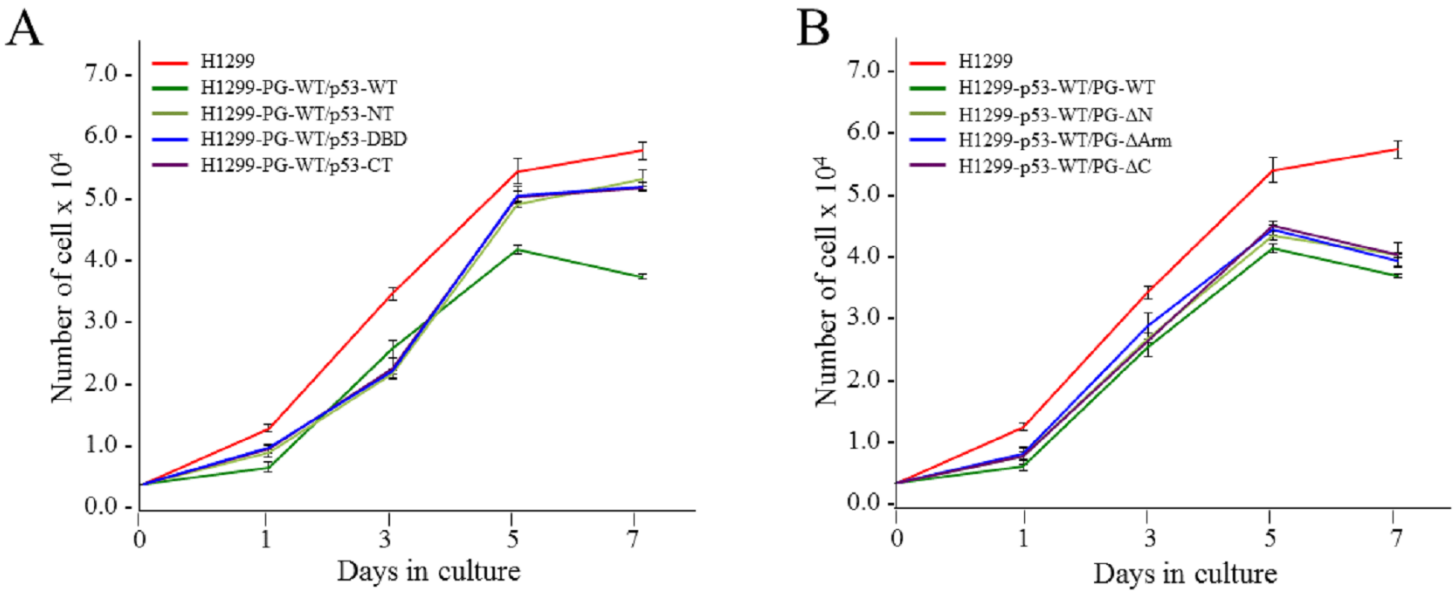
In a HA-p53-WT background, while cells expressing FLAG-PG deletions were less migratory than H1299 cells, they were significantly more migratory than FLAG-PG-WT transfectants. When compared, H1299-HA-p53-WT/-FLAG-PG-WT double transfectants were >70% less migratory than H1299 cells. H1299-HA-p53-

WT/-FLAG-PG-ΔN, -ΔArm, -ΔC double transfectants showed reduced migration by 18%, 25% and 29%, respectively (Figure 3.1B, 3.10B, Table 3.3).

Invasion assays showed that H1299-HA-p53-WT/FLAG-PG-WT double transfectants, were 75% less invasive than H1299 cells (Figure 3.1C, 3.10C, Table 3.3). The expression of any of the HA-p53 fragments in a FLAG-PG-WT background (H1299-FLAG-PG-WT/p53-NT, -DBD, -CT) showed increased invasiveness (Figure 3.10C, Table 3.3), although these transfectants were still less invasive than the H1299 cells (Figure 3.10C, Table 3.3).

Finally, HA-p53-WT/FLAG-PG-ΔN and -ΔArm double transfectants showed a decrease in invasiveness that was comparable to the FLAG-PG-WT transfectants (67% and 70% vs. 73%), whereas HA-p53-WT/FLAG-PG-ΔC transfectants were significantly more invasive (27% vs. 74%) (Figure 3.10D, Table 3.3).

Together, the results in Figures 3.1, 3.9 and 3.10, and Table 3.3 suggested that: 1) individual expression of either p53 or PG reduced the growth, migration and invasion of H1299, 2) p53 alone was more effective than PG alone, 3) the greatest reduction was attained when both proteins were expressed, 4) the PG C-terminus domain was necessary for the inhibition of invasion.



Cell line	Day			
	1	3	5	7
H1299 vs. H1299-p53-WT/PG-WT	**	*	*	**
H1299 vs. H1299-PG-WT/p53-NT	*	**	*	*
H1299 vs. H1299-PG-WT/p53-DBD	*	*	*	*
H1299 vs. H1299-PG-WT/p53-CT	*	**	*	*

Cell line	Day			
	1	3	5	7
H1299 vs. H1299-p53-WT/PG-WT	**	*	*	**
H1299 vs. H1299-p53-WT/PG-ΔN	*	**	*	*
H1299 vs. H1299- p53-WT/PG-ΔArm	*	*	*	**
H1299 vs. H1299- p53-WT/PG-ΔC	*	*	*	**

Figure 3.9. Contribution of various p53 and plakoglobin domains to their growth inhibitory function. H1299 and H1299 transfectants co-expressing PG-WT and p53-WT, NT, -DBD, or -CT (**A**), or p53-WT and PG-WT, -? N, -? Arm or -? C **(B)** were plated at single cell density (5×10^4) in replicate cultures and allowed to grow for 7 days. At days 1, 3, 5, and 7 cultures were trypsinized and cells counted. Each time point represents the average of three independent experiments. The absence of error bars at some time points is due to the small differences among the experiments. * <0.05, ** < 0.001.

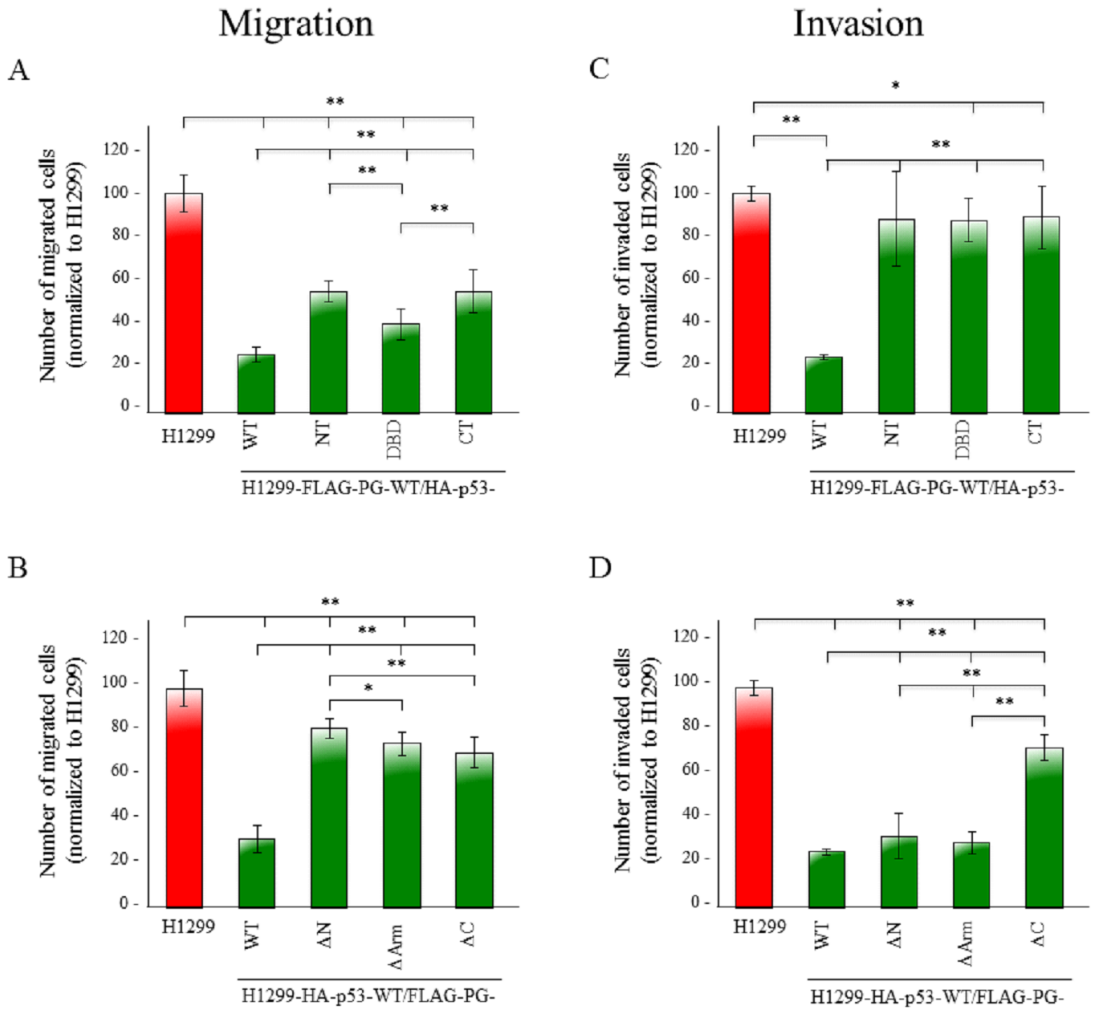


Figure 3.10. Contribution of various p53 and plakoglobin domains to their synergistic inhibition of migration and invasion. H1299 and H1299 transfectants expressing FLAG-PG-WT and HA-p53-WT, -NT, -DBD, or -CT **(A, C)**, or HA-p53-WT and FLAG-PG-WT, -?N, -?Arm or -?C **(B, D)**, were processed for migration **(A, B)** and invasion **(C, D)** assays as described in the legends to Figure 3.1C. All values were normalized to H1299 cells. *p* values, * <0.05, ** < 0.01.

Table 3.3. Summary of changes in the growth, migration and invasion of H1299 transfectants expressing various combinations of p53 and PG constructs. *p* values, * <0.05, ** < 0.001.

Cell line	% Decreased growth (day 7) Relative to H1299	% Decreased migration Relative to H1299	% Decreased invasion Relative to H1299
H1299-HA-p53-WT	32**	40**	34**
H1299-FLAG-PG-WT	28**	21**	18**
H1299-HA-p53-WT/FLAG-PG-WT	40**	73**	75**
H1299-FLAG-PG-WT/HA-p53-NT	9**	45**	12*
H1299-FLAG-PG-WT/HA-p53-DBD	10**	60**	12*
H1299-FLAG-PG-WT/HA-p53-CT	9**	45**	11*
H1299-HA-p53-WT/FLAG-PG-ΔN	35**	18**	67**
H1299-HA-p53-WT/FLAG-PG-ΔArm	31**	25**	70**
H1299-HA-p53-WT/FLAG-PG-ΔC	28**	29**	27**

3.4. Discussion

We showed that p53 and plakoglobin cooperatively reduced growth and acted synergistically to decrease cellular migration and invasion. The two proteins interacted with each other via the DNA-binding domain of p53 and the transactivation domain of plakoglobin.

p53 prevents cancer development and progression by transcriptionally regulating genes involved in cell cycle arrest, senescence and cell death/apoptosis [26,27]. p53 also has transcription/nuclear-independent growth inhibitory functions, the most well-characterized of which is the induction of apoptosis [4, 28-32].

H1299-HA-p53-WT transfectants showed significantly less growth, migration and invasion. These effects are mediated by the p53 regulation of expression of various tumor suppressors, signaling molecules and oncogenic and tumor suppressor miRNAs [33-41]. p53 also promotes stable junction formation and cadherin-mediated contact inhibition by downregulating transcriptional repressors of E-cadherin and regulating cytoskeleton remodeling [42-45].

H1299-FLAG-PG-WT cells also showed significant reductions in growth, migration and invasion. Unlike H1299-HA-p53 transfectants in which growth was decreased from day 1, the H1299-FLAG-PG cells showed similar growth kinetics to that of H1299 cells until day 5, when cell numbers declined due to the induction of contact inhibition of growth. Plakoglobin can also suppress tumor growth by inducing apoptosis [46-49]. These findings are consistent with the role of plakoglobin as an essential regulator of cell-cell adhesion and growth [19, 46-49].

Numerous *in vitro* and *in vivo* studies have shown that plakoglobin has tumor/metastasis suppressor activities. The loss of heterozygosity and low frequency mutations in the plakoglobin gene was shown to predispose patients to familial breast and ovarian cancers [50]. Plakoglobin knockdown in human umbilical vein endothelial cells promoted migration, tubular formation and angiogenesis [51]. Since these early studies, plakoglobin's role in the inhibition of migration and invasion has been demonstrated in many carcinoma cell lines [15, 19,22,52-56]. Consistent with these *in vitro* observations, loss/changes in plakoglobin levels and localization are associated with increased metastasis and poor prognosis *in vivo* [19].

Plakoglobin also acts as a tumor/metastasis suppressor independent of its role in cell-cell adhesion. Plakoglobin null keratinocytes expressing exogenous PG-WT, -ΔN or -ΔC showed similar adhesiveness but different migratory properties. Although PG-WT and -ΔN transfectants were not migratory, PG-ΔC transfectants became migratory via activation of Src signaling [54], suggesting that the TA is essential for the tumor/metastasis suppressor activity of plakoglobin. Plakoglobin may regulate gene expression independent of its role in cell-cell adhesion via interaction with transcription factors including TCF/LEF, CBP, SOX4 and p53 [14, 21, 57-62]. We previously showed that plakoglobin interacted with both WT and several mutant p53s in various carcinoma cell lines, leading to the induction of a non-transformed phenotype. This phenotypic transition coincided with changes in the expression of several p53 target genes, the promoters of which interacted with both p53 and plakoglobin [14, 15]. Recently, Sechler *et al.* (2015) reported that

plakoglobin overexpression in NSCLC cells reduced cell migration via HAI-1 induction, in a p53-dependent manner [22]. These observations are consistent with the dramatic decreases in the migration and invasion of H1299-HA-p53-WT/ FLAG-PG-WT co-transfectants vs. cells expressing either HA-p53 or FLAG-PG alone [15, 24, 56].

Co-immunoprecipitation experiments revealed that p53 interacted with the TA domain of plakoglobin via its DBD. Immunofluorescence staining showed colocalization of FLAG-PG-WT and HA-p53-DBD within the cytoplasm, consistent with the absence of nuclear localization signal in p53-DBD. Similar experiments with cells expressing p53-WT and various plakoglobin deletions showed a lack of interaction between p53 and PG-ΔC. In HA-p53-WT-FLAG-PG-ΔC cells, plakoglobin distribution was primarily at the membrane, whereas p53 was exclusively nuclear, further confirming that plakoglobin interacted with p53 via its C-terminal domain.

We also examined the changes in growth, migration and invasion of H1299 cells co-expressing various HA-p53 fragments with FLAG-PG-WT or various FLAG-PG deletions with HA-p53-WT. These results showed that only cells co-expressing p53-WT and PG-WT exhibited maximum inhibition of cell growth, migration and invasion. This finding is novel and has not been previously reported. In contrast, the co-expression of HA-p53-NT, -DBD and -CT with FLAG-PG-WT reduced cell growth and invasiveness by only ~10-12%. Interestingly, however, all p53 fragments were effective in reducing the migration of H1299 double transfectants noticeably, albeit not to the level of p53-WT.

The NT domain regulates the p53-mediated transcription via interaction with the basal transcription machinery, but also has transcription-independent functions. The NT also regulates the stability of p53 by binding to Hdm-2, and its regulation of growth by interactions with apoptotic proteins and FAK [30, 63-66]. However, both the DBD and the CT are necessary for proper functioning of the NT domain [67-75], consistent with the limited capacity of NT to reduce the growth and invasiveness of H1299 transfectants observed in our study.

The DBD construct used in this study also includes the TAD2 domain. The DBD has a tightly regulated, sequence-specific DNA binding activity and plays a critical role in p53 transcriptional activity and also mediates the cytosolic function of p53 in regulating apoptosis [5, 72, 76]. Here, we showed that DBD plus TAD2, which is involved in senescence induction [65], is not sufficient to significantly reduce the growth and invasiveness of H1299 transfectants.

H1299-HA-p53-CT cells expressed a peptide comprising the oligomerization and transcriptional regulatory domains [71, 77-80]. The CT domain contains many phosphorylation and acetylation sites which confer the proper conformation, localization, stability, DNA binding and transcriptional activity on p53 [5, 75, 81-84]. Our data showed almost exclusive nuclear localization of p53-CT, while p53-NT and -DBD proteins were localized entirely within the cytoplasm. However, while properly localized, the CT domain alone was not sufficient to reduce the growth and invasiveness of H1299 cells to the same extent as WT-p53.

Surprisingly, the co-expression of p53-NT, -DBD or -CT with PG-WT decreased the migratory properties of the respective H1299 transfectants, albeit to a lesser

extent than p53-WT. A number of studies have shown interactions between NT, DBD and CT with various kinases involved in migration including FAK, JNK, PLK1 and GSK3β [39, 52, 85- 91]. Our results clearly suggest that the NT, DBD and CT fragments of p53 retain some ability to inhibit cell migration. Whether the expressed fragments could act as dominant negative peptides to sequester these kinases is not clear and warrants further investigation.

In a p53-WT background, various plakoglobin deletions exhibited reduced growth similar to H1299-HA-p53 cells, suggesting that the inhibition of growth by plakoglobin was primarily mediated by its role in the induction of contact inhibition. Moreover, p53 may have a larger contribution to the significantly reduced growth of H1299 cells coexpressing p53 and plakoglobin.

When plakoglobin deletions were coexpressed with p53-WT, these transfectants were less migratory than H1299 cells (~25% reduction). However, their migration was significantly higher than H1299-HA-p53-WT/FLAG-PG-WT cells (~75% reduction). This is consistent with the inability of PG-ΔN and ΔArm to interact with α-catenin and cadherins, respectively, mediate stable junction formation and inhibit migration. However, while PG-ΔC expressing cells exhibited extensive cell-cell contact, they also showed increased migration. This observation is also in keeping with previous studies demonstrating the involvement of the C-terminal domain of plakoglobin in inhibition of migration independent of its adhesive properties [54]. Consistent with this observation, PG-ΔC expressing cells exhibited extensive cell-cell contact, but increased migration. The invasiveness of H1299-HA-p53-WT/FLAG-PG-ΔN and -ΔArm (with intact TA domain) was similar to that of

H1299-HA-p53-WT/FLAG-PG-WT cells (~70%), whereas invasiveness was reduced by only ~27% in H1299-HA-p53-WT/FLAG-PG-ΔC. These results may be explained by the loss of interaction between plakoglobin and p53 due to the absence of TA domain of plakoglobin.

In conclusion, our data indicated that 1) p53 and plakoglobin cooperated to reduce the growth and acted synergistically to decrease migration and invasiveness of H1299 cells and 2) the C-terminal domain of plakoglobin interacted with the DBD of p53, and this interaction was necessary for the maximum inhibition of invasion by p53 and plakoglobin. The data presented also raises the possibilities that the NT, CT and DBD fragments of p53 may act in a dominant negative manner to inhibit signaling pathways involved in migration. Furthermore, the differences in the migratory properties of the transfectants expressing various p53 fragments relative to the WT-p53 cells may suggest that the genes/pathways involved in inhibition of migration by p53 may be different than those involved in its inhibition of growth and invasion. Future studies will be focused on determining the exact amino acids involved in p53/plakoglobin interactions and examining the interactions between p53 fragments and various signaling molecules that regulate cell migration. Since more than 50% of all tumors and 80% of metastatic tumors have mutations in p53 [1], our observations provide the exciting possibility that plakoglobin may be a potential therapeutic target for cancers with non-functional mutant p53s.

3.5. References

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Chapter four

Plakoglobin Partially Restores *in Vitro* Tumor Suppressor Activities of $p53^{R175H}$ Mutant by Sequestering the Oncogenic Potential of β-catenin

PREFACE

After submission of this thesis to the examining committee, a revised version of this chapter was accepted for publications as: Alaee M, Nool K, Pasdar M. Plakoglobin Partially Restores in Vitro Tumor Suppressor Activities of p53R175H Mutant by Sequestering the Oncogenic Potential of β-catenin. Cancer Sciences. 2018 Apr 16. doi: 10.1111/cas.13612.

Contributions:

I participated in conceiving and designing the experiments, performed the experiments and wrote the manuscript. KN performed replicates of the migration and invasion assays for H1299 cells co-expressing plakoglobin and $p53^{R175H}$.

4.1. Introduction

p53 is a sequence-specific transcription factor with tumor and metastasis suppressor activities [1, 2]. It plays pivotal roles in the regulation of cell cycle, DNA repair, senescence, apoptosis, and metabolism by responding to various cellular stress signals such as DNA damage, hypoxia, mitotic stress, oncogenic signaling etc. [3-5]. As a transcription factor, p53 down-regulates the expression of genes involved in tumor development and cancer progression [6-8]. p53 is mutated or lost in over half of all cancers and more than 80% of metastatic tumors [3,4]. Furthermore, many tumors that express wild type p53 frequently display aberrations in their p53 pathway [3,4]. In addition to the loss/partial loss of the tumor suppressor activities, some p53 mutants also gain oncogenic functions (GOF) that contribute to tumor cell growth, aggressiveness, metastasis and drug resistance [9]. p53 inactivation can result from genetic alterations, decreased stability, defective post-translational modifications and interaction with intracellular partners [10].

There are over 30,000 somatic mutations in *TP53*, including missense, nonsense, deletions, frameshifts and temperature sensitive [11]. Most of these changes occur within the DNA-binding domain with more than 75% single missense mutations, 40% of which are represented by six hot spot mutations (Arg175, Gly245, Arg248, Arg249, Arg273 and Arg282) that are highly frequent in tumors of different origins [11]. The hot spot mutations are further classified into two groups: contact mutations (Arg248, Arg273) that inhibit the direct interaction between p53 and DNA leading to a loss of sequence-specific transactivation and structural mutations (Arg175, Gly245, Arg249, and Arg282) that alter the local or global conformation

of p53 causing indirect loss of DNA binding [12]. Among the hot spot structural mutations, Arg175His (R175H), is the most frequent GOF p53 mutant [13,14] that increases cancer cell proliferation, migration and invasion by deregulating different signaling pathways involved in tumorigenesis and metastasis.

p53 functions are regulated by posttranslational modifications and protein-protein interactions [15]. We have identified plakoglobin (γ-catenin) as an endogenous interacting partner of wild type as well as a number of most frequent mutant p53s (mp53) in various carcinoma cell lines of different origins and, have shown that its interaction with mp53s restores their tumor suppressor activities *in vitro* [16-19]. Plakoglobin is an Armadillo protein family member and a paralog of β-catenin with similar dual cell-cell adhesion and signaling activities [20,21]. However, unlike β-catenin that acts as an oncogene via its interaction with the transcription factors TCF/LEF, and activation of Wnt signaling pathway [22,23], plakoglobin generally acts as a tumor and metastasis suppressor [20,21,24-27]. We have shown that plakoglobin can act as a tumor and metastasis suppressor by at least three mechanisms: regulation of stability and subcellular localization of growth regulating molecules [19,25,28], interaction with transcription factors involved in the regulation of cell growth and metastasis [16-19,25] and sequestration of β-catenin oncogenic activities [29, also see 30-34].

p53 GOF mutations can induce aberrant accumulation and increased transcriptional activation of β-catenin in cancer cells [35-37]. In the absence of Wnt, the excess cytoplasmic β-catenin is degraded via phosphorylation by the destruction complex and subsequent ubiquitination and proteasomal degradation [38-40]. Upon Wnt

activation, the destruction complex is dissociated and β-catenin translocates into the nucleus, binds to TCF/LEF transcription factors and activates the expression of Wnt targets including cyclin D1, c-Myc, MMPs, S100A4, and survivin, etc. [38-43]. Activation of these genes triggers an epithelial to mesenchymal phenotypic transition, cell proliferation, cell migration and invasion and metastasis [41,43]. β-catenin is also degraded via its ubiquitination by Siah-1, an E3 ubiquitin ligase that enhances β-catenin's proteasomal degradation independent of the canonical Wnt signaling pathway [44,45].

In the present work, our goal was to assess the effects of GOF $p53^{R175H}$ mutant (herein referred to as $p53^{R175H}$) alone or together with plakoglobin on β-catenin accumulation in the nucleus and its transcriptional activation. To this end, plakoglobin deficient and p53 null H1299 cells were transfected with wild type p53 (herein referred to as p53) or $p53^{R175H}$ with or without plakoglobin. $p53^{R175H}$ expressing H1299 cells showed significantly higher levels of total and nuclear β-catenin relative to the p53 expressing transfectants. H1299 cells expressing plakoglobin or co-expressing plakoglobin and p53 or $p53^{R175H}$ had significantly lower levels of total and nuclear β-catenin. Plakoglobin and β-catenin interacted with TCF-4 and expression of plakoglobin decreased the β-catenin/TCF interaction. $p53^{R175H}$ cells showed significant increase in β-catenin/TCF luciferase reporter activity, whereas co-expression of plakoglobin in these cells significantly decreased the luciferase activity. β-catenin target genes, *c-MYC* and *S100A4* were upregulated in $p53^{R175H}$ cells and were significantly downregulated when plakoglobin was co-expressed. $p53^{R175H}$ expression also increased the *in vitro* migration and invasion

of H1299 cells, which were significantly reduced when plakoglobin was co-expressed.

4.2. Material and methods

4.2.1. Cell lines and culture conditions

The non-small cell lung carcinoma (NSCLC) cell line H1299 have been described [18] and were grown in Minimum Essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin-kanamycin (PSK) antibiotics. SW620 colon carcinoma cells were grown in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 10% FBS and 1% PSK.

4.2.2. Plasmid construction and transfection

HA-tagged p53 has been described previously [18,46]. The pcDNA3.1/hygro-plakoglobin construct was generated using the previously described FLAG-tagged-plakoglobin as a template [29]. The $p53^{R175H}$ expression construct was a gift from Dr. Giovanni Blandino [47].

Cultures of H1299 cells were established in 60 mm petri dishes and transfected at 60% confluency with 9 μg of DNA using calcium phosphate. Twenty hours after transfection, cells were rinsed with media and allowed to recover for 24 hours in complete MEM. Forty-eight hours post transfection, stable transfectants were selected by placing cultures in selection media containing 500 μg/ml Hygromycin B (plakoglobin transfectants) or 400 μg/ml G418 ($p53^{R175H}$ transfectants) or both (double transfectants) for 2-3 weeks. Resistant clones were screened for p53 and plakoglobin expression by immunofluorescence and immunoblot assays and

maintained in maintenance selection media containing 350 μg/ml Hygromycin B or 200 μg/ml G418 or both. Positive clones were subcultured by limiting dilution. Both parental and multiple single cell isolated clones were tested for plakoglobin and p53 expression using various assays and the results are presented for one representative clone.

4.2.3. Cell fractionation, preparation of cell extracts and immunoblot analysis

To extract total cellular proteins, confluent 100 mm culture plates were rinsed with cold PBS and solubilized in SDS sample buffer (10 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 50 mM DTT, 2 mM EDTA, 0.5 mM PMSF, 1 mM NaF, 1 mM Na3VO4). Equal amounts of total cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Biorad). Membranes were incubated in specific primary antibodies overnight at 4°C followed by the appropriate secondary antibodies at room temperature (Table 4.1). Membranes were scanned using an Odyssey CLx infrared imaging system.

Nuclear fractions were prepared with Thermo Fisher Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol. The purity of nuclear fractions was verified by immunoblotting with nuclear lamins antibodies (Table 4.1).

Table 4.1. Antibodies and their respective dilutions in specific assays

Primary antibodies	Species	Assay			Source/ catalog number
		WB	IP	IF	
p53- DO-1	Mouse	1:1000	1:100	-	Santa Cruz Biotechnology/ sc-126
Plakoglobin (γ-catenin)	Mouse	1:1000	1:100	1:100	BD Transduction Laboratories/ 610254
β-catenin	Mouse	1:1000	1:100	1:100	Sigma Aldrich/ C-7207
β-catenin (nuclear)	Mouse	1:1000	-	-	Abcam/ ab 19451-50
TCF-4	Mouse	1:500	1:100	-	Upstate Biotechnology/ 05-511
β-actin	Mouse	1:1000	-	-	Santa Cruz Biotechnology/ sc-47778
Lamin B1	Rabbit	1:1000	-	-	Abcam/ ab 16048
Secondary antibodies					
Anti-mouse, light chain IgG	Goat	1:20000	-	-	Jackson Immuno Research/ 115-625-174
Anti-rabbit, light chain IgG	Goat	1:20000	-	-	Jackson Immuno Research/ 211-652-171
Alexa fluor 488	Mouse	-	-	1:2000	Molecular Probes Biotechnology/ A11029
Alexa fluor 546	Rabbit	-	-	1:3000	Molecular Probes Biotechnology / A11035

4.2.4. Immunoprecipitation

Cultures (100 mm) were washed with cold PBS containing 1 mM NaF, Na3VO4 and CaCl2 and extracted in 1 ml of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 100 mM NaF [48], and protease inhibitor cocktail (1 tablet/10 ml; Roche Diagnostics) for 30 minutes at 4ºC on a rocker. Cells were then scraped and centrifuged at 48000 xg for 10 minutes. Supernatants were divided into equal aliquots and processed for immunoprecipitation with p53, plakoglobin and β-catenin antibodies (Table 4.1) and 40 µl protein G agarose beads (Pierce Biotechnology) overnight at 4°C on a rocker rotator. To ensure complete depletion, immunoprecipitates were centrifuged at 14000 xg for 2 min and supernatants were separated processed for a second immunoprecipitation for 3 hours. Beads from the two immunoprecipitations were combined and washed three times with the lysis buffer. Immune complexes were solubilized in 60 µl SDS sample buffer, separated by SDS-PAGE and processed for western blot as described above.

4.2.5. Immunofluorescence and confocal microscopy

Confluent cell cultures were established on glass coverslips and rinsed with cold PBS containing 1 mM each of NaF, Na3VO4 and CaCl2. Cells were fixed with 3.7% formaldehyde in PBS for 20 minutes and extracted with cytoskeleton (CSK) extraction buffer ([48]; 50 mM NaCl, 300 mM Sucrose, 10 mM PIPES pH 6.8, 3 mM MgCl2, 0.5% Triton X-100, 1.2 mM PMSF, and 1 mg/ml DNase and RNase) for 10 minutes. Coverslips were blocked with 4.0% goat serum and 50 mM NH4Cl

in PBS containing 0.2% BSA for 1 hour. Coverslips were then incubated in the specific primary antibodies for 1 hour followed by the secondary antibodies for 30 minutes at concentrations indicated in Table 4.1. Following nuclei staining with DAPI (1:2000), coverslips were mounted in elvanol containing paraphenylene diamine [PPD, 0.2% (w/v)] and viewed using a 63X objective lens of a Zeiss confocal microscope.

4.2.6. RNA Isolation, RT-PCR and real-time PCR

Total RNA was isolated from cells in 100 mm culture dishes with Trizol reagent (Invitrogen-Thermo Fisher Scientific), treated with DNase I and reverse-transcribed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For real-time PCR, Syber green mastermix (Thermo Fisher Scientific) and specific forward and reverse primers for *MYC, S100A4* and *ACTB* (β-actin) (Table 4.2) were used as per manufacturer's instructions.

Table 4.2. Oligos/primer sequences used for RT-qPCR

Construct	Primers		Size (nucleotide)
c-MYC	Forward	5'-CAGCTGCTTAGACGCTGGATT-3'	21
	Reverse	5'-GTAGAAATACGGCTGCACCGA-3'	21
S100A4	Forward	5'-GATGAGCAACTTGGACAGCAA-3'	21
	Reverse	5'-CTGGGCTGCTTATCTGGGAAG-3'	21
ACTB	PrimePCR SYBR Green Assay ACTB Human, Cat No. 10025636		

4.2.7. Proteasome inhibition assay

Replicate cultures remained untreated or were treated with 1μM of proteasome inhibitor MG132 (Sigma) for 16 hours. Untreated and treated cells were then lysed and total cell lysates were used for western blot with β-catenin antibodies as described above.

4.2.8. Luciferase reporter assay

To measure β-catenin-driven transactivation, parental H1299 cells and H1299 transfectants in confluent 35 mm cultures were co-transfected with 5 μg of pTOPFLASH plasmid [49] and 3 μg of Renilla luciferase plasmid (pRL-TK) serving as a control for transfection efficiency [50]. Forty-eight-hour post-transfection, luciferase activities were measured and normalized to Renilla activities. Each experiment was repeated 4 times and the mean and standard errors were calculated.

4.2.9. *In vitro* migration and invasion assays

For cell migration assays, 2×10^5 cells were resuspended in 0.5 ml serum-free media and plated in the upper chamber of transwell inserts (3 μm pore, 6.5 mm diameter; BD Biosciences). Normal media containing 10% FBS was added to the lower chamber and cultures were incubated for 24 hours at 37°C. Inserts were then transferred into new dishes, rinsed with PBS to remove unattached cells, fixed with 3.7% formaldehyde for 2 minutes, permeabilized with 100% methanol for 20 minutes and stained with Giemsa stain for 15 minutes at room temperature.

Following staining, membranes were viewed under an inverted microscope using a 20X objective lens and photographed.

For Matrigel invasion assays, cells were starved in serum-free media for 24 hours prior to the assay. For each cell line, 5×10^4 cells in 0.2 ml serum-free media were plated in the top compartment of Matrigel-coated invasion chambers (8 μm pore PETE membrane; BD Biosciences). Fibroblast conditioned media (0.8 ml) was added to the bottom chambers and plates were incubated overnight at 37°C. After 24 hours, membranes were recovered and processed as described for the migration assay.

The migrated/invaded cells were counted in 5 random fields for each membrane using the NIH ImageJ Cell Counter program. Numbers for each cell line were averaged and normalized to those of the parental untransfected cells and histograms constructed. Histograms represent the average ± standard deviation of 3-6 independent assays for each cell line.

4.2.10. Statistical analysis

Values are presented as means ± standard deviation. Statistical differences between groups were assessed by Student's t-tests. *P*-value <0.05 was considered significant.

4.3. Results

4.3.1. Plakoglobin interacted with $p53^{R175H}$ and decreased β-catenin protein levels

We first validated the expression of plakoglobin, p53 and $p53^{R175H}$ in single and double H1299 transfectants by processing total cell extracts from all transfectants for western blots with plakoglobin and p53 antibodies (Figure 4.1A).

Previously, we have shown that plakoglobin interacted with p53 and several mp53 proteins using different carcinoma cell lines [16-19]. To verify plakoglobin interaction with $p53^{R175H}$, we processed H1299 transfectants co-expressing plakoglobin and $p53^{R175H}$ for reciprocal co-immunoprecipitation and immunoblotting with plakoglobin and p53 antibodies (Figure 4.1B). Immunoprecipitation of the double transfectants total cell extracts with p53 antibodies precipitated p53 (Figure 4.1B, lane 2; IP: p53, IB: p53) and co-precipitated PG (Figure 4.1B, lane 6, IP: p53, IB: PG). The reciprocal co-immunoprecipitation using PG antibodies confirmed this finding, as PG antibodies co-precipitated p53 (Figure 4.1B, lane 3, IP: PG, IB: p53) and pulled down PG lane 6, IP: PG, IB: PG) from cells co-expressing both proteins.

Figure 4.1C shows plakoglobin and β-catenin protein expression in H1299 transfectants expressing p53 or $p53^{R175H}$ with or without plakoglobin. Relative to H1299 cells, β-catenin levels were decreased in p53 expressing H1299 cells (Figure 4.1C, β-catenin, lane 2), whereas it was increased when $p53^{R175H}$ was expressed (Figure 4.1C, β-catenin, lane 3). Plakoglobin expression significantly reduced β-catenin levels in parental as well as p53 or $p53^{R175H}$ expressing H1299 transfectants (Figure 4.1C, β-catenin, lanes 4-6).

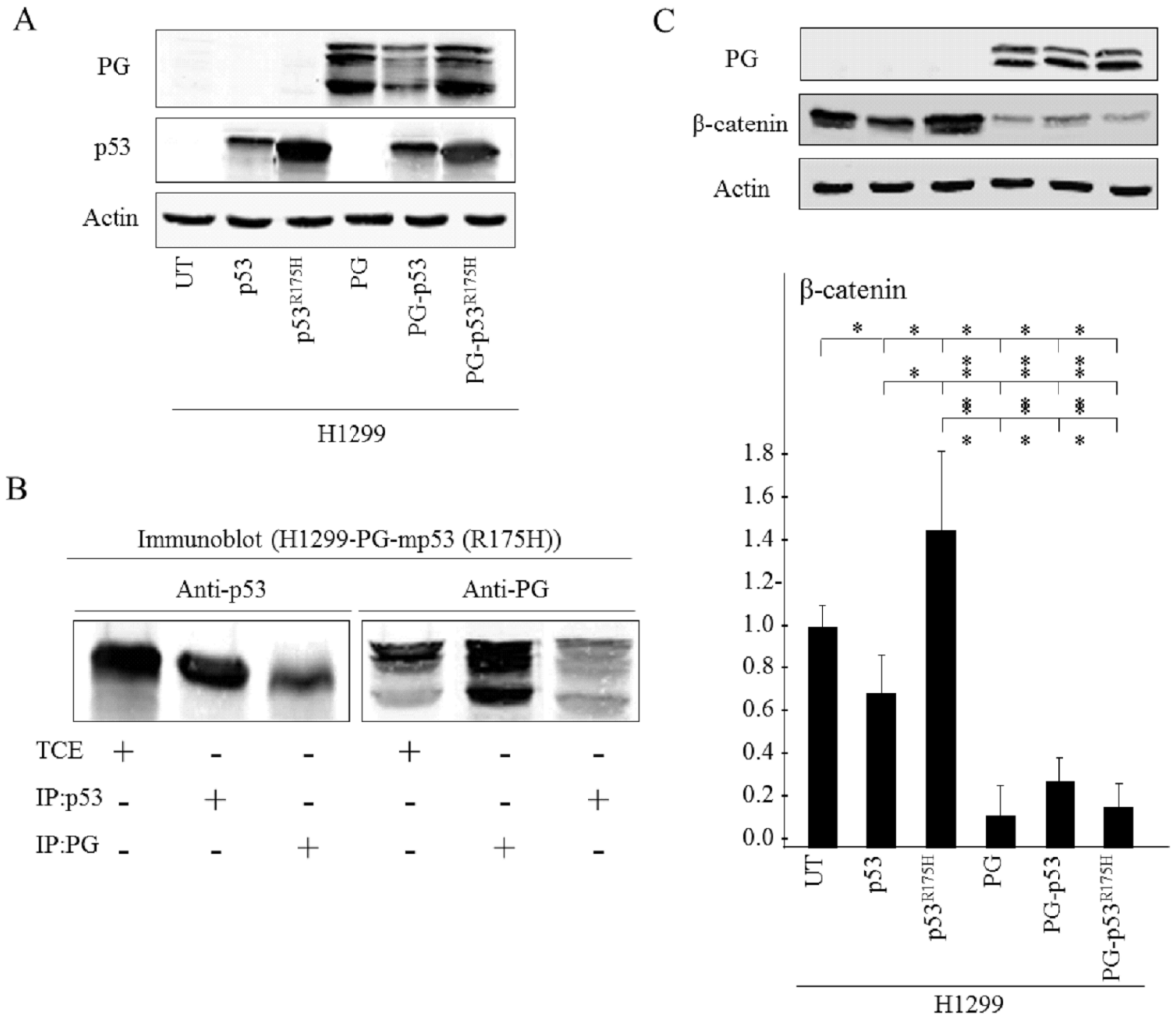

Figure 4.1. Plakoglobin interacts with the mutant p53^{R175H} and decreases β-catenin levels.
(A) Total cell extracts from untransfected H1299 cells and H1299 cells transfected with **p53, p53^{R175H}, PG, PG and p53 (PG-p53) or PG and p53^{R175H} (PG- p53^{R175H})** were processed for immunoblot using plakoglobin and p53 antibodies. β-actin levels in the same extracts was used as an internal control. PG, plakoglobin; UT, untransfected. **(B)** Total cell extracts from H1299 cells co-expressing **plakoglobin** and **p53^{R175H}** were processed for reciprocal and sequential co-immunoprecipitation (IP) and immunoblotting (IB) using p53 and plakoglobin antibodies. PG, plakoglobin, TCE, total cell extracts. **(C)** Total cell extracts from parental H1299 cells and H1299 transfectants expressing **p53** or **p53^{R175H}** with or without **plakoglobin** were processed for immunoblot using plakoglobin and β-catenin antibodies. β-actin levels were used as loading control. Histograms represent the average ± standard deviation of four separate experiments. All values are normalized to H1299 untransfected cells. PG, plakoglobin; UT, untrasfected. *P* values * <0.05 ** <0.001.

4.3.2. Expression of plakoglobin decreased β-catenin protein levels by promoting its proteasomal degradation

Western blot analysis of the nuclear extracts from parental H1299 cells and H1299 transfectants was used to examine the cytoplasmic and nuclear levels of plakoglobin and β-catenin (Figure 4.2A, B). The highest level of nuclear and cytoplasmic β-catenin was detected in H1299-p53^{R175H} transfectants (Figure 4.2A, B, β-catenin (N, C) lane 3). In contrast, significantly lower amounts of β-catenin were detected in parental as well as in p53 or p53^{R175H} cells when plakoglobin was co-expressed (Figure 4.2A, B, β-catenin (N) and (C) lanes 4-6).

Examination of β-catenin's mRNA levels in various transfects showed no difference (Figure 4.2C) suggesting that changes in β-catenin levels in plakoglobin expressing cells occurs post-transcriptionally. This led us to assess whether downregulation of β-catenin is due to the increased proteasomal degradation upon plakoglobin expression. To address this possibility, we used MG132, which inhibits 20S proteasome activity and degradation of ubiquitinated proteins [29]. Replicate cultures of H1299 cells and H1299 transfectants remained untreated or were treated with 1 μM MG132 for 16 hours and processed for western blot using β-catenin antibodies. As shown in Figure 4.2D, β-catenin was detected as multiple bands in MG132 treated cultures, which was consistent with the inhibition of degradation of the ubiquitinated protein. The quantitation of the β-catenin protein levels in untreated and treated cell lines showed that in untreated cultures, plakoglobin expression increased β-catenin degradation by ~ 5.5-, 3- and 4.6-fold in H1299, H1299-p53 and H1299-p53^{R175H} cells, respectively (Figure 4.2E).

MG132 treatment decreased β-catenin degradation by >2- fold in the absence of plakoglobin, whereas this reduction was significantly higher (up to 7-fold) in plakoglobin expressing transfectants (Figure 4.2E). These results suggested that plakoglobin decreased β-catenin levels by promoting its proteasomal degradation.

We also examined the subcellular distribution of β-catenin in H1299 cells and H1299 transfectants by confocal double immunofluorescence microscopy (Figure 4.3). Confluent cultures of various cell lines were fixed with formaldehyde, extracted with CSK buffer and processed for double immunofluorescence staining with plakoglobin and β-catenin antibodies [48]. There was no detectable plakoglobin staining in H1299, H1299-p53 and H1299-p53^{R175H} cells whereas it was distributed throughout the cytoplasm and at the membrane in H1299-PG, -PG-p53 and -PG-p53^{R175H} transfectants. β-catenin was expressed in all cell lines, although with different intensity. Relative to H1299 cells, β-catenin staining was significantly reduced in H1299-p53 transfectants, whereas it was significantly increased in H1299-p53^{R175H} cells. Plakoglobin co/expression dramatically reduced β-catenin's staining (nuclear and cytoplasmic) in all transfectants, particularly in H1299-p53^{R175H} cells (Figure 4.3, H1299-PG-p53^{R175H}). These results were consistent with the western blot studies and further confirmed that plakoglobin expression reduced β-catenin levels in both cytoplasmic and nuclear pools.

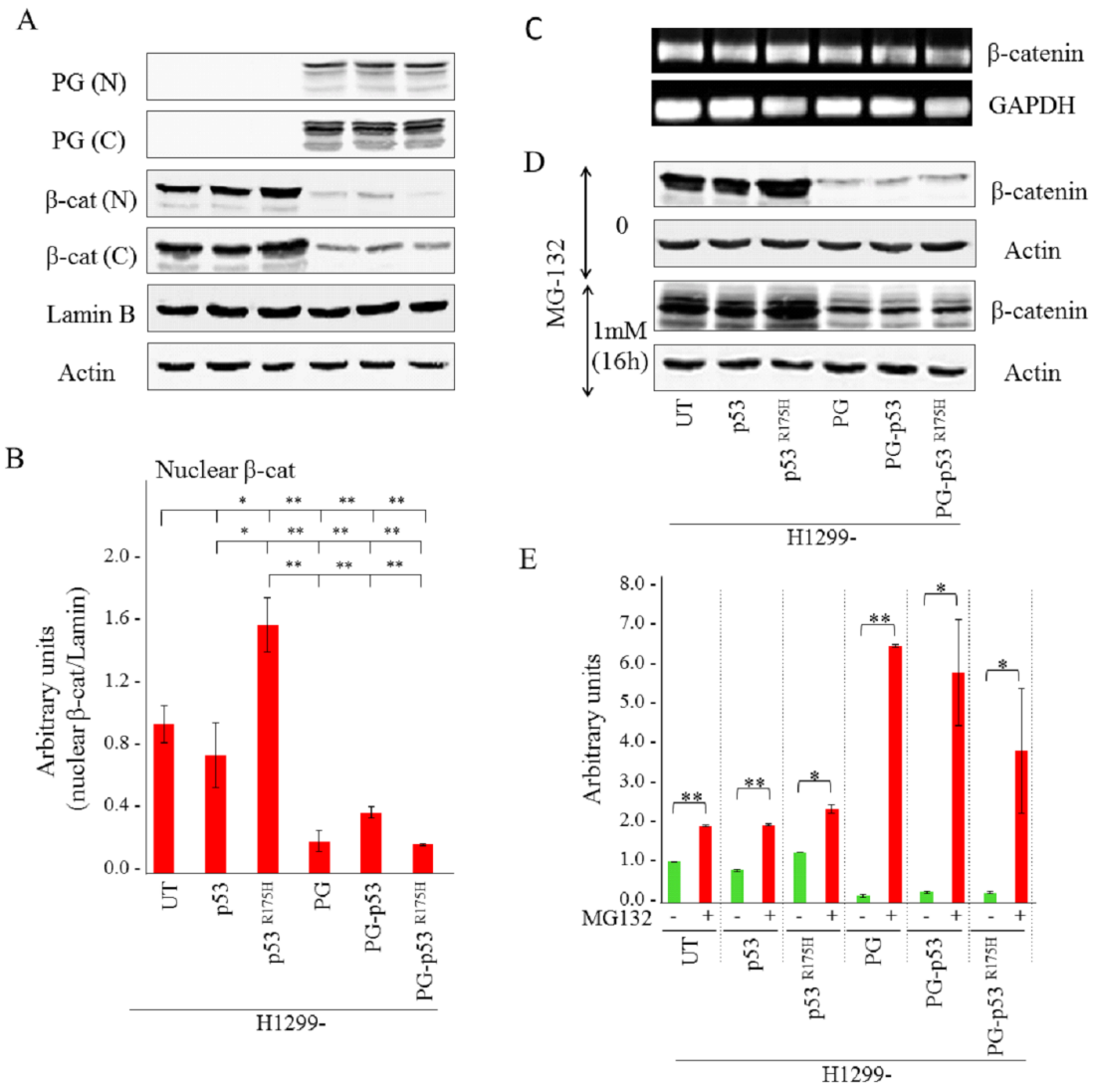

Figure 4.2. Plakoglobin expression decreases cytoplasmic and nuclear β-catenin levels by promoting its proteasomal degradation in H1299-p53^{R175H} cells. (A) Equal amounts of nuclear **(N)** and cytoplasmic **(C)** extracts from parental H1299 cells and H1299 transfectants (**p53, PG, p53^{R175H}, PG-p53, PG- p53^{R175H}**) were processed for immunoblot using plakoglobin and β-catenin antibodies. Lamin-B and actin levels were probed from the nuclear and cytoplasmic extracts of different transfectants, respectively, to confirm equal loadings. **(B)** Nuclear β-catenin blots in A were quantitated by NIH ImageJ software. Histograms represent the average ± standard deviation of four experiments. All values were normalized to untransfected H1299 cells. PG, plakoglobin; N, nuclear; C, cytoplasmic; β-cat, β-catenin. *P* values * <0.05. **(C)** Total RNA was extracted from parental H1299 cells and H1299 transfectants and subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers for *CTNNB1* (β-catenin). *GAPDH* expression levels were used as an internal control. **(D)** Replicate cultures of H1299 cells and H1299 transfectants (**p53, p53^{R175H}, PG, PG-p53, PG- p53^{R175H}**) remained untreated or were treated with 1μM MG132 for 16 hours. Total cell extracts from the untreated and treated cells were processed for immunoblot using β-catenin antibodies. Blots were also probed with β-actin antibodies to confirm equal protein loadings. UT, untransfected; PG, plakoglobin. **(E)** Blots in (C) were quantitated and histograms were generated that represent the average ± standard deviation of four separate experiments. All values are normalized to the untreated cells for untransfected and transfected cells. PG, plakoglobin; UT, untrasfected. *P* values * <0.05 ** <0.001.

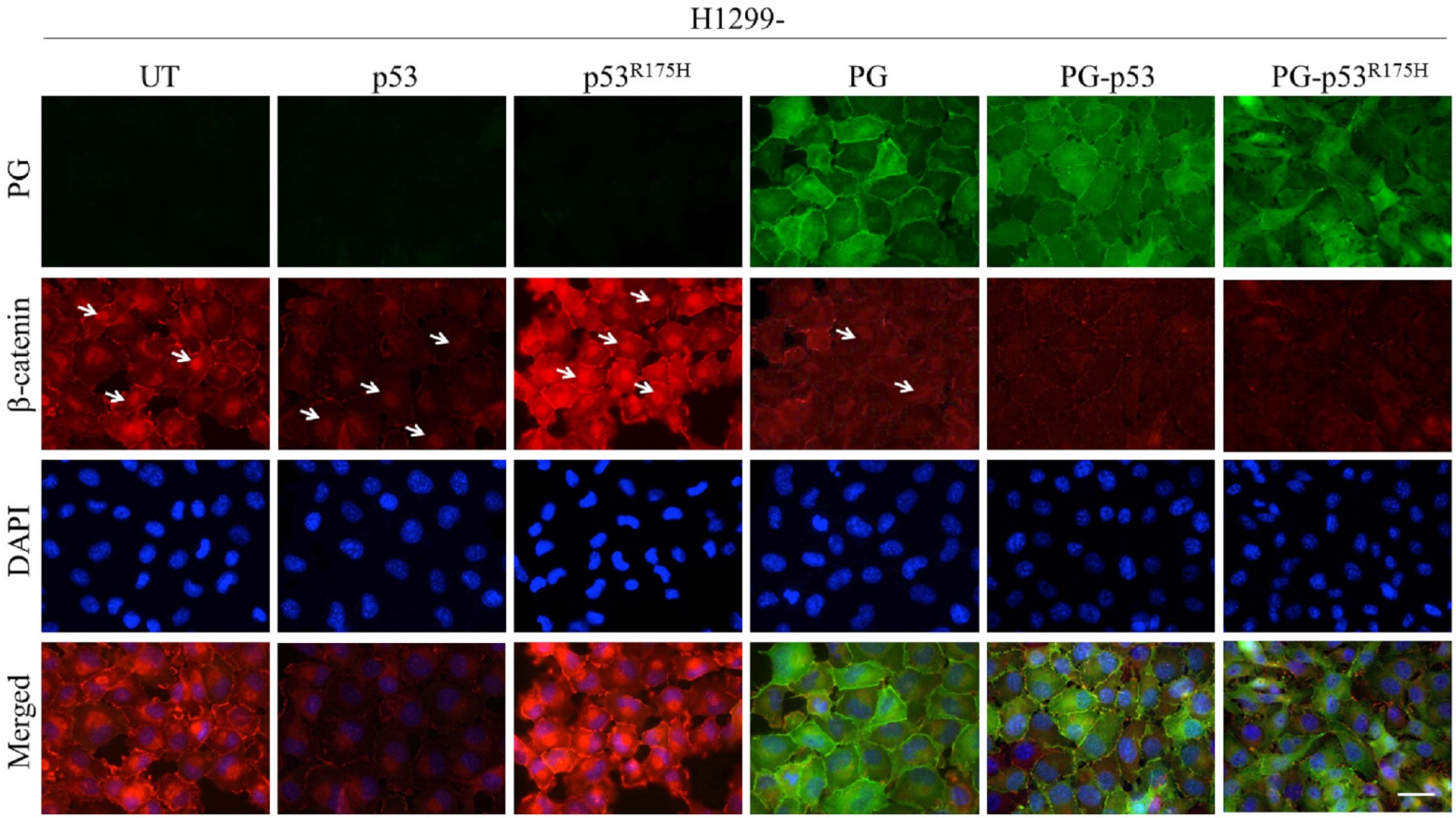

Figure 4.3. Subcellular localization of plakoglobin and β-catenin in H1299 transfectants. H1299 cells and H1299 transfectants (**p53, p53^{R175H}, PG, PG-p53, PG- p53^{R175H}**) were grown to confluency on coverslips, fixed with formaldehyde and permeabilized with CSK buffer as described in Materials and Methods. Coverslips were processed for confocal microscopy using **plakoglobin** (green) and **β-catenin** (red) antibodies. Nuclei were stained with **DAPI** (blue). Arrows indicate nuclei with β-catenin staining. Bar, 25μM. UT, untransfected; PG, plakoglobin.

4.3.3. Plakoglobin expression decreased β-catenin interaction with TCF-4, reduced β-catenin/TCF-4 reporter activity and down-regulated target gene expression

So far, the results showed that plakoglobin expression decreased the nuclear pool of β-catenin, which may influence the interaction between β-catenin and its cognate transcription factor TCF. Therefore, we used co-immunoprecipitation experiments to examine the interactions between plakoglobin and β-catenin with TCF in parental H1299 cells and H1299 transfectants. In Figure 4.4A, equal amounts of total cellular protein from parental H1299 cells and H1299 transfectants were processed for sequential co-immunoprecipitation and immunoblotting with β-catenin, plakoglobin and TCF antibodies. TCF was co-precipitated with β-catenin in H1299, H1299-p53 and H1299-$p53^{R175H}$ cells and its level was significantly lower in H1299-p53 transfectants (Figure 4.4A, IP: β-cat, IB: TCF). Interestingly, very little/no TCF was detected in β-catenin precipitates from the plakoglobin expressing transfectants. In these cells, TCF was only detected in association with plakoglobin (Figure 4.4A, H1299-PG, -p53, -$p53^{R175H}$; IP: PG, IB: TCF). Consistent with the earlier results, β-catenin levels were decreased in p53 and in all plakoglobin expressing transfectants (H1299-p53, -PG, -PG-p53, -PG-$p53^{R175H}$) relative to parental H1299 cells (Figure 4.4A; TCE, β-catenin). In contrast, TCF level was not notably different among various cell lines (Figure 4.4A; TCE, IB: TCF). These results indicated that plakoglobin co-expression significantly reduced the interactions between β-catenin and TCF in H1299, H1299-p53 and H1299-$p53^{R175H}$ transfectants.

We next assessed if decreased β-catenin/TCF association was reflected in β-catenin-dependent TCF reporter activity (Figure 4.4B). Parental H1299 cells and H1299 transfectants were transiently transfected with pTOPFLASH and pRL-TK Renilla reporter constructs and luciferase activities were measured in all cell lines. As a positive control, SW620, a colon carcinoma cell line expressing mutant APC and signaling-competent β-catenin [51] was included in these studies (Figure 4.4B). The luciferase activities of all cell lines were normalized to that of parental H1299 cells. The results showed no significant differences in the luciferase activity among H1299, H1299-p53 and H1299-PG cells (Figure 4.4B). In contrast, relative to parental H1299 cells, H1299-p53^{R175H} transfectants showed over 60% higher luciferase activity (Figure 4.4B), which was significantly reduced when plakoglobin was co-expressed in these cells (Figure 4.4B, H1299-PG-p53^{R175H}). Based on these observations, we reasoned that decreased β-catenin/TCF transactivation should result in decreased expression of their target genes. Specifically, we focused on *c-MYC* and *S100A4*, two β-catenin/TCF target genes that are known to participate in tumorigenesis and metastasis [41-43, 52-54]. RT-qPCR experiments showed that the levels of *c-MYC* and *S100A4* mRNA were significantly increased in H1299-p53^{R175H} transfectants compared to H1299 cells (Figure 4.4C). Co-expression of plakoglobin in these cells (H1299-PG-p53^{R175H}) led to over 3- and 5-fold decrease in the *c-MYC* and *S100A4* mRNA levels, respectively (Figure 4.4C). Together, the results of the experiments in Figure 4.4 suggested that plakoglobin expression in p53^{R175H} cell reduced β-catenin/TCF

association and the activation of at least two of their target genes involved in tumorigenesis and metastasis.

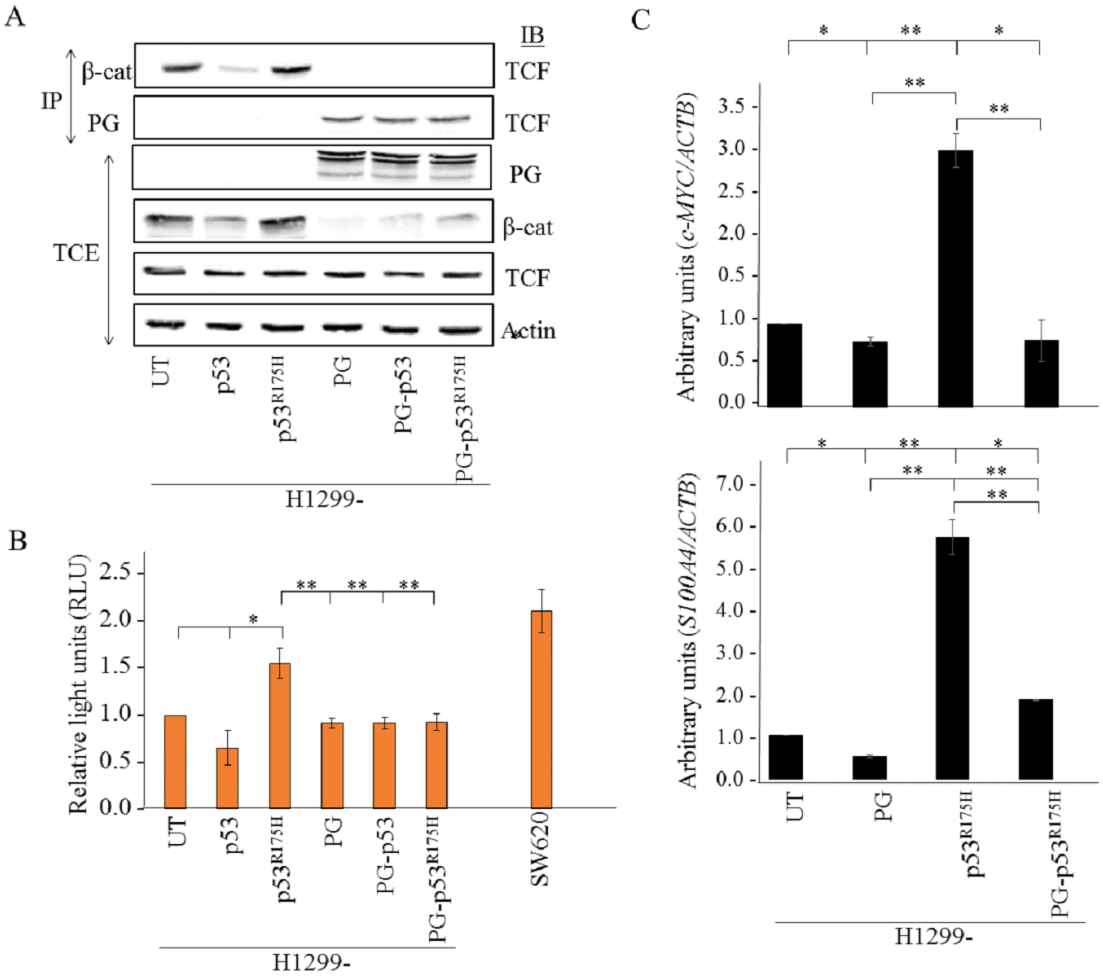

Figure 4.4. Plakoglobin expression reduces β-catenin interaction with TCF-4, decreases β-catenin/TCF-4 reporter activity and target gene expression. **(A)** Equal amounts of total cellular extracts (TCE) from H1299 cells and H1299 transfectants were processed for sequential immunoprecipitation (IP) and immunoblotting (IB) using plakoglobin, β-catenin (β-cat) and TCF-4 (TCF) antibodies. Equal loadings were confirmed by processing total cell extracts from all cell lines for immunoblotting using β-actin antibodies. **(B)** Untransfected H1299 cells and H1299 transfectants (**p53, p53^{R175H}, PG, PG-p53, PG- p53^{R175H}**) were co-transfected with pTOPFLASH β-catenin/TCF reporter construct and p-RK-TK Renilla reporter plasmid and luciferase activities were measured 48-hour post-transfection. The levels of luciferase activities from pTOPFLASH were normalized to those of Renilla plasmid. Four independent experiments were performed and the results were normalized to H1299 parental cells. SW620 cells were included as a positive control. RLU, relative light unit. UT, untrasfected; PG, plakoglobin. *P* values * <0.05, ** <0.001. **(C)** Total cellular RNA was extracted from untransfected H1299 cells and H1299 transfectants (**PG, p53^{R175H}, PG-p53^{R175H}**), reverse transcribed and processed for real-time PCR for *C-MYC* (top) and *S100A4* (bottom) using specific primers (Table 4.2). Expression levels were first normalized to the amount of *ACTB* in the same cell line and then to H1299 untransfected cells. The histograms were constructed based on the average ± standard deviations. UT, untransfected; PG, plakoglobin. *P* values * <0.05, ** <0.001.

4.3.4. Plakoglobin expression decreased migratory and invasive properties of $p53^{R175H}$ expressing H1299 cells

To assess the biological significance of decreased β-catenin/TCF transactivation, we examined the *in vitro* migration and invasion of parental H1299 cells and H1299 transfectants.

As demonstrated in Figure 4.5, p53 and plakoglobin expression decreased migration by 45% and 34% relative to H1299 cells, respectively, whereas co-expression of both proteins reduced migration by 73% (Figure 4.5, migration; H1299-p53, H1299-PG and H1299-PG-p53; also see [18]). In contrast, expression of $p53^{R175H}$ increased the migration of H1299 cells by ~ 20% (Figure 4.5, migration, H1299- $p53^{R175H}$), which was reduced by >40% when plakoglobin was co-expressed (Figure 4.5, H1299-PG-$p53^{R175H}$).

Similarly, the invasiveness of H1299-p53 and H1299-PG cells was decreased by 60% and 33% respectively, whereas the invasiveness of H1299-PG-p53 cells was decreased by 68% relative to parental H1299 cells (Figure 4.5, invasion; H1299-p53, H1299-PG and H1299-PG-p53, also see [18]). In contrast, $p53^{R175H}$ expression in H1299 cells increased their invasiveness by 30%, and the co-expression of plakoglobin in these cells reduced their invasiveness by >60% (Figure 4.5, invasion, H1299-$p53^{R175H}$ and H1299-PG-$p53^{R175H}$). These results indicated that plakoglobin acted synergistically with p53 to decrease migration and invasion and significantly reduced the migration and invasion promoting effects of $p53^{R175H}$.

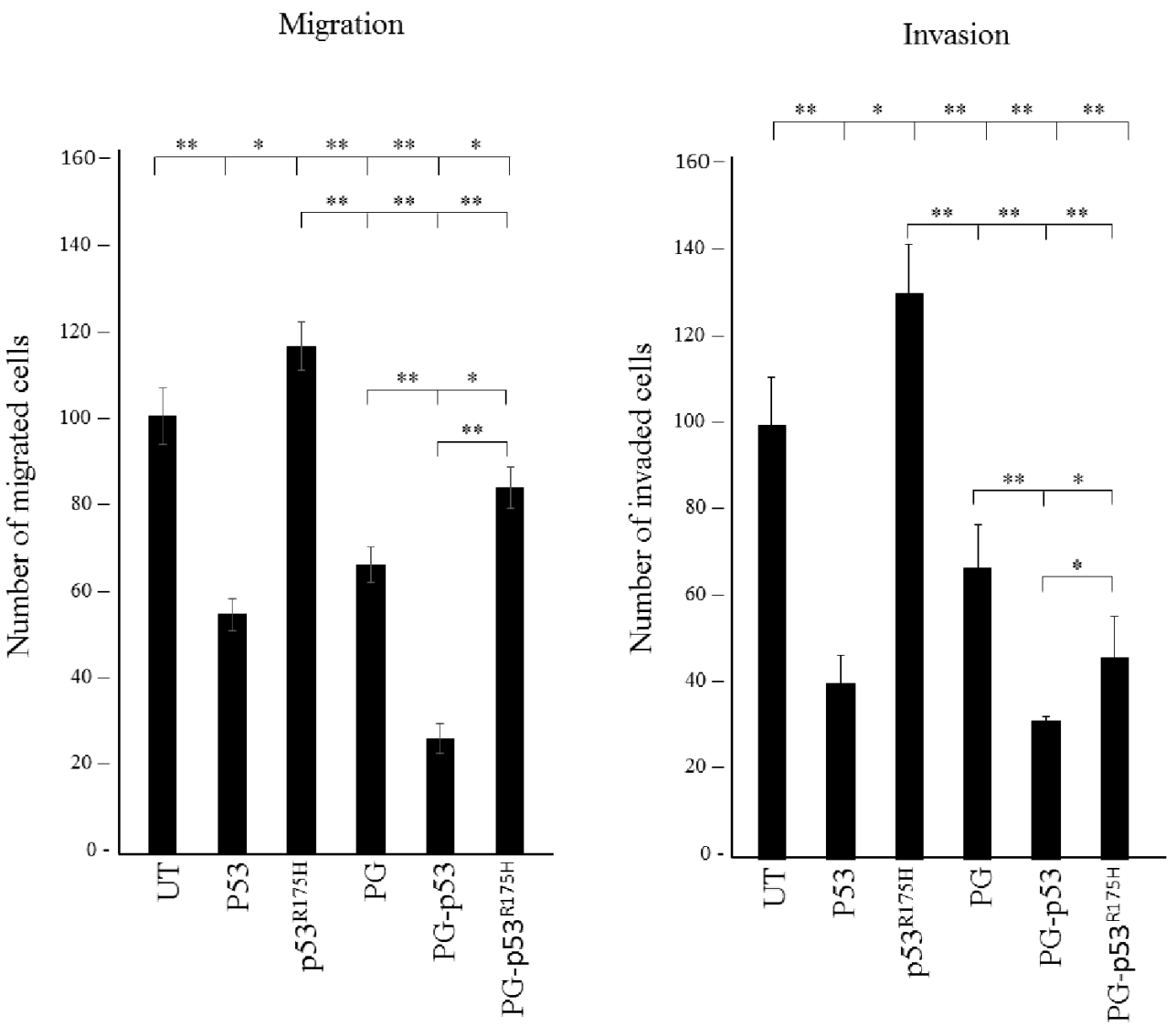

Figure 4.5. Plakoglobin reduced *in vitro* migration and invasion of p53R175H expressing H1299 cells. **H1299 and H1299 transfectants (p53, $p53^{R175H}$, PG, PG-p53, PG-$p53^{R175H}$)** were processed for 24-hour Transwell migration and invasion assays as described in Materials and Methods. The number of migrated/invaded cells in five random fields were counted using ImageJ cell counter program. Four separate experiments were performed for each cell line and the histograms represent the average ± standard deviation of the number of the migrated/invaded cells for each cell line. All values were normalized to H1299 untransfected cells. UT, untransfected; PG, plakoglobin. *P* values * <0.05, ** <0.001.

4.4. Discussion

$p53^{R175H}$ is one of the most common hot spot mutations that are frequently expressed in many cancers [13,14]. $p53^{R175H}$ expression has been shown to increase genomic instability [55], induce the expression of oncogenic miRNAs [56,57] and promote the expansion of cancer stem cell population [58], epithelial to mesenchymal transition [59-61] and drug resistance [62-64]. Furthermore, $p53^{R175H}$ mice models exhibit tumor formation and metastasis characteristics of the inherited Li-Fraumeni syndrome, the disease that is associated with the germline mutations in the *TP53* gene [65,66].

In the present study, we used the invasive and metastatic H1299 cells with activated Wnt/β-catenin pathway [67]. This cell line is plakoglobin deficient and p53 null and has been extensively used to assess the function of p53 and p53 mutants. We showed that the expression of $p53^{R175H}$ increased β-catenin levels, its interaction with TCF and activation of *c-MYC* and *S100A4,* two known β-catenin/TCF target genes [41-43, 52-54]. Increased β-catenin levels and activation were concurrent with increased migration and invasion of $p53^{R175H}$ expressing cells. We further demonstrated that the oncogenic effects of $p53^{R175H}$ were counteracted by the co-expression of plakoglobin in these cells. Plakoglobin interacted with $p53^{R175H}$ and reduced β-catenin level, its interaction with TCF and the expression *c-MYC* and *S100A4*. These changes were concurrent with decreased migration and invasion of these transfectants and are consistent with the previously reported activated Wnt/β-catenin pathway in these cells [67].

β-catenin is the main downstream effector of the canonical Wnt signaling pathway [38-45]. In the absence of Wnt signal, Axin/APC/GSK3β/CKI form the destruction complex that recruits and phosphorylates the excessive cytoplasmic β-catenin, which is subsequently ubiquitinated and degraded by the proteasome pathway [38-40]. In the presence of Wnt, the destruction complex becomes dissociated and the stabilized β-catenin translocates into the nucleus, binds to TCF/LEF transcription factors and induces the expression of Wnt target genes involved in tumorigenesis and metastasis [38,39]. β-catenin can also be activated independent of the Wnt signal. Mutations that interfere with β-catenin's interaction with the components of the destruction complex or with the phosphorylation of its N-terminal serine/threonine residues (S33, S37, S45, T41] that are required for degradation also activate β-catenin in the absence of the Wnt signal [68,69].

In agreement with previous reports, we showed that wild type p53 expression in H1299 cells reduced the total and nuclear β-catenin levels and its transcriptional activity. There are several mechanisms by which p53 reduces β-catenin protein levels and activation. p53 interacts with and activates GSK3β and/or accelerates the movement of the scaffolding protein, Axin, into the destruction complex both of which lead to increased phosphorylation of β-catenin and its subsequent degradation [70,71]. p53 also inhibits the activity of CK2, which phosphorylates and protects β-catenin from proteasomal degradation [72]. Furthermore, p53 can inhibit Wnt pathway by inducing the expression of the Wnt antagonizer, Dickkopf-1 [73] and the E3 ubiquitin ligase Siah-1 that mediates the degradation of β-catenin independent of the GSK3β [74-76]. In contrast GOF mutant p53s have been shown

to inhibit β-catenin degradation and promote its oncogenic activities in various tumor cell lines and tissues [35-37,77]. Here, we showed that $p53^{R175H}$ expression significantly reduced β-catenin degradation via the proteasome pathway. $p53^{R175H}$ expressing cells had increased total and nuclear β-catenin and β-catenin/TCF reporter activity and showed upregulation of the Wnt target genes [41-43, 52-54, 78-80].

Previously, we have shown that plakoglobin interacted with both wild type and a number of mutant p53 proteins and this interaction was direct (data not shown) and mediated by the DNA binding domain of p53 and the C-terminal transactivation domain of plakoglobin [16-19]. We showed that plakoglobin and p53 associated with promoters of a number of p53 target genes including tumor suppressors *SFN* (14-3-3δ) and *NME1* and the oncogenic genome organizer *SATB1* [16-19]. Furthermore, plakoglobin expression in plakoglobin deficient and mp53 expressing cells reduced the *in vitro* growth, migration and invasion of these cells [16,17,19]. Plakoglobin has also been shown to regulate the expression of HAI-1 and to reduce migration in a p53 dependent manner in NSCLC cells [27]. Co-immunoprecipitation experiments revealed that plakoglobin interacted with $p53^{R175H}$. Furthermore, expression of plakoglobin in $p53^{R175H}$ cells promoted β-catenin's proteasomal degradation and significantly reduced its total and nuclear levels, as had been reported previously [81]. Plakoglobin expression also reduced β-catenin/TCF-4 interaction, and the expression of c-*MYC* and *S100A4*. These observations are supported by our previous microarray studies, which identified p44 and p65 subunits of the 26S proteasome and S100A4 as transcripts that were

upregulated and downregulated, respectively, in plakoglobin expressing cells relative to their plakoglobin deficient and mutant p53 expressing parental cells [29]. S100A4, a recently identified target of β-catenin/TCF [82] was shown to be an early factor in EMT and its elevated level in various carcinoma cells and cancers was correlated with poor prognosis [83,84].

Apart from increasing β-catenin's proteasomal degradation, plakoglobin may inhibit β-catenin's transcriptional activity. β-catenin and plakoglobin interact with two sequential and non-overlapping domains in the N-terminus of TCF [32]. However, whereas binding of the β-catenin/TCF complex to DNA is highly efficient; plakoglobin/TCF binding to DNA is inefficient with significantly weaker transcriptional activities [85-87]. Our co-immunoprecipitation studies showed significant reduction in β-catenin-TCF association in plakoglobin expressing cells. These results further support the decreased c-Myc and S100A4 expression in H1299-PG- $p53^{R175H}$ transfectants and are consistent with our previous observations in another mutant p53 expressing carcinoma cell line [29]. Studies from other groups have also demonstrated that transcriptional activity of β-catenin downstream of the Wnt signaling was significantly reduced upon increased accumulation of plakoglobin in the nucleus [32,33]. Plakoglobin also repressed the Wnt/β-catenin signaling and target genes expression (*DICER* and *AXIN2)* via its assocoation with the transcription factor SOX4 and inhibition of β-catenin-SOX4 interaction [26].

In addition to upregulating the Wnt pathway, $p53^{R175H}$ has been shown to activate other signaling pathways including EGFR/PI3K/AKT, TGF-β and c-Met leading to

enhanced migratory and invasiveness of cancer cells [88-92]. Our results also showed increased migration and invasion of H1299 cells expressing $p53^{R175H}$ and their significant decrease when plakoglobin was co-expressed in these cells. While the effects of mp53s on enhancing migratory and invasive properties of cancer cells has been studied extensively, the effects of plakoglobin on hindering the *in vitro* metastatic features of $p53^{R175H}$ is novel and has not been previously reported.

In conclusion, our data suggest plakoglobin promoted β-catenin's proteasomal degradation and reduced its transcriptional activation independent of p53 status. Furthermore, its co-expression with $p53^{R175H}$ clearly counteracted the gain of function activities of this mutant, which is mediated, at least in part, by activating the oncogenic function of β-catenin. These observations together with our previous studies suggest that plakoglobin may counteract oncogenic functions of mutant p53 by at least two different mechanisms: plakoglobin augments β-catenin proteasomal degradation and reduces Wnt pathway activation and, it associates with mutant p53s and may either interfere with the expression of mutant p53 target genes and/or enable them to interact with and regulate wild type p53 target genes (Figure 4.6). The latter possibility is supported by our previous studies that have shown activation of p53 target genes in plakoglobin deficient and mutant p53expressing cell lines upon plakoglobin expression as well as our microarray experiments that have identified a number of growth/metastasis inhibiting and oncogenic promoting targets that are up- and down regulated, respectively, in mutant p53 expressing cells when plakoglobin is expressed [16,17]. Overall, these results suggest that plakoglobin may act as a tumor and metastasis suppressor protein in mutant p53

expressing cells by down-regulating Wnt/β-catenin axis and oncogenic activation of mutant p53s, two pathways that are known to be frequently dysregulated in many cancers. These findings provide insight into the possibility of developing therapeutic drugs that can mimic plakoglobin to concurrently inhibit the oncogenic effects of β-catenin and restore wild type tumor suppressor activities of mutant p53s in cancer.

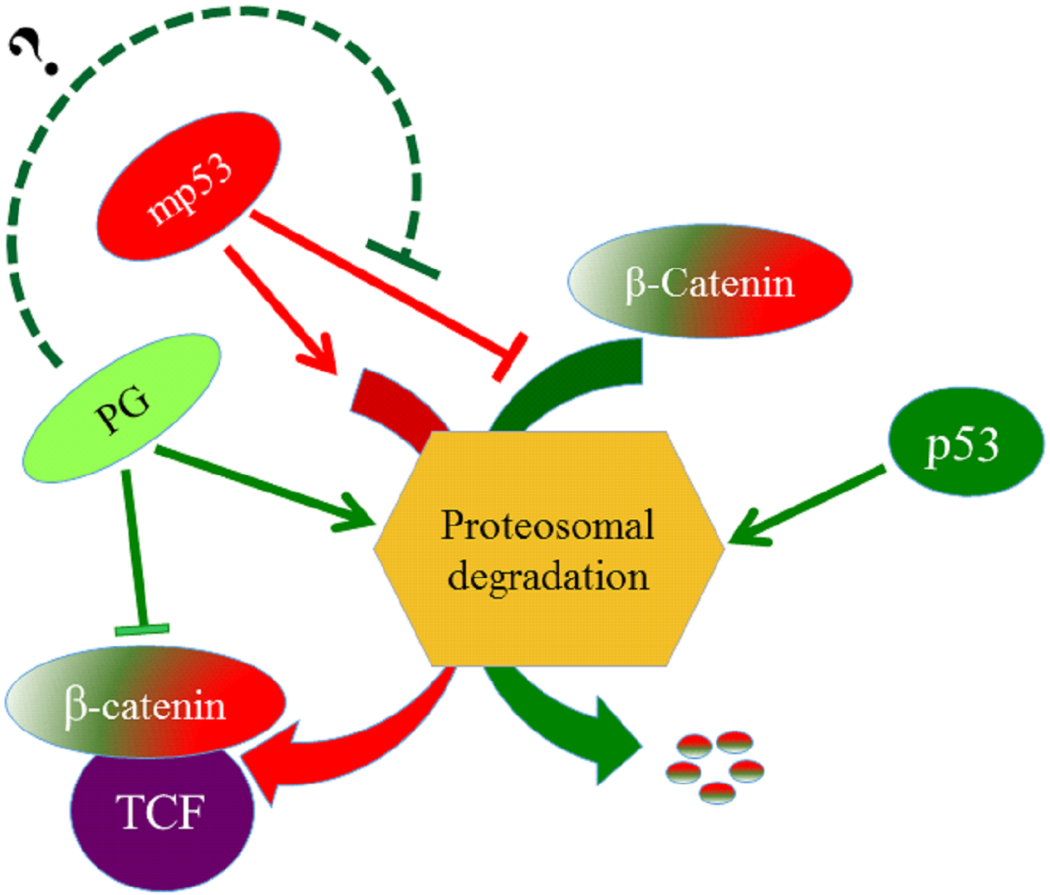

Figure 4.6. Potential model for restoration of tumor suppressor activity of mutant p53 by plakoglobin. p53 enhances β-catenin's proteasomal degradation by increasing the assembly of the destruction complex and/or inducing the expression of Siah-1, whereas mp53s inhibit β-catenin degradation (see the text for details). This would lead to the increased stability and translocation of β-catenin to the nucleus and transactivation of target genes in mp53 expressing cells. Based on the current study and our previous findings, plakoglobin may counteract oncogenic function of mutant p53s by increasing β-catenin's proteasomal degradation and by restoring tumor suppressor activities of mutants p53 via enabling their interaction with promoters of wild type p53 target genes [16,17] or modifying mutant p53s target genes expression [16]. PG, plakoglobin.

4.5. References

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Chapter five

Conclusion and future directions

5.1. Thesis overview

Plakoglobin was initially discovered as a junctional protein with essential roles in regulating cell-cell adhesion [1-3]. Further studies suggested that plakoglobin may participate in various signaling pathways [4,5]. While many studies focused on deciphering the role of other catenin proteins in tumor development and cancer progression, little was known on how plakoglobin regulated malignant transformation of cells.

The earliest clue as to the tumor suppressor activity of plakoglobin came from a study that demonstrated its loss of heterozygosity and low frequency mutations in sporadic breast and ovarian cancers [6]. Following this initial study, it was shown that combinatory loss of plakoglobin and other junctional proteins promoted tumor development, cancer metastasis, and resulted in poor clinical outcome [7-14]. Further investigations have demonstrated that the sole loss of plakoglobin also triggers tumor formation and cancer progression [15-20]. In esophageal cancer, concurrent decrease in the expression of E-cadherin and plakoglobin led to poor differentiation and decreased survival rate, whereas reduced plakoglobin expression promoted lymph node metastasis [21]. Decreased expression levels of plakoglobin has also been correlated with poor survival rate and metastasis in renal carcinomas [15], lymph node metastasis in oral squamous carcinomas [20] and bladder cancer [22], pulmonary metastasis in Wilms' tumor [19] and soft tissue sarcomas [18] as well as myometrial metastasis in endometrial cancer [23]. Furthermore, in prostate tumors plakoglobin expression was lost due to the hypermethylation of its gene [24]. Reduced expression of plakoglobin and its

altered cellular distribution were detected in thyroid tumors [25] and oropharynx squamous cell carcinomas [17].

Taken together, while loss or decreased expression of plakoglobin and/or altered cellular distribution of this protein have been identified as one of the contributing factors to tumor development and metastatic progression of different cancers. However, very little was known about the mechanisms underlying these effects of plakoglobin. Work from our lab has provided experimental evidence that suggest three mechanisms by which plakoglobin may exert its tumor and metastasis suppressor functions: sequestration of β-catenin oncogenic function, regulation of levels and localization of growth regulating molecules and interaction with transcription factors involved in regulation of cell growth and metastasis. Our laboratory has shown that tumor and metastasis suppressor effects of plakoglobin may, at least partially, be mediated by its interaction with mutant p53 proteins. In this thesis, I have examined plakoglobin interaction with wild type and several mutant p53s and investigated the molecular and functional significance of this interaction.

5.1.1. Tumor and metastasis suppressor functions of plakoglobin in ovarian cancer cell lines

The ovarian high grade serous carcinoma is the most common and aggressive form of ovarian tumors with epithelial origin [26,27]. Unlike the low grade serous carcinoma, more than 80% of ovarian high grade serous tumors harbor mutations in *TP53* [26,27]. In general, inactivation of *TP53* occurs at later stages in tumor

progression, however in these tumors *TP53* mutations occur at early stages of cancer development and contribute to both initiation and metastatic progression of this cancer [28-30].

Like any other carcinoma, loss of cell-cell adhesion and aberrant expression of cadherins and catenins are critical steps during the development of ovarian tumors [31-35]. Disruption of cadherin-catenin complexes leads not only to the loss of cell-cell adhesion but also the liberation of catenins and their subsequent interaction with other cellular proteins and activation of signaling pathways involved in tumorigenesis and metastasis [36,37]. In this context, β-catenin has oncogenic function, whereas plakoglobin acts as a tumor and metastasis suppressor protein [36,37]. The oncogenic activation of β-catenin is well documented in ovarian cancer [38]. In contrast, very little is known about the functional significance plakoglobin in this cancer. However, the loss of heterozygosity of the plakoglobin gene has been reported in sporadic ovarian cancer, whereas its nuclear accumulation was correlated with better survival rate in ovarian cancer patients [6, 39]. In the second chapter, we have investigated the tumor and metastasis suppressor functions of plakoglobin in epithelial ovarian cancer cell lines with mutant p53 expression and different adhesion profiles.

We showed that plakoglobin-deficient ovarian cancer cells that express N-cadherin and mutant p53 were highly migratory and invasive, whereas those that express mutant p53 and plakoglobin were not. The exogenous expression of plakoglobin or knockdown of N-cadherin significantly reduced migration and invasion. Plakoglobin interacted with wild type and mutant p53 proteins and associated with

E- or N-cadherins in adhesion complexes. N-cadherin is a mesenchymal marker that promotes tumorigenesis and metastasis. Our laboratory has shown that plakoglobin was able to induce a mesenchymal to epithelial phenotypic transition by sequestering and stabilizing N-cadherin at the membrane (40). Consistent with these studies, we detected a significant reduction in growth, migration and invasion of plakoglobin expressing and N-cadherin knockdown cells (41). Our data suggested that plakoglobin induced growth and metastasis inhibitory effects in ovarian cancer cells expressing N-cadherin and mutant p53.

5.1.2. Functional significance of plakoglobin and p53 interaction

Previous work form our laboratory has demonstrated that plakoglobin interacted with wild type and a number of mutant p53s in both the cytoplasm and nucleus and this interaction enhanced wild type p53's transcriptional activity and restored mutant p53s tumor suppressor function. These experimental evidence suggested that plakoglobin mediated its tumor and metastasis suppressor functions, at least partially, by interacting with mutant p53 proteins and changing their target genes [42,43]. In Chapter 4, we showed that the individual expression of wild type p53 and plakoglobin in p53 null and plakoglobin deficient H1299 cells significantly decreased their growth, migration and invasion *in vitro*. Furthermore, this inhibitory effect was significantly augmented when the two proteins were co-expressed. These observations strongly suggested that p53 and plakoglobin cooperated to promote a non-transformed and non-invasive phenotype *in vitro*.

In this Chapter, we mapped the interacting domain of p53 and plakoglobin. H1299 cells were co-transfected with a combination of constructs encoding wild type plakoglobin with wild type or various fragments of p53. Reciprocal co-transfectants, were generated expressing wild type p53 with wild type or different deletions of plakoglobin. Characterization of transfectants using confocal immunofluorescence microscopy and co-immunoprecipitation showed that: 1) p53 and plakoglobin interaction was mediated by the DNA binding domain of p53 and the C-terminal transactivation domain of plakoglobin, and 2) p53 and plakoglobin cooperatively decreased growth whereas they acted synergistically to significantly reduce *in vitro* migration and invasion and 3) the C-terminal domain of PG was important for its invasion inhibitory function via its interaction with p53.

5.1.3. Plakoglobin counteracts mutant p53 tumor promoting activity by suppressing β-catenin's oncogenic potential

Work from our laboratory and other researchers have shown that one way that plakoglobin may act as a tumor and metastasis suppressor protein is by changing β-catenin levels/subcellular localization and/or its oncogenic potential [44-47]. p53 GOF mutants have been shown to activate β-catenin oncogenic function [48-50].

In Chapter 4, we examined the effects of $p53^{R175H}$, one of the most frequently expressed p53 mutations [51], on β-catenin accumulation and transcriptional activation and their modification by plakoglobin expression. Our results showed that $p53^{R175H}$ expression in H1299 cells increased total and nuclear levels of β-catenin and its transcriptional activity concurrent with increased *in vitro* migration

and invasion of the transfectants. Co-expression of plakoglobin in $p53^{R175H}$ cells promoted β-catenin's proteasomal degradation, decreased its total and nuclear levels and its transactivation activity. β-catenin target genes, *Myc* and *S100A4* were upregulated in $p53^{R175H}$ expressing cells suggesting that the oncogenic function of this mutant is, at least in part, mediated by β-catenin activation. Co-expression of plakoglobin in $p53^{R175H}$ transfectants significantly decreased the expression of *MYC* and *S100A4* concurrent with a significant reduction in their migration and invasion *in vitro*. Together, these results suggest that plakoglobin may act as a tumor and metastasis suppressor protein by down-regulating the oncogenic effects of $p53^{R175H}$ and Wnt/β-catenin axis, two pathways that are known to be frequently dysregulated in many cancers.

5.2. Potential model for the tumor and metastasis suppressor functions of plakoglobin

Experimental findings from this thesis and the previous work in our laboratory and other research groups suggest that plakoglobin may act as a tumor and metastasis suppressor protein by several mechanisms (Figure 5-1). First, plakoglobin participates in the formation of adhesive junctions that are necessary for the maintenance of tissue integrity and induction of contact inhibition of growth and motility [52,53]. Second, plakoglobin interacts with various cellular protein partners and regulates their levels, subcellular localization and functions [54,55]. Our laboratory has shown that plakoglobin expression in plakoglobin deficient carcinoma cells increased the metastasis suppressor protein Nm23 protein levels [54]. Plakoglobin interacted with Nm23 and increased its stability by sequestering

it at the membrane [54]. Plakoglobin interacted with the dual tumor suppressor and oncogene NPM, promoting its tumor suppressor function [55]. Third, plakoglobin may regulate gene expression via its interaction with transcription factor p53 [56]. The focus of our laboratory is to study the functional significance of plakoglobin and p53 interaction. We showed that plakoglobin interacted with wild type and a number of mutant p53 proteins, which led to the induction of a less proliferative, migratory and invasive phenotype [41-43,56]. This phenotypic transition may be partially mediated by regulation of wild type p53 target genes by mutant p53s and/or transcriptional repression of mutant p53 targets when plakoglobin is expressed. Lastly, plakoglobin may sequester oncogenic functions of β-catenin by increasing its proteasomal degradation and decreasing its transcriptional activity [44,57,58 and Chapter 4] (Figure 5-1).

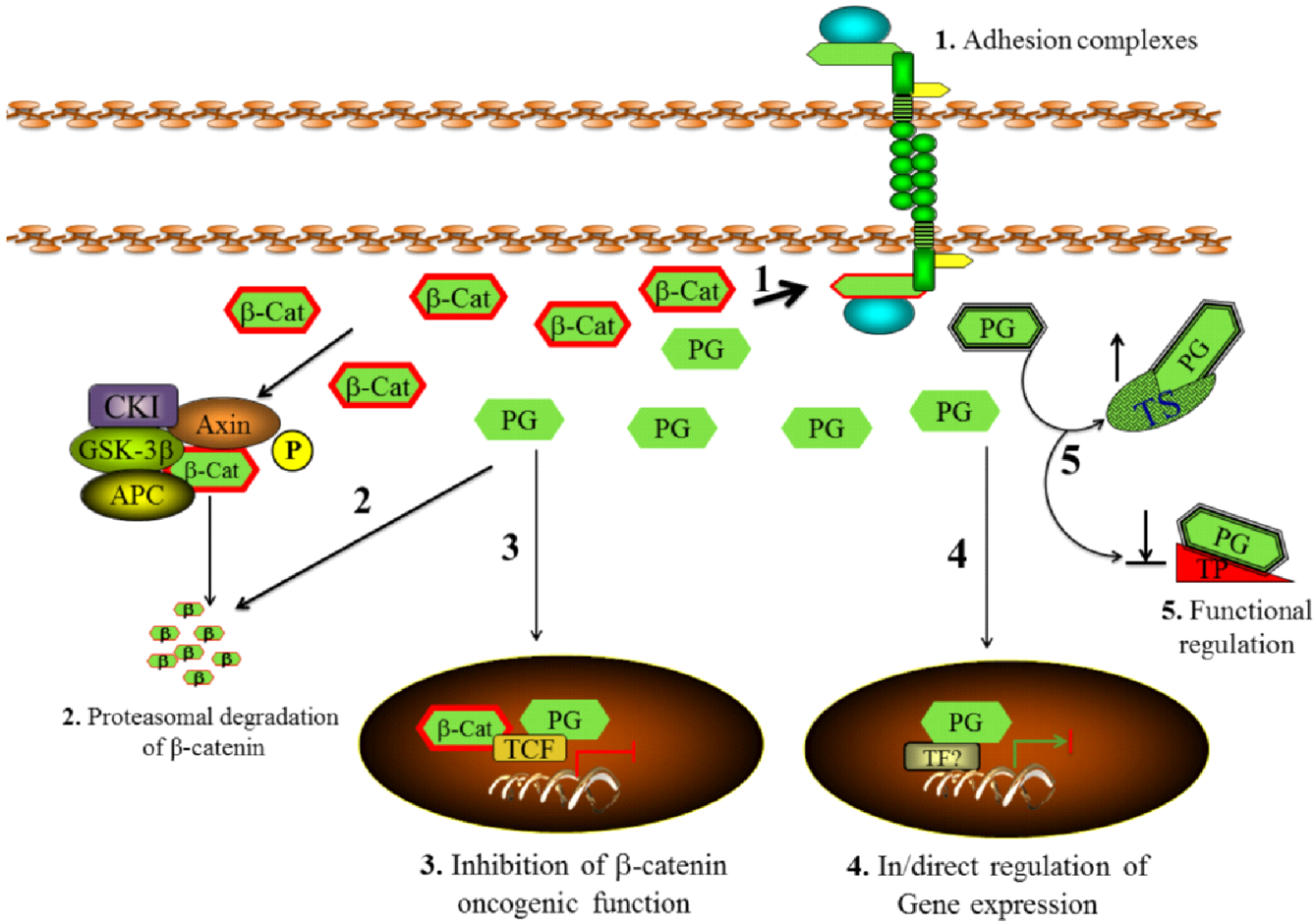

Figure 5.1. A potential model for tumor and metastasis suppressor activities of plakoglobin. (1) Upon synthesis both plakoglobin and β-catenin participate in the formation of junctional complexes at the membrane. **(2)** Excessive cytoplasmic pool of β-catenin gets phosphorylated by the destruction complex (Axin, APC, GSK3β, CKI) and subsequently is degraded via the proteasomal pathway. Plakoglobin enhances proteasomal degradation of β-catenin. **(3)** Plakoglobin interacts with β-catenin/TCF complexes and inhibits their interaction with DNA. **(4)** Plakoglobin may also interact with transcription factors in the nucleus to regulate the expression of target genes. **(5)** Furthermore, plakoglobin interacts with protein partners and regulates their levels, localization and therefore their tumor suppressive or promoting functions. α-cat, α-catenin; β-cat, β-catenin; APC, Adenomatous polyposis coli; CKI, Casein kinase I; GSK3β, Glycogen synthase kinase 3 β; PG, plakoglobin; TCF, T-cell factor; TF, transcription factor; TS, tumor suppressor; TP, tumor promoting

Modified from Pasdar, M.

5.3. Future directions

5.3.1. Identifying the exact amino acids involved in plakoglobin/p53 interaction

Previously, we performed *in vitro* pull down assays and confirmed that plakoglobin directly interacted with wild type p53. We successfully mapped the interacting domains of plakoglobin and p53. Subsequently, our collaborators in Dr. Jack Tuszynski group (Department of Oncology, University of Alberta), performed 3D modeling of PG and p53 interaction interface and tentatively identified the amino acids involved in this interaction. To verify the results of computational modeling, site-directed mutagenesis should be used to mutate each identified amino acid in wild type plakoglobin and p53. To verify the results of computational modeling, site-directed mutagenesis should be used to mutate each identified amino acid in wild type plakoglobin and p53 and subsequently *in vitro* pull down assay will need to be carried out to examine plakoglobin/p53 interaction.

Furthermore, similar 3-dimensional modeling experiments followed by site-directed mutagenesis and *in vitro* pull down assays should be used to identify the amino acid sequences involved in plakoglobin and mutant p53 proteins interaction.

The results from the *in vitro* pull down assays should be further validated by expressing the mutant plakoglobin and p53 constructs in human cell lines and confirming their interaction by co-immunoprecipitation studies.

5.3.2. Identifying mutant p53 target genes regulated by Plakoglobin

In this study, we have shown that plakoglobin down-modulates the oncogenic function of $p53^{R175H}$ by inhibiting tumorigenic effects of β-catenin. However, plakoglobin may also regulate the expression of mutant p53 target genes independent of the Wnt pathway. As a starting point to identify mutant p53 target genes that are regulated by plakoglobin, microarray analyses should be performed using H1299 cells expressing $p53^{R175H}$ with or without plakoglobin. The results of microarray studies should be further validated by performing biochemical assays including quantitative RT-PCR and western blot.

ChIP experiments should be carried out to assess the association of $p53^{R175H}$ with the promoter of the identified target genes in the presence or absence of plakoglobin expression. Luciferase reporter assays can be performed to further validate the effects of plakoglobin on the transcriptional activity of $p53^{R175H}$.

5.3.3. Investigating the role of plakoglobin in modulating the oncogenic effects of p53 contact mutations

Here, we have looked at the effects of structural mutant $p53^{R175H}$ on modulating the oncogenic potential of β-catenin in the presence and absence of plakoglobin expression. Future studies can focus on determining whether contact p53 mutants (R273H, R248Q and R248W) may induce oncogenic functions of β-catenin and if plakoglobin may inhibit their tumor growth and metastasis by inhibiting β-catenin's oncogenic activity. These studies can be performed as described in Chapter 5 and section 5.3.2. of this chapter.

5.3.4. Assessing the role of plakoglobin in regulating mutant p53 interaction with p63 and p73

As described in Chapter 1 (section 1.7.2), one mechanism by which GOF mutant p53 proteins act as oncogenes is by interacting with and inhibiting tumor suppressor activities of p63 and p73 [59-61]. As a future direction, the effects of plakoglobin expression on the mutant p53 proteins interaction with p63 or p73 can be assessed. Co-immunoprecipitation studies should be performed to assess the association of mutant p53 with p63 or p73 in the presence or absence of plakoglobin. ChIP experiments can be performed to examine the association of p63 with the promoter of its target genes in cells expressing mutant p53 proteins and plakoglobin individually or in pair. Transcriptional activity of p63 and p73 can be assessed using luciferase reporter in cells expressing mutant p53 proteins with or without plakoglobin.

5.3.5. Assessing the effects of plakoglobin/p53 interaction on *in vivo* tumorigenesis and metastasis

The studies presented in this thesis and previous work from our laboratory have shown plakoglobin expression significantly reduced *in vitro* tumorigenesis and metastasis in breast, ovarian, squamous cell, and non-small cell lung carcinoma cell lines expressing mutant p53 proteins. To validate the functional significance of these observations *in vivo*, potential tumor and metastasis suppressor effects of plakoglobin should be further assessed using xenograft animal models. Plakoglobin and p53 deficient cancer cell lines expressing plakoglobin, wild type and mutant

p53s individually or in pair can be used to inject immunodeficient mice models to assess *in vivo* tumor growth and metastasis.

5.4. Concluding remarks

In summary, my thesis further supports the earlier findings that have shown plakoglobin interacts with wild type and mutant p53 proteins to regulate tumorigenesis and metastasis. Here, for the first time we identified the interacting domains of plakoglobin and p53 and showed their cooperation in reducing *in vitro* growth, migration and invasion of cancer cells. Furthermore, we showed that plakoglobin down-regulated the oncogenic functions of $p53^{R175H}$ structural mutant at least partially by sequestering the oncogenic effects of β-catenin.

Experimental evidence from this work elucidated some of the underlying mechanisms involved in tumor and metastasis suppressor effects of plakoglobin via its interaction with wild type and mutant p53s and modulation of Wnt/β-catenin axis, two pathways that are known to be frequently dysregulated in many cancers. The larger implication of these studies is the potential for the development of therapeutic drugs that can mimic plakoglobin in order to simultaneously target the Wnt/β-catenin pathway and restoring wild type activities of mutant p53 proteins. Designing drugs based on the plakoglobin/p53 interaction has the advantage of mimicking the interaction of an endogenous cellular protein that normally interacts with p53 and therefore, may have less toxicity and side effects.

5.5. References

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