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Molecular characterization of the tumor/metastasis suppressor activity of plakoglobin

by

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To my baby girl Madina, whose bright eyes and beautiful smile light up my life

ABSTRACT

Plakoglobin (γ-catenin) is a member of the Armadillo family of proteins and a homolog of β-catenin with similar dual adhesive and signaling functions. The adhesive function of these proteins is mediated by their interactions with cadherins and their signaling function by association with various intracellular proteins, from signaling molecules to transcription factors. However, while β-catenin has well-documented oncogenic potential, plakoglobin signaling capabilities are typically associated with tumor/metastasis suppression through mechanisms that have remained unclear. The focus of this thesis was to elucidate the molecular mechanisms by which plakoglobin regulates tumorigenesis and metastasis. To this end, we expressed plakoglobin in plakoglobin-null human carcinoma cells and compared the mRNA and protein profiles of plakoglobin expressing cells with those of parental cells. We identified a number of oncogenes and tumor/metastasis suppressors whose mRNA/protein levels were decreased and increased, respectively, upon plakoglobin expression. Extensive characterization of the plakoglobin expressing cells showed that plakoglobin regulates tumorigenesis and metastasis by interacting with and altering the levels, localization and/or function of growth/metastasis regulating proteins and/or by associating with transcription factors that regulate the expression of genes involved in these processes.

Plakoglobin interacted with and increased both the protein and mRNA levels of the metastasis suppressor Nm23-H1 while only increasing the protein levels of Nm23-H2. Furthermore, in plakoglobin expressing cells, Nm23-H1/H2 complex was redistributed from the cytoplasm to the adherens junction at the membrane.

We also showed that plakoglobin interacted with p53 and together they regulated the expression of a number of p53-target genes, including tumor suppressors *SFN* and *NME1* and the tumor promoter *SATB1*. Concurrent with these changes, there was a significant decrease in cell proliferation and *in vitro* migration and invasion of plakoglobin expressing cells.

These results clearly demonstrate that plakoglobin plays an active role in suppressing tumorigenesis/metastasis through both the regulation of gene expression and by interacting with and altering the levels, localization and function of various intracellular proteins involved in these processes. The larger implication of this work is that plakoglobin may be a useful marker for diagnosis and prognosis as well as a therapeutic target for the treatment of various cancers.

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In the name of Allah, the most Gracious, the most Merciful.

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who at times was going crazy. I wish them continued success in their studies.

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14-3-3σ	Tumor suppressor protein
3-D	3-Dimensional
α-cat	α-catenin
A431	Human vulvar carcinoma cell line
ABL1	Gene encoding c-Abl
AML	Acute myeloid leukemia
ANKRD11	Ankyrin repeat domain 11
APC	Adenomatous polyposis coli, tumor suppressor protein
ARF	Alternate reading frame, tumor suppressor encoded by INK4a-ARF locus
ATM	Ataxia telangiectasia mutated, serine/threonine protein kinase
ATR	Ataxia telangiectasia and Rad3-related, serine/threonine protein kinase
AZA	5-aza-2'-deoxycytidine
β-cat	β-catenin
BCL2	Gene encoding Bcl-2 protein
BCL2A1	Gene encoding Bcl-2 related protein A1
Bcl-2	B-cell lymphoma-2, antiapoptotic
Bcl-xL	B-cell lymphoma-extra large, antiapoptotic
BEX5	Brain expressed, x-linked 5
BRCA1	Gene encoding BRCA1 tumor suppressor
BRMS1	Gene encoding BRMS1 protein

BRMS1	Breast cancer metastasis suppressor-1
BSA	Bovine serum albumin
BUR	Base-unpairing regions
c-Abl	Abelson murine leukemia viral oncogene homolog 1, oncogene
c-Fos	Oncogene
cad	Cadherin
cat	Catenin
Cdc42	Small GTPase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-chip	Chromatin immunoprecipitation and microarray technology
Chk2	Checkpoint kinase-2
CKI	Casein kinase-1
CLDN1	Gene encoding claudin-1
Claudin-1	Tight junction protein
CML	Chronic myeloid leukemia
CpG	DNA sequences prone to methylation
CSK	Cytoskeleton-extraction buffer
CtBP	C-terminal binding protein-1, transcriptional repressor
CXCL2	Gene encoding chemokine ligand-2
dI-dC	Poly(deoxyinosinic-deoxycytidylic) acid
DNA	Deoxyribonucleic acid

DNase	Deoxyriobnuclease
DP	Desmoplakin
DSC2	Gene encoding desmocollin-2
Dsc	Desmocollin, desmosomal cadherin
Dsg	Desmoglein, desmosomal cadherin
Dsh	Dishevelled
DTT	Dithiothreitol
E2F1	Transcription factor
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EDG2	Gene encoding lysophosphatidic acid receptor
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
ERBB2	Gene encoding ErbB2 protein
ErbB2	Growth factor receptor, oncogene
FBS	Fetal bovine serum
Fzd	Frizzled, Wnt receptor
G418	Geneticin antibiotic
GTP	Guanosine 5’-triphosphate
GTPase	Enzyme that hydrolyzes GTP
GSK-3β	Glycogen synthase kinase 3β
H1299	p53-null human NSCLC cell line

HCT116	Human colon carcinoma cell line
HDAC1	Histone deacetylase-1
HDAC5	Gene encoding histone deacetylase-5
Hdm2	Human double minute protein
hGH	Human growth hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HME1	Human mammary epithelial-specific marker; alternate name for 14-3-3σ
HRP	Horseradish peroxidase
HT1080	Human fibrosarcoma cell line
HUVEC	Human umbilical vascular endothelial cell line
IB	Immunoblot
IF (assay)	Immunofluorescence
IF	Intermediate filaments
IgG	Control non-specific antibodies
IHC	Immunohistochemistry
IL-2	Interleukin-2 gene
IP	Immunoprecipitate
JUN	Gene encoding jun oncogene
JUP	Gene encoding plakoglobin
KAI1	Metastasis suppressor
KISS1	Gene encoding Kiss-1
Kiss-1	Kisspeptin-1, metastasis suppressor

KpnI	Restriction enzyme
KTCTL 60	Human renal carcinoma cell line
LEF	Lymphoid enhancer factor
LRP	Low density lipoprotein-receptor-related protein, Wnt co-receptor
MCF-10-2A	“Normal” human mammary epithelial cell line
MCF-7	Non-invasive human breast carcinoma cell line
MCF-7-shPG	MCF-7 cells in which plakoglobin has been knocked down
MDA-231	Invasive human breast carcinoma cell line
MDA-231-PG	MDA-231 cells expressing plakoglobin
MDA-MB-435	Invasive human breast carcinoma cell line
MDCK	Madin-Darby Canine Kidney cell line
MF	Actin microfilaments
MMP1	Gene encoding MMP1 protein
MMP3	Matrix metalloproteinase-3
MMP7	Matrix metalloproteinase-7
mRNA	Messenger RNA
MUT	Mutant sequence
MYC	Gene encoding Myc oncogene
Myc	Oncogene
NDPK	Nucleoside diphosphate kinase
NES	Nuclear export signal
NIH3T3	Rat fibroblast cell line

NL9980	Human large cell lung cancer cell line
NLS	Nuclear localization signal
Nm23	Nonmetastatic protein 23
NME1	Gene encoding Nm23-H1
NME2	Gene encoding Nm23-H2
NPM	Nucleophosmin
NSCLC	Non-small cell lung carcinoma
^{32}P-ATP	Radioactive adenosine 5'-triphosphate
p120	p120-catenin
p53	Tumor suppressor, transcription factor
pBK-CMV	Expression vector
PBS	Phosphate buffered saline
pBV-Luc	Luciferase vector
PC3	α-catenin null human prostate cancer cell line
PCR	Polymerase chain reaction
PG	Plakoglobin (γ-catenin)
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PKP	Plakophilin
PML	Gene encoding promyelocytic leukemia protein
PMSF	Phenylmethylsulfonyl fluoride
PPD	Paraphenylene diamine
PTN	Gene encoding pleotrophin
qRT-PCR	Quantitative RT-PCR
R	Repressor

Rac	Small GTPase
RE	Response element
Rho	Small GTPase
RIPA	Radioimmunoprecipitation assay buffer
RK3E	Rat kidney cell line
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase-polymerase chain reaction
Sac1	Restriction enzyme
SATB1	Gene encoding SATB1 oncogene
SATB1	Special AT-rich sequence binding protein 1
SCC9	Human tongue squamous carcinoma cell line
SCC9-E	E-cadherin-expressing SCC9 cells
SCC9-PG	SCC9 cells expressing physiological levels of plakoglobin
SCC9-PG-Flag	SCC9 cells expressing flag-tagged wild-type plakoglobin
SCC9-PG-Flag-WT	Same as SCC9-PG-Flag
SCC9-PG-Flag-ΔN123	SCC9 cells expressing flag-tagged plakoglobin with N-terminal deletion (first 123 amino acids)
SCC9-PG-WT	Same as SCC9-PG
SCC9-PG-NES	SCC9 cells with restricted cytoplasmic distribution of plakoglobin
SCC9-PG-NLS	SCC9 cells with restricted nuclear distribution of plakoglobin

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFN	Stratifin, gene encoding the tumor suppressor 14-3-3σ
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNA1	Gene encoding Snail protein
Snail	Transcriptional repressor
SOX4	Transcription factor
Src	Non-receptor tyrosine kinase
SV40	Simian virus
SW620	Human colon carcinoma cell line
SYBR Green	Cyanine nucleic acid staining dye
T47D	Human breast carcinoma cell line
TCF	T-cell factor
TCL	Total cell lysate
TF	Transcription factor
TGF-β	Transforming growth factor-β
TP53	Gene encoding tumor suppressor p53
TSA	Trichostatin A
UTR	Untranslated Region
UV	Ultraviolet
VWF	Gene encoding Von Willebrand factor

WB	Western blot
Wnt-1	Signaling protein
WT	Wild-type; wild-type sequence

CHAPTER ONE: INTRODUCTION

1.1. Cadherin-mediated cell adhesion[1]

Epithelial tissues cover the surface of the body and line the internal cavities (McCaffrey and Macara, 2011). The structural integrity of these tissues requires extensive cell-cell adhesion and interactions mediated by the adhesive junctional complexes consisting of the adherens junction and desmosomes (Halbleib and Nelson, 2006; Jeanes et al., 2008; Makrilia et al., 2009; Yilmaz and Christofori, 2010, Saito et al., 2012; David and Rajasekaran, 2012). Adherens junctions are a ubiquitous type of intercellular junction and are present in both epithelial and non-epithelial cells (Halbleib and Nelson, 2006; Jeanes et al., 2008; Yonemura, 2011), whereas desmosomes are adhesive junctions that confer tensile strength and resilience to cells and are present not only in epithelial cells but also in non-epithelial cells that endure mechanical stress, such as cardiac muscle and meninges (Garrod and Chidgey, 2008; Kowalczyk and Green, 2013). Both adherens junctions and desmosomes are cadherin based. Cadherins are single-pass transmembrane glycoproteins that form homotypic interactions with cadherin proteins on neighboring cells and intracellularly interact with proteins of the catenin family (Saito et al., 2012; David and Rajasekaran, 2012). At the adherens junction, the C-terminal domain of E-cadherin interacts, in a mutually exclusive manner, with β-catenin or γ-catenin (plakoglobin), which then interacts with α-catenin, an actin-binding protein. A fourth catenin protein, p120-catenin, interacts with the

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juxtamembrane domain of E-cadherin and stabilizes the cadherin dimers at the membrane (Figure 1-1; Meng and Takeichi, 2009; Harris and Tepass, 2010; Brieher and Yap, 2013). At the desmosome, the desmosomal cadherins (desmocollins and desmogleins) interact intracellularly with plakophilin and plakoglobin, which in turn are associated with desmoplakin, an intermediate filament binding protein that connects the complex to the cytoskeleton (Figure 1-1; Garrod and Chidgey, 2008; Dusek and Attardi, 2011; Brooke et al., 2012).

Although originally identified as structural proteins with a “glue-like” function, subsequent studies have shown significant interactions between the cadherin-based cell adhesion complexes and elements of signal transduction pathways regulating growth and morphogenesis (Qian et al., 2004; Lilien and Balsamo, 2005; Nelson, 2008). More specifically, cadherin-independent β-catenin, plakoglobin and p120 have been shown to have signaling functions through their interactions with an array of functionally diverse proteins including receptor tyrosine kinases and phosphatases, tumor suppressors and transcription factors (Aktary and Pasdar, 2012; Pieters et al., 2012; Kim et al., 2013).

1.2. Signaling through catenins

Catenin-mediated cell signaling has been the focus of many studies, most of which have concentrated on β-catenin and p120-catenin, overlooking both α-catenin and plakoglobin. These studies have

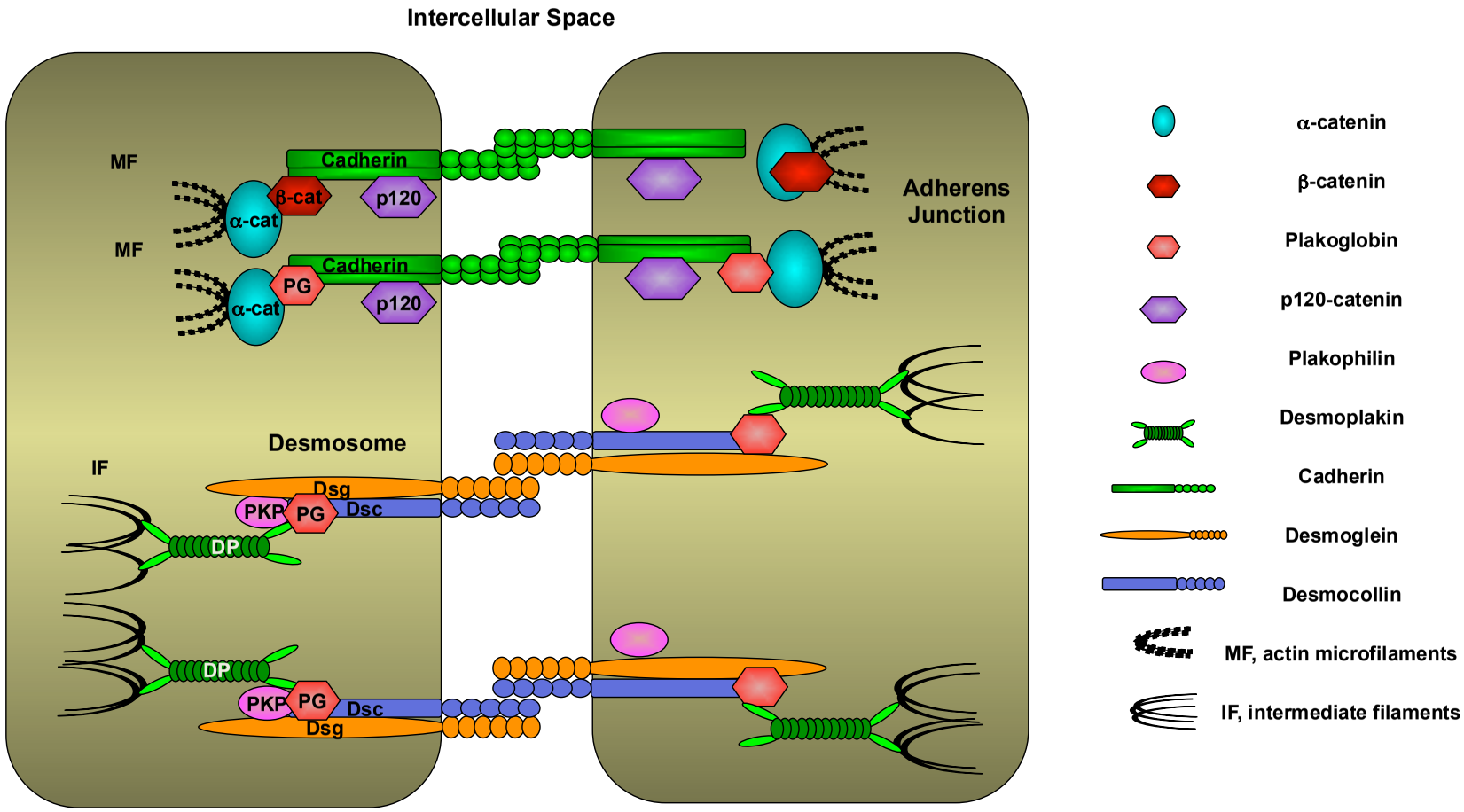

Figure 1-1. Cell adhesion complexes in epithelial cells. Cell-cell adhesion is maintained in epithelial tissues by the adherens junction and desmosomes. At the adherens junctions, E-cadherin forms extracellular interactions with E-cadherin molecules on neighboring cells. Intracellularly, E-cadherin interacts with either β-catenin or plakoglobin, which then interact with α-catenin, an actin binding protein. A fourth catenin, p120-catenin, also interacts with E-cadherin and regulates its stability at the membrane. At the desmosome, the desmosomal cadherins (desmoglein and desmocollin) interact with plakoglobin and plakophilin, which interact with desmoplakin, which in turn associates with the intermediate filament cytoskeleton. The basic, core protein composition of the desmosomes is represented here: the exact protein constituents of the desmosomes and their interactions vary between different types of cells and tissues.

suggested that both β-catenin and p120-catenin have oncogenic signaling activities through well-defined pathways, whereas plakoglobin acts as a tumor/metastasis suppressor through mechanisms that have just begun to be deciphered. Unlike β-catenin and p120, the role of α-catenin in cell signaling has not been examined to any significant detail. Only recently, a few studies have suggested that α-catenin may regulate the MAPK, Hedgehog and Hippo signaling pathways, although at this point it remains unclear as to how this regulation may occur (Vasioukhin et al., 2001; Lien et al., 2006; Schlegelmilch et al., 2011; Silvis et al., 2011; Maiden and Hardin, 2011).

On the contrary, the oncogenic signaling potential of p120-catenin has been well documented. Several studies have shown that cadherin-independent p120-catenin can translocate into the nucleus and interact with the transcription factor Kaiso, promoting the expression of tumor promoters, e.g. Siamois, c-Fos, Myc, cyclin D1, MMP7 (Figure 1-2; Pieters et al., 2012; Menke and Giehl, 2012). In addition, p120-catenin promotes cell migration by activating the Rho GTPases Rac and Cdc42 and inhibiting Rho, resulting in actin cytoskeleton remodeling and increased cell motility, migration and invasion (Pieters et al., 2012; Menke and Giehl, 2012).

β-catenin is the most extensively studied component of the cadherin catenin-complex with respect to signaling. β-catenin has a well-known

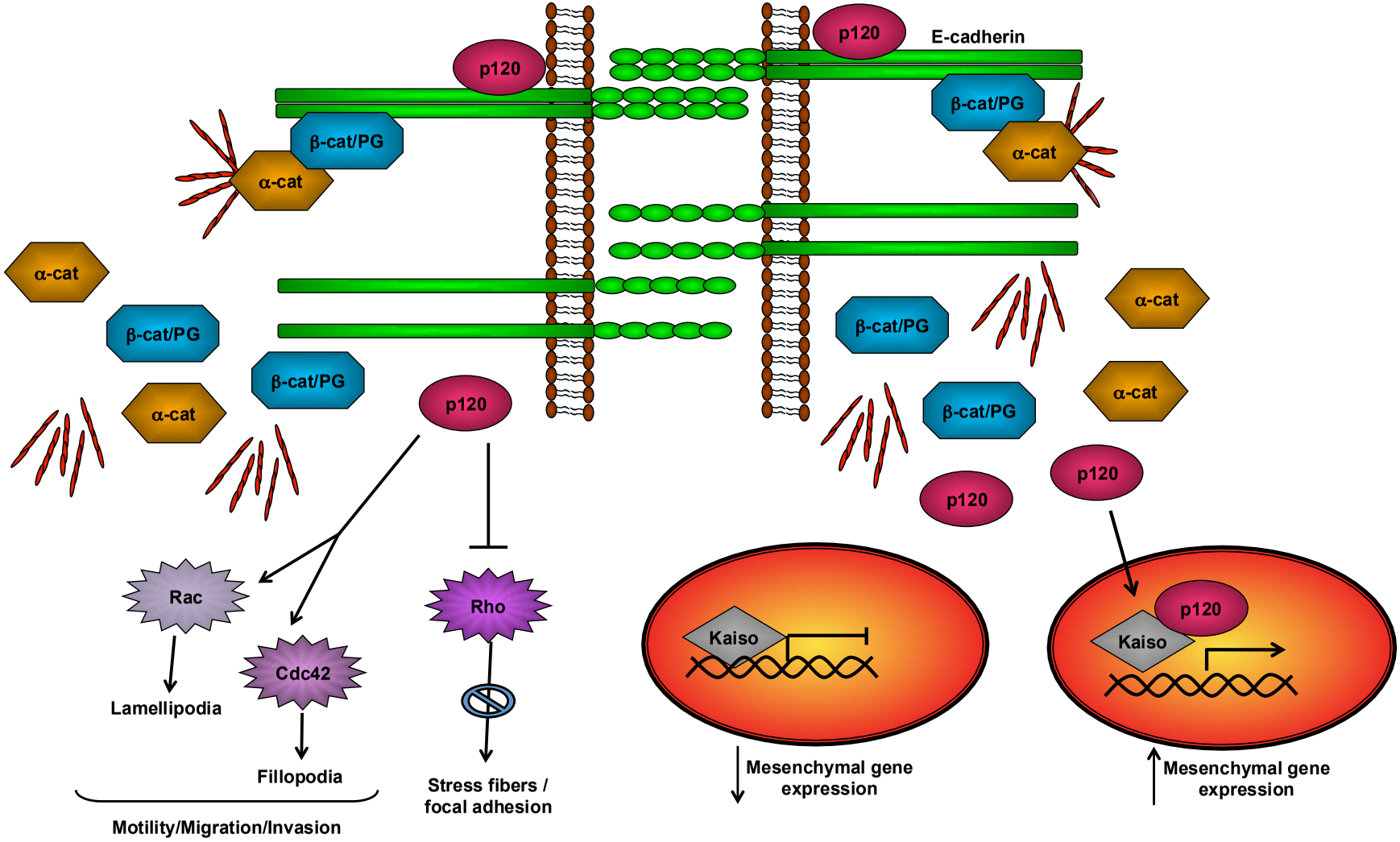

Figure 1-2. Cadherin independent p120-catenin signaling function. Under normal cellular conditions, p120-catenin is found as part of the cadherin-catenin complex and associates with the juxtamembrane domain of E-cadherin. During tumorigenesis, cadherin-mediated adhesion is disrupted and catenin proteins are released into the cytoplasm. Cadherin-independent p120-catenin can function in cell signaling in multiple ways. First, p120-catenin activates Rac and Cdc42 while inhibiting Rho, which results in the formation of fillopodia and lamellipodia and increased cell motility, migration and invasion. Second, p120-catenin can translocate into the nucleus, interact with the transcription factor Kaiso and promote the expression of genes involved in proliferation, migration and invasion. α-cat, α-catenin; β-cat, β-catenin; p120, p120-catenin; PG, plakoglobin.

oncogenic role as the terminal component of the Wnt signaling pathway and will be discussed in further detail.

1.3. β-catenin and the Wnt signaling pathway

The Wnt pathway is a signaling cascade with fundamental roles in the regulation of cell proliferation, cell polarity and cell fate determination during embryonic development and in tissue homeostasis. Deregulation of this pathway results in birth defects and various diseases, including cancer (Clevers, 2006; MacDonald et al., 2009; Niehrs, 2012; Clevers and Nusse, 2012; Kim et al. 2013).

Under normal conditions and in the absence of Wnt, cytoplasmic, cadherin-independent β-catenin levels are kept low through the action of the destruction complex, which consists of the tumor suppressor adenomatous polyposis coli (APC), the scaffolding protein Axin, and the kinases casein kinase I (CKI) and glycogen synthase kinase (GSK)-3β . The formation of this complex results in the phosphorylation of β-catenin and its subsequent degradation via the proteasome pathway (Figure 1-3; Huang and He, 2008; MacDonald et al., 2009; Valenta et al., 2012; Kim et al. 2013). During development and tumorigenesis, Wnt binds to its co-receptors frizzled and low-density lipoprotein receptor-related protein (LRP) 5/6. This binding leads to the activation of dishevelled protein and the relocalization of the components of the destruction complex to the membrane. As a result, the destruction complex does not form, which allows β-catenin to accumulate in the cytoplasm and translocate into the

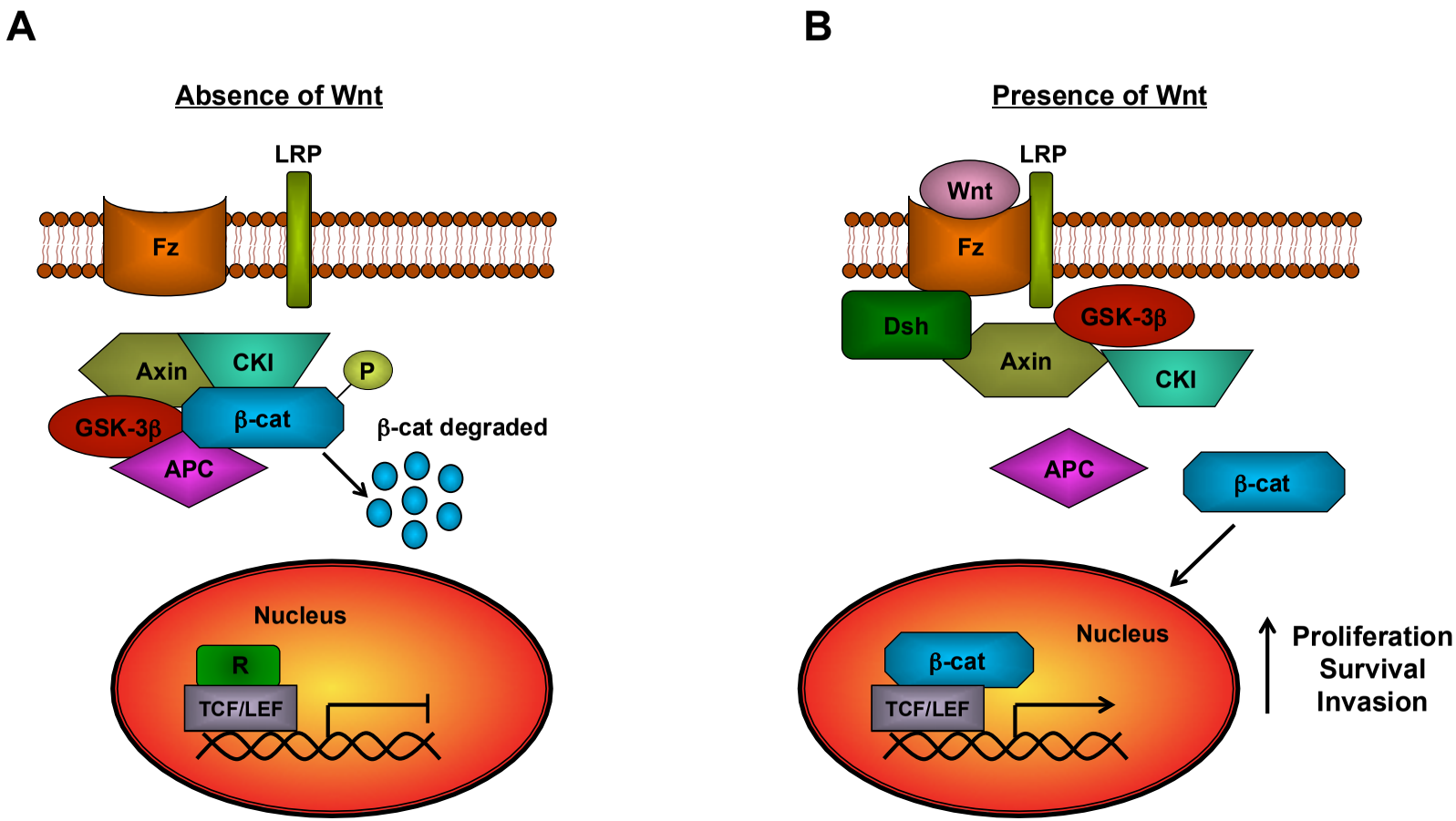

Figure 1-3. The Wnt signaling pathway. (A) Under normal conditions, excess cytoplasmic (cadherin-independent) β-catenin is actively degraded by the destruction complex, which consists of the tumor suppressor APC, the scaffolding protein Axin, and two kinases, GSK-3β and CKI. β-catenin is phosphorylated and degraded by the proteasome. **(B)** In the presence of Wnt, Wnt binds to its receptor, Frizzled, and recruits its co-receptor LRP. These activated receptors then activate dishevelled protein, which leads to the re-distribution of the components of the destruction complex to the membrane. Therefore, cytosolic β-catenin is not degraded, but rather can translocate into the nucleus. In the nucleus, β-catenin can regulate gene expression by interacting with the TCF/LEF family of transcription factors and promoting the expression of genes involved in proliferation, survival and invasion, which are otherwise repressed by these transcription factors. APC, adenomatous polyposis coli; CKI, casein kinase I; Dsh, dishevelled; FZ, frizzled; GSK, glycogen synthase kinase; LPR, low density lipoprotein receptor-related protein; TCF/LEF, T-cell factor/Lymphoid enhancer factor; TF, transcription factor. R, repressor.

nucleus where it interacts with the TCF/LEF family of transcription factors. The β-catenin-TCF complex can then activate the expression of genes involved in survival, proliferation, migration and invasion (Figure 1-3; Huang and He, 2008; MacDonald et al., 2009; Valenta et al., 2012; Kim et al. 2013).

1.4. β-catenin and plakoglobin: structural and functional homologs

Plakoglobin and β-catenin are structural and functional homologs and members of the Armadillo family of proteins (Peifer et al., 1992). As such, they share common intracellular partners, including classical cadherin, α-catenin, Axin, APC and TCF/LEF (Figure 1-4; Butz et al., 1992; Shibata et al., 1994; Kodama et al., 1999; Zhurinsky et al., 2000a; Aktary and Pasdar, 2012). Despite their structural similarities and their common interacting partners, plakoglobin and β-catenin appear to play opposite roles with respect to cell signaling in tumorigenesis and metastasis. As discussed above, β-catenin has a well-defined oncogenic potential as the terminal component of the Wnt signaling pathway, whereas plakoglobin has been typically associated with tumor and metastasis suppressor activities through mechanisms that have, up until recently, remained unknown (Simcha et al., 1996; Parker et al., 1998; Pantel et al., 1998; Charpentier et al., 2000; Winn et al., 2002; Rieger-Christ et al., 2005; Yin et al., 2005; Kanazawa et al., 2008; Narkio-Makela et al., 2009; Todorovic et al., 2010; Bailey et al., 2012; Holen et al., 2012; Franzen et al., 2012).

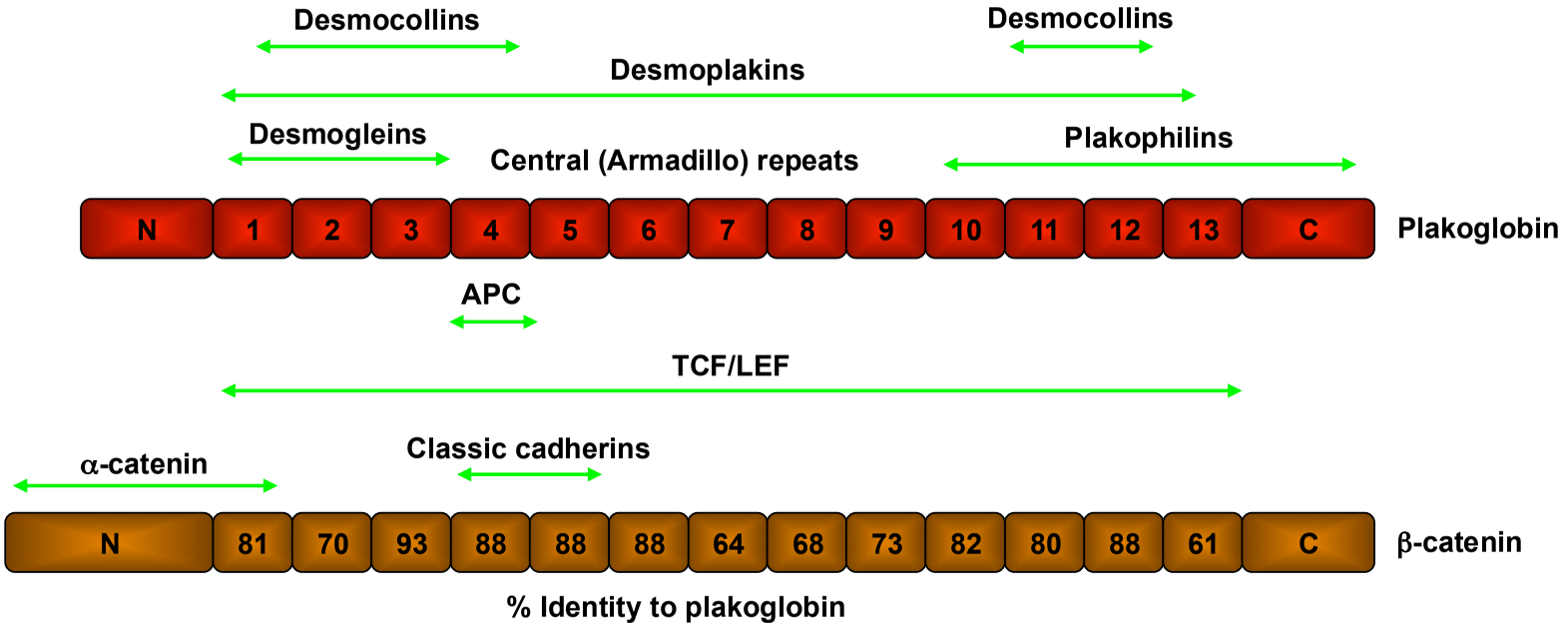

Figure 1-4. Schematic structure of β-catenin and plakoglobin. Both β-catenin and plakoglobin contain 13 Armadillo repeats that are flanked by N- and C-terminal domains, respectively. The degree of homology between β-catenin and plakoglobin for each Armadillo domain is indicated. Protein partners that interact with plakoglobin and the domains involved in these interactions are indicated. APC, adenomatous polyposis coli; TCF/LEF, T-cell factor/Lymphoid enhancer factor.

This thesis is focused on characterizing the mechanisms by which plakoglobin regulates tumorigenesis and metastasis.

1.5. Plakoglobin initial identification and early characterization

Plakoglobin was initially identified as an 83 kDa protein component of the desmosomal plaque (Franke et al., 1983). Subsequently, experiments using monoclonal antibodies, cDNA cloning and a combination of biochemical, morphological and molecular approaches demonstrated that this 83 kDa protein was present in both desmosomes and the adherens junction and was given the name plakoglobin (Cowin et al., 1986).

Although plakoglobin was identified as a junctional protein, the role that it played in junctional complexes was unclear, and the partners with which plakoglobin interacted were not identified. It was not until several years later that coimmunoprecipitation experiments showed that plakoglobin interacted with the desmosomal cadherin desmoglein, thereby confirming plakoglobin as a constituent of the desmosomes (Korman et al., 1989). Around the same time, several groups showed that E-cadherin (initially known as uvomorulin) immunoprecipitates contained three distinct proteins, named α-, β- and γ-catenin with molecular weights of approximately 102, 88 and 80 kDa, respectively, which interacted with the cytoplasmic domain of E-cadherin (Vestweber and Kemler, 1984; Peyrieras et al., 1985; Ozawa and Kemler, 1992). Further work analyzing the formation and stability of the E-cadherin-catenin complexes suggested that the E-cadherin-β-catenin complex was formed immediately after E-

cadherin synthesis and was very stable. Interestingly, it was also determined that α-catenin could not be found in association with E-cadherin independent of β-catenin, suggesting that β-catenin was a physical link between E-cadherin and α-catenin. However, since γ-catenin was found to be only loosely associated with E-cadherin, it was suggested that the main adhesive complexes consisted of E-cadherin, β-catenin and α-catenin, although the existence of a separate E-cadherin- γ-catenin complex could not be ruled out (Ozawa and Kemler, 1992).

Soon after this initial characterization, work from several groups demonstrated that plakoglobin and γ-catenin were the same E-cadherin interacting protein and that this protein was homologous to, yet a different protein from, β-catenin (McCrea et al., 1991; Knudsen and Wheelock, 1992; Piepenhagen and Nelson, 1993). Further studies demonstrated that plakoglobin and β-catenin are homologues of the *Drosophila* Armadillo protein with similar properties and together constituted the Armadillo multigene family (Peifer et al., 1992).

Subsequent analysis of the kinetics of plakoglobin synthesis and associations with cadherins demonstrated that following synthesis, plakoglobin interacted with both desmoglein and E-cadherin in both the soluble and cytoskeleton-associated pools of cellular proteins. Furthermore, a distinct, cadherin-independent pool of plakoglobin was observed, suggesting that in addition to cell-cell adhesion, plakoglobin may have an adhesion-independent role in the cell (Pasdar et al., 1995).

Finally, phosphorylation experiments revealed that whereas the insoluble (cadherin-associated) pool of plakoglobin was serine phosphorylated, the soluble pool was serine, threonine and tyrosine phosphorylated, suggesting that these different pools of plakoglobin are differentially regulated and perform varying functions (Pasdar et al., 1995). Collectively, these studies demonstrated that plakoglobin is a homolog of β-catenin and a unique protein in that it is the only component common to both the adherens and desmosomal junctions.

1.6. Plakoglobin and cell-cell adhesion

The most documented role of plakoglobin within the cell is in cell-cell adhesion. The identification of plakoglobin as a constituent of both the adherens junction and desmosomes suggested that it plays an important role in regulating cell-cell adhesion. However, the observation that the adherens junctions could exist as a complex containing E-cadherin, β-catenin and α-catenin, independent of plakoglobin (Ozawa and Kemler, 1992), questioned the necessity of plakoglobin, at least at the adherens junctions. Regardless, it soon became apparent that plakoglobin does have an essential role in regulating cell-cell adhesion.

Earlier *in vitro* studies had shown that the disruption of E-cadherin based cell-cell adhesion led to a transformed and/or invasive phenotype while re-expression of E-cadherin in E-cadherin-null cells resulted in a mesenchymal to epithelial phenotypic transition (Nagafuchi et al., 1987; Nose et al., 1988; Mege et al., 1988; Behrens et al., 1989; Vleminckx et

al., 1991; Chen and Obrink, 1991). Similarly, a number of *in vivo* studies demonstrated that the reduced expression of E-cadherin was inversely correlated with the differentiation grade of tumors (Shimoyama et al., 1989; Schipper et al., 1991; Gamallo et al., 1993). Thus, while it was clear that the E-cadherin based junctions were important for the maintenance of an “epithelial” phenotype, the role of plakoglobin in this phenomenon was not discerned until it was shown that the re-expression of E- or P-cadherin in cadherin-null murine spindle cell carcinomas with very low levels of plakoglobin was not sufficient to modify the morphology or tumorigenicity of the cells (Navarro et al., 1993). In these cells, although the exogenously expressed cadherins were localized to the cell membrane and interacted with both α- and β-catenin, they did not interact with plakoglobin and there was no desmosome formation. From this work, the authors concluded that the presence of plakoglobin in the E-cadherin complex may be necessary for proper cell-to-cell adhesion.

The role of plakoglobin in regulating junction formation was also demonstrated when it was shown that A431 epithelial cells treated with dexamethasone (which decreased E-cadherin and plakoglobin levels) were unable to form adherens junctions and desmosomes and exhibited a fibroblastic morphology. Following the expression of E-cadherin in these cells, the adherens junction was formed but the fibroblastic morphology of the cells remained unchanged. The authors then expressed an E-cadherin-plakoglobin chimeric protein in these cells, which led to the

formation of stable adherens junctions and desmosomes as well as an induction of an epithelioid morphology. Together, these results suggested that E-cadherin-plakoglobin interactions were necessary for the formation of stable adhesive complexes and provided the first indication that plakoglobin served as a molecule involved in cross-talk between the adherens junctions and desmosomes (Lewis et al., 1997).

Following this study, our laboratory demonstrated the role of plakoglobin in junction formation by expressing plakoglobin in SCC9 cells, a human tongue squamous cell carcinoma cell line that lacks the expression of both plakoglobin and E-cadherin but expresses N-cadherin (Parker et al., 1998; Li et al., 1998). Transfectants expressing E-cadherin (SCC9-E) or low/physiological levels of plakoglobin (SCC9-PG) or both were generated and showed that the independent expression of either E-cadherin or plakoglobin induced a mesenchymal (transformed) to epidermoid (normal) phenotypic transition. This phenotypic transition was associated with decreased cell proliferation and increased cell-cell adhesion and only SCC9-PG cells were able to form desmosomes. E-cadherin or plakoglobin expression also coincided with decreased soluble β-catenin levels however, while E-cadherin expression downregulated N-cadherin, plakoglobin expression increased N-cadherin levels and stability (Li et al., 1998; Parker et al., 1998). Since then, numerous subsequent studies identified the switch from E- to N-cadherin as a major contributing factor in epithelial to mesenchymal phenotypic transition and metastatic

progression. Significantly, our results clearly demonstrated that in the absence of E-cadherin, plakoglobin was able to inhibit N-cadherin tumor promoting activities and that the cadherin switch by itself cannot explain the transformed phenotype of SCC9 cells. Furthermore, the induction of the mesenchymal to epidermoid phenotype by E-cadherin and plakoglobin may occur via a common pathway that also involves β-catenin (Li et al., 1998; Parker et al., 1998).

Other studies have further characterized the role of plakoglobin in desmosome assembly and function, demonstrating the essential role of plakoglobin for the proper assembly of the desmosomal plaque and the efficient binding of desmoplakins to the intermediate filaments (Palka and Green, 1997; Acehan et al., 2008). Finally, work from Birchmeier's laboratory showed that plakoglobin double knockout mice died during embryogenesis as a result of disrupted heart function due to the loss of stable desmosomes in the intercalated discs of cardiac muscle, further confirming the essential role of plakoglobin in desmosome formation and the adhesive properties of cells (Ruiz et al., 1996; Ruiz and Birchmeier, 1998).

1.7. Plakoglobin and cell signaling

While the majority of plakoglobin is found in association with desmosomes and adherens junction, there is a considerable amount (~ 35%) of non-junctional plakoglobin that can potentially participate in cell signaling (Pasdar et al., 1995). However, when studying the signaling

activity of plakoglobin one must take into account the fact that due to its structural and functional homology to β-catenin, plakoglobin can participate in cellular signaling in four ways (Figure 1-5). First, plakoglobin may potentially displace β-catenin from the adherens junction, causing increased cytoplasmic β-catenin levels and its subsequent nuclear translocation, ultimately leading to the activation of the Wnt pathway and changes in the expression of TCF/β-catenin target genes. In this scenario, plakoglobin would exhibit an oncogenic potential. Second, plakoglobin may compete with β-catenin signaling by inhibiting TCF/β-catenin-DNA interactions and Wnt target genes expression. Third, plakoglobin may interact with transcription factors and regulate gene expression independent of β-catenin. Finally, plakoglobin may interact with various cellular partners involved in signaling and alter their levels, localization and/or function (Figure 1-5). In the latter three cases, plakoglobin would exhibit growth inhibitory function. Experimental evidence from our laboratory and other groups suggests that plakoglobin participates in cell signaling through all of these mechanisms (see below).

1.8. Plakoglobin oncogenic signaling

The first clue that plakoglobin might participate in cell signaling came from studies of the exogenous expression of Wnt-1 in PC12 cells. In these cells, plakoglobin levels were increased and it underwent membrane redistribution, suggesting that in addition to β-catenin levels, Wnt-1 can modulate plakoglobin levels and localization (Bradley et al., 1993).

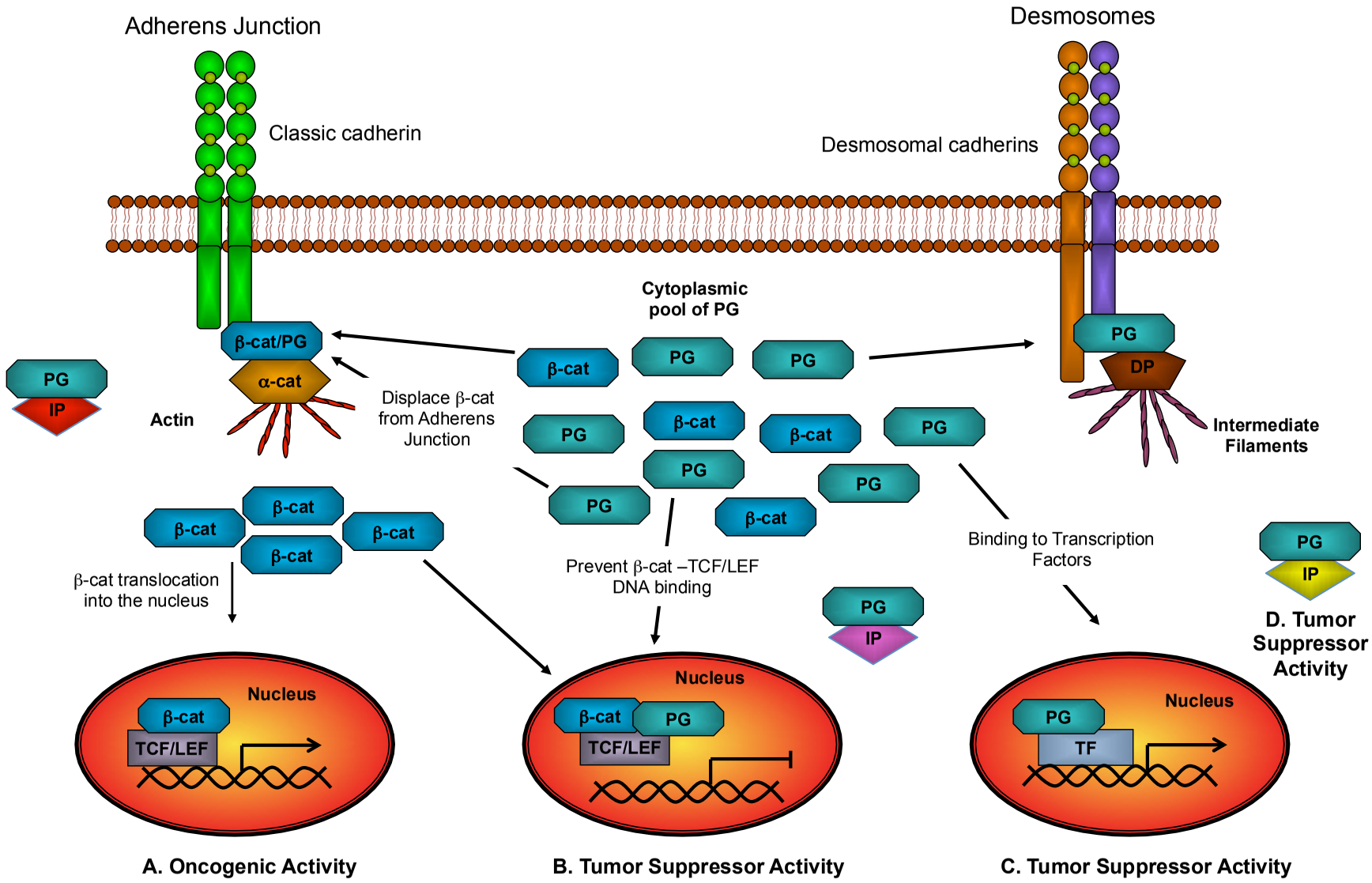

Figure 1-5. Potential mechanisms of plakoglobin-mediated signaling. Cytoplasmic, junction-independent plakoglobin may function in cell signaling in one of four possible ways. **(A)** Cytoplasmic plakoglobin may participate in oncogenic signaling, since plakoglobin can displace β-catenin from the adherens junction. The increased cytoplasmic β-catenin can then translocate into the nucleus and increase the expression of oncogenes. **(B)** Alternatively, plakoglobin may act as a tumor suppressor by translocating into the nucleus, interacting with β-catenin/TCF complexes and inhibiting the complex from binding to and promoting the expression of oncogenes. **(C)** Plakoglobin may also regulate the expression of tumor and metastasis suppressor genes through its interactions with various other transcription factors. **(D)** In addition, plakoglobin may interact with other intracellular partners involved in tumorigenesis and metastasis and alter their levels, localization or function. α-cat, α-catenin; β-cat, β-catenin; DP, desmoplakin, PG, plakoglobin; TCF/LEF, T-cell factor/Lympoid enhancer factor; TF, transcription factor; IP, interacting protein.

Subsequently, Karnovsky and Klymkowsky (1995) demonstrated plakoglobin signaling activity by microinjecting mRNAs encoding plakoglobin into fertilized *Xenopus* embryos, resulting in dorsalized gastrulation and anterior axis duplication. In this study, the exogenously expressed plakoglobin localized both at the plasma membrane and in punctate nuclear aggregates. Furthermore, the co-injection of mRNAs encoding plakoglobin and the cytoplasmic domain of desmoglein suppressed both dorsalized gastrulation and anterior axis duplication. In these embryos, plakoglobin was localized primarily to the plasma membrane with some peri-nuclear distribution. These results suggested that plakoglobin has signaling ability similar to β-catenin, but when it is sequestered at the plasma membrane (as part of junctional complexes), plakoglobin is unable to participate in cell signaling.

Following these initial observations, the same group showed that this outcome does not depend on the nuclear localization of plakoglobin, since membrane-anchored forms of this protein produced the same axis duplication (Merriam et al., 1997). This demonstrated that nuclear plakoglobin was inconsequential in inducing a Wnt-like phenotype, since this phenotype was induced even when plakoglobin was sequestered in the cytoplasm.

Later on, Kolligs et al. (2000) proposed that plakoglobin has oncogenic signaling activity and showed that the tumor suppressor adenomatous polyposis coli (APC), which was already known to regulate the levels of β-

catenin, could also regulate plakoglobin protein levels. In this study, the authors also showed that exogenous overexpression of plakoglobin in rat RK3E cells, which express considerable amounts of endogenous plakoglobin and β-catenin (Kolligs et al., 2000; Bommer et al., 2005), resulted in a transformed phenotype, which they suggested was dependent on the upregulation of the oncogene c-Myc and activation of TCF/LEF signaling. More recently, Pan et al. (2007) showed that the exogenous expression of plakoglobin in HCT116 colon carcinoma cells, which express a mutant β-catenin protein that cannot be degraded (Morin et al., 1997), resulted in genomic instability and increased invasion and migration.

Although these studies concluded that plakoglobin possessed oncogenic activity, evidence suggests that this activity may be indirectly achieved, through modulation of β-catenin protein levels and activation of its signaling function (Figure 1-5A). Since plakoglobin and β-catenin interact with some of the same proteins and display high sequence homology (Butz et al., 1992; Shibata et al., 1994; Kodama et al., 1999; Zhurinsky et al., 2000a; also see Figure 1-4), overexpressed plakoglobin may be able to promote tumorigenesis by interacting with proteins that would normally sequester β-catenin (e.g. E-cadherin, Axin, APC), resulting in increased levels of cytoplasmic and nuclear β-catenin and in turn, its enhanced signaling. Indeed, Salomon et al. (1997) showed that overexpression of plakoglobin in HT1080 fibrosarcoma cells resulted in the

replacement of β-catenin by plakoglobin in the cadherin-catenin complexes and subsequent translocation of the excess cytoplasmic β-catenin into the nucleus. This was also supported by the overexpression of plakoglobin in NIH3T3 cells, which resulted in the nuclear accumulation of β-catenin (Simcha et al., 1998). Furthermore, overexpression of the Wnt co-activator LEF-1 in MDCK cells resulted in its preferential interaction with β-catenin (instead of plakoglobin) and the subsequent localization of the β-catenin-LEF-1 complexes to the nucleus, suggesting that when both plakoglobin and β-catenin were present within the cell, β-catenin-LEF-1 complexes were more readily formed and transcriptionally active (Simcha et al., 1998). Additional studies examining the ability of plakoglobin and β-catenin to signal via interactions with the TCF/LEF family of transcription factors showed that although plakoglobin interacted with LEF-1, this complex was inefficient in binding to DNA, whereas β-catenin-LEF-1 complexes were readily associated with DNA. Furthermore, β-catenin was a much stronger activator of TCF/LEF target genes than plakoglobin and overexpression of plakoglobin resulted in increased β-catenin-LEF-1 complex formation and its activation (Zhurinsky et al., 2000b; Williams et al., 2000). Consistent with these observation, work from our laboratory has shown that while the expression of low/physiological levels of plakoglobin in plakoglobin deficient SCC9 cells induced a mesenchymal to epidermoid change in phenotype, its overexpression led to a more transformed phenotype concurrent with upregulation of the pro-survival protein Bcl-2,

foci formation and decreased apoptosis (Hakimelahi et al., 2000). Using cDNAs encoding plakoglobin fused to nuclear localization (NLS) or nuclear export (NES) signals, we subsequently showed that Bcl-2 levels were upregulated in plakoglobin overexpressing-SCC9 cells regardless of plakoglobin localization. Furthermore, in these cells, β-catenin-N-cadherin interactions were decreased, and β-catenin accumulated in the nucleus, interacted with TCF and became transcriptionally active, confirming that the overexpressed plakoglobin acted indirectly by enhancing the signaling capability of β-catenin (Li et al., 2007a).

Finally, recent studies in leukemia cells have shown that plakoglobin was overexpressed in both acute and chronic myeloid leukemias (AML and CML, respectively) and that this overexpression resulted in the increased stability and nuclear localization of β-catenin. In AML, TCF-dependent reporter activity was increased in the presence of plakoglobin and in CML, knock down of plakoglobin resulted in decreased β-catenin nuclear localization, suggesting that plakoglobin promoted tumorigenesis in leukemia by increasing β-catenin signaling activity (Morgan et al., 2012; Niu et al., 2012).

The above studies support the notion that the oncogenic activity of plakoglobin is indirect and mediated by β-catenin. Therefore, it is likely that in the studies reported by Kolligs et al. (2000) and Pan et al. (2007), the oncogenic potential of plakoglobin resulted from increased β-catenin signaling. In the Kolligs's study where plakoglobin was overexpressed in

RK3E cells (which express endogenous β-catenin and plakoglobin; Bommer et al., 2005), it was not determined if plakoglobin could activate c-Myc expression in the absence of β-catenin or whether either of these catenins was detected in the nucleus in association with the c-Myc promoter. In Pan's study in which HCT116 cells showed increased genomic instability and migration and invasion upon plakoglobin expression, the endogenous β-catenin was a mutant protein that was unable to be degraded (Morin et al., 1997). Furthermore when these HCT116 cells were induced to overexpress plakoglobin they showed increased expression of the oncogenes securin and c-Myc and decreased expression of E-cadherin, all of which are well-documented β-catenin target genes (He et al., 1998; Zhou et al., 2005; ten Berge et al., 2008). Taken together, the evidence suggests that although plakoglobin expression may lead to a transformed phenotype, it is highly likely that this outcome is due to the increased levels of signaling competent β-catenin rather than plakoglobin's oncogenic activity (Figure 1-5A).

1.9. Plakoglobin tumor suppressor activity

The first demonstration of the tumor suppressor activity of plakoglobin was when Simcha et al. (1996) found that plakoglobin expression in SV40-transformed NIH3T3 cells decreased the ability of these cells to form tumors in syngeneic mice. This growth suppressive effect of plakoglobin was augmented by co-transfection with N-cadherin. The authors also expressed plakoglobin in the tumorigenic renal carcinoma cell line KTCTL

60, which lacks endogenous expression of E-cadherin and desmosomal cadherins, α-catenin, β-catenin, plakoglobin and desmoplakin. Plakoglobin expression in KTCTL 60 cells also inhibited the tumorigenicity of these cells in syngeneic mice. Notably, the majority of the plakoglobin in these cells was Triton X-100 soluble, suggesting that it was not junction associated. This observation was clearly significant because it demonstrated that plakoglobin could suppress tumorigenesis independent of its role in cell-cell adhesion.

We previously showed that plakoglobin expression in plakoglobin-null SCC9 cells resulted in a mesenchymal to epidermoid phenotypic transition. This phenotypic transition of plakoglobin-expressing SCC9 cells was concurrent with the downregulation of β-catenin, stabilization of N-cadherin, formation of desmosomes and decreased growth, migratory and invasive properties of these cells (Parker et al., 1998, Aktary and Pasdar 2013). These results suggested that plakoglobin may act as a tumor and potentially a metastasis suppressor protein.

The ability of plakoglobin to inhibit cell growth and proliferation was also observed by tissue specific expression of plakoglobin in epidermal basal cells and hair follicles of transgenic mice (Charpentier et al., 2000). In these animals, plakoglobin expression reduced the proliferative potential of the epidermal cells and the growth phase of the hair follicles and furthermore, hairs were shorter by roughly 30%.

Further evidence suggesting a growth suppressive activity for plakoglobin was provided in lung cancer, when it was shown that while β-catenin was uniformly expressed in various Non-small cell lung cancer (NSCLC) cell lines and lung primary tumors, plakoglobin expression was very low or completely absent (Winn et al., 2002). The authors showed that exogenous expression of plakoglobin in the low-plakoglobin expressing NSCLC cells resulted in decreased β-catenin-TCF signaling, which was concurrent with decreased cell and anchorage-independent growth. This result also supported the idea that plakoglobin can act as a tumor suppressor by inhibiting the oncogenic activity of β-catenin.

Interestingly, when the authors treated these NSCLC cells with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (AZA) or the histone deacetylase inhibitor trichostatin A (TSA), plakoglobin levels were increased. Previous analysis of the plakoglobin promoter had described CpG islands within the promoter (Potter et al., 2001), and while it had been observed that inhibition of DNA methylation could result in increased plakoglobin protein levels in at least one thyroid carcinoma cell line (Husmark et al., 1999), this was the first indication that both DNA methylation and histone deacetylation played important roles in regulating plakoglobin expression.

The occurrence of methylated CpG islands within the plakoglobin promoter as well as histone deacetylation has not been limited to NSCLC cell lines. Various groups have shown that the plakoglobin promoter is

methylated in prostate, bladder, trophoblastic and mammary carcinomas (Shiina et al., 2005; Canes et al., 2005; Rahnama et al., 2006; Shafiei et al., 2008), which is concurrent with a transformed phenotype. Canes et al. (2005) have shown that treatment of bladder carcinoma cells with TSA resulted in increased plakoglobin expression and a decreased ability of these cells to form tumors in mice, once again suggesting a growth inhibitory activity of plakoglobin. Similarly, when mammary carcinoma cell lines were treated with AZA, plakoglobin levels were increased while soft agar colony formation and overall cell growth were decreased (Shafiei et al., 2008).

Consistent with its growth suppressor activity, several lines of evidence suggest that plakoglobin plays a role in regulating apoptosis. In their work describing the effects of plakoglobin on epithelial proliferation and hair growth in transgenic mice, Charpentier et al. (2000) also showed that plakoglobin expression resulted in premature apoptosis since the inner root sheath of the plakoglobin-expressing transgenic follicles underwent apoptosis two days earlier than in normal hair follicles. In agreement with these findings, we have previously shown that SCC9 cells expressing physiological levels of plakoglobin were more prone to undergo staurosporine-induced apoptosis relative to parental SCC9 cells (Hakimelahi et al., 2000). We have also observed that SCC9 cells expressing plakoglobin exclusively in the nucleus (SCC9-PG-NLS) showed decreased Bcl-2 levels compared to cells with overexpressed

wild-type plakoglobin (Li et al., 2007a), which suggests that plakoglobin may play a more direct role in regulating the expression of apoptotic genes. More recently, it has been shown that mouse keratinocytes that lack endogenous plakoglobin expression were protected from etoposide-induced apoptosis, whereas plakoglobin-expressing keratinocytes readily underwent apoptosis upon etoposide treatment (Dusek et al., 2007). In this study, the authors demonstrated that plakoglobin-null keratinocytes were unable to release cytochrome c from the mitochondria and activate caspase 3, suggesting that plakoglobin may play a role in regulating the apoptotic cascade. Furthermore, the mRNA levels of the anti-apoptotic protein Bcl-X_L were higher in the plakoglobin-null keratinocytes, which could potentially have prevented the translocation of cytochrome c from the mitochondria. Finally, the expression of plakoglobin in the null keratinocytes resulted in decreased Bcl-X_L levels, caspase 3 activation and apoptosis induction following etoposide treatment. Taken together, these studies have demonstrated that plakoglobin does have some role in apoptosis signaling and potentially may exert part of its tumor suppressor activity through the modulation of apoptosis.

1.10. Plakoglobin metastasis suppressor activity

As the tumor suppressor activity of plakoglobin began to be revealed, it soon became evident that in addition to inhibiting the growth properties of carcinoma cell lines, plakoglobin also plays a role in regulating the invasive and migratory properties of cancer cells. The initial observation of

plakoglobin's metastasis suppressor activity was documented in human umbilical vascular endothelial (HUVEC) cells, where plakoglobin is typically associated with sites of cell-cell contact (Nagashima et al., 1997). Plakoglobin anti-sense oligonucleotides increased HUVEC migration, suggesting that the loss of plakoglobin expression led to an increased migratory phenotype. Concurrent with increased migration, the anti-sense treated HUVEC cells also became more prone to forming tubular structures in Matrigel, suggesting that plakoglobin knock down also promoted angiogenesis.

The metastasis suppressor activity of plakoglobin was next described using MCF-7 cells, which express membrane localized E-cadherin and plakoglobin, and stable cell junctions. In this study, the authors treated MCF-7 cells with human growth hormone (hGH) and observed a downregulation of plakoglobin, a cytoplasmic distribution of E-cadherin and an increased migratory and invasive phenotype, which was accompanied by an increase in matrix metalloproteinase levels. They demonstrated that hGH-mediated invasiveness was dependent on Src kinase and that chemical inhibitors of Src resulted in increased plakoglobin levels and in turn, decreased invasion and migration. To discern the specific role of plakoglobin in these processes, the authors expressed plakoglobin in the hGH-treated MCF-7 cells, which resulted in both the decreased migration and invasiveness of these cells (Mukhina et al., 2004). Similarly, knockdown of plakoglobin in MCF-7 and T47D breast

cancer cells resulted in decreased cell-cell contact, increased *in vitro* invasion and *in vivo* tumor formation and spread (Holen et al., 2012).

The metastasis suppressor activity of plakoglobin has also been described in bladder carcinomas, where the expression of plakoglobin in plakoglobin-null cell lines resulted not only in decreased growth and tumorigenicity (as assessed by colony formation in soft agar and tumor formation in nude mice, respectively), but also in decreased invasive and migratory capabilities of the transfectants (Rieger-Christ et al., 2005). Similarly, knock down of plakoglobin using siRNAs resulted in the increased tumorigenic and invasive properties of bladder carcinoma cells relative to their plakoglobin-expressing parental cell lines. This study further demonstrated that plakoglobin expression did not affect Wnt/β-catenin signaling in these cells, which suggested that plakoglobin possessed tumor and metastasis suppressor activities independent of β-catenin.

The ability of plakoglobin to act as a metastasis suppressor independent of its role in cell-cell adhesion has been demonstrated using plakoglobin-null keratinocytes (Yin et al., 2005), which were shown to be less adherent and more migratory than plakoglobin expressing keratinocytes. However, when plakoglobin-null keratinocytes were induced to express plakoglobin, they became more adherent and less migratory. Using colloidal gold-coated coverslips, the authors were able to assess the migratory abilities of individual cells, and observed that individual

plakoglobin-null keratinocytes were more migratory than their plakoglobin-expressing counterparts. They also showed that plakoglobin may regulate single keratinocyte migration by inhibition of Src signaling, which had been previously shown to promote migration and invasion of mammary carcinomas by downregulation of plakoglobin (see above; Mukhina et al., 2004). These results suggested that plakoglobin could suppress migration through the modulation of cell-cell adhesion, as had been previously suggested. However, to determine whether plakoglobin could have an effect in migration independent of its role in cell-cell adhesion, plakoglobin-null keratinocytes were transfected with cDNAs encoding mutant plakoglobin, missing either its N- or C-terminus (α-catenin binding and transactivation domain, respectively). The expression of either of these mutant proteins resulted in increased keratinocyte adhesiveness when compared to the plakoglobin-null cells, demonstrating that these domains were dispensable for the adhesive function of plakoglobin. Importantly, the authors showed that whereas individual keratinocytes expressing the N-terminal deleted plakoglobin were not migratory, those that expressed the C-terminal deleted plakoglobin were migratory. This showed that plakoglobin could indeed suppress migration independent of its adhesive function (since keratinocytes expressing C-terminal deleted plakoglobin were as adhesive to one another as wild-type plakoglobin expressing keratinocytes). Subsequent work using these same keratinocytes has suggested that plakoglobin affected individual cell motility by regulating the

deposition of the extracellular matrix (ECM) protein fibronectin, actin cytoskeleton organization (which in turn regulates Src signaling) and RhoGTPases (Todorovic et al., 2010).

More recently, the metastasis suppressor activity of plakoglobin was demonstrated in a study that showed that plakoglobin expression was repressed in triple negative breast cancer cells by the transcriptional repressor slug. In this study, the authors showed that slug bound to the plakoglobin gene (*JUP*) promoter and recruited the co-repressors CtBP and HDAC1, resulting in the silencing of gene expression. Furthermore, plakoglobin knock down in non-invasive MDA-MB-468 breast cancer cells resulted in actin reorganization, formation of membrane extensions (invadopodia) and increased cell migration, consistent with a migratory phenotype (Bailey et al., 2012). Collectively, these observations clearly demonstrate tumor/metastasis suppressor activity of plakoglobin independent of its role in cell-to-cell adhesion.

1.11. Plakoglobin expression in human tumors

The initial characterization of *JUP*, the gene encoding plakoglobin, mapped it to chromosome 17q21, proximal to the *BRCA1* gene (Aberle et al., 1995). In this study, the authors also analyzed RNA isolated from ovarian and breast cancer tumors and showed that loss of heterozygosity in these tumors and low frequency mutations in the plakoglobin gene predisposed patients to familial breast and ovarian cancer. Since then, the loss of plakoglobin expression has been reported in a wide range of

tumors, with the majority of these reports examining plakoglobin in conjunction with other adhesive junctional proteins. These studies have demonstrated that loss of plakoglobin expression in conjunction with the lack of expression of other cell-cell adhesion proteins such as E-cadherin, α-catenin, β-catenin, desmoglein or desmoplakin resulted in increased tumor formation and size and was correlated with increased tumor stage, poor patient survival and increased metastasis in bladder, pituitary, oral, pharyngeal, skin, prostate and NSCLC tumors (Syrigos et al., 1998; Depondt et al., 1999; Morita et al., 1999; Lo Muzio et al., 1999; Tada et al., 2000; Tziortzioti et al., 2001; Bremnes et al., 2002; Clairotte et al., 2006; Ueda et al., 2006). However, several studies (described below) have found that decreased levels of plakoglobin alone also occur in various tumors.

The loss of plakoglobin expression has been observed in melanocytic and thyroid tumors (Sanders et al., 1999; Cerrato et al., 1998). Cerrato et al. (1998) found that nearly 90% of papillary and follicular tumors showed decreased or loss of membrane plakoglobin localization. Decreased expression of the plakoglobin gene was also observed in prostate tumors, where methylation of the plakoglobin gene was prevalent in localized prostate cancer relative to benign prostatic hyperplasia, suggesting that loss of plakoglobin expression was an early step in prostate tumorigenesis (Shiina et al., 2005). In oropharynx squamous cell carcinomas, decreased plakoglobin expression and its abnormal cytoplasmic distribution was

correlated with increased tumor size and poor clinical outcome (Papagerakis et al., 2004).

In colon carcinomas, Lifschitz-Mercer et al. (2001) showed that β-catenin accumulated in the nuclei of cells of primary and metastatic adenocarcinoma and adenoma lesions, while the levels of nuclear plakoglobin were decreased in these tumors, suggesting that nuclear plakoglobin did not promote tumorigenesis in the colon. In esophageal cancers, while decreased levels of E-cadherin and plakoglobin were associated with poor differentiation and decreased patient survival, reduced plakoglobin levels alone correlated with lymph node metastasis (Lin et al., 2004). The finding that reduced plakoglobin levels alone correlated with increased metastasis was also observed in renal carcinomas in which patients with tumors expressing plakoglobin showed significantly higher survival rates than those that did not (Buchner et al., 1998). Aberrant or decreased plakoglobin levels have also been reported in Wilms' tumors and soft tissue sarcomas, where the decrease in plakoglobin levels were associated with increased risk of pulmonary metastasis (Basta-Jovanovic et al., 2008; Kanazawa et al., 2008). In endometrial tumors, the aberrant expression of plakoglobin was correlated with myometrial invasion (Kim et al., 2002), whereas medulloblastoma tumors expressing plakoglobin were non-metastatic, with no evidence of subarachnoid or hematogenous metastasis (Misaki et al., 2005). Finally, reduced plakoglobin expression was also correlated with increased lymph

node metastasis in oral squamous cell and bladder tumors (Baumgart et al., 2007; Narkio-Makela et al., 2009). Collectively, these observations confirm the *in vitro* studies described earlier and suggest that lack or decreased expression of plakoglobin due to genetic or epigenetic causes in tumors of different origins is associated with poor clinical outcome and increased tumor formation and metastasis.

1.12. Plakoglobin-mediated regulation of gene expression

When discussing roles for plakoglobin during tumorigenesis and metastasis, it is important to consider that while plakoglobin may function as both a regulator of cell-cell adhesion and an intracellular signaling molecule, it may also affect these processes through the regulation of gene expression. Evidence supporting plakoglobin-mediated regulation of gene expression has started to emerge. Work from our laboratory and several other groups has suggested that plakoglobin interacts with transcription factors and regulates the expression of genes involved in cell-cycle control, apoptosis, cell proliferation and invasion (Figure 1-5C).

Shtutman et al. (2002) showed that the exogenous expression of plakoglobin in renal carcinoma cells lacking both β-catenin and plakoglobin resulted in the increased expression of the tumor suppressor gene *PML*, a nuclear protein involved in the regulation of p53 activity. Importantly, the increased *PML* levels due to plakoglobin expression were independent of β catenin and TCF, since β-catenin was not detected in the plakoglobin-expressing cells and deletion of TCF/LEF sites in the *PML*

promoter did not affect the ability of plakoglobin to increase *PML* gene expression. Together, these observations suggested that plakoglobin may regulate gene expression independent of TCF/LEF.

Williamson et al. (2006) have shown that plakoglobin acts as a repressor of the c-Myc (*MYC*) gene. Using mouse keratinocytes and reporter assays, the authors of this study showed that plakoglobin suppressed *MYC* expression in a LEF-1 dependent manner, suggesting that when plakoglobin interacted with LEF-1, this complex was unable to promote gene expression, confirming previous results demonstrating the inefficiency of plakoglobin-TCF/LEF complexes in binding DNA (Simcha et al., 1998; Zhurinsky et al., 2000b; Williams et al., 2000; Miravet et al., 2002). This study further showed that the plakoglobin-mediated suppression of *MYC* was similar in both wild-type and β-catenin-null keratinocytes, demonstrating that plakoglobin could regulate gene expression independent of β-catenin. Finally, using chromatin immunoprecipitation with plakoglobin antibodies, the authors demonstrated that plakoglobin and LEF-1 associated with the *MYC* promoter in keratinocytes undergoing growth arrest, which implicated the downregulation of c-Myc gene expression as a possible reason for the suppression of cell growth by plakoglobin.

As described earlier, Todorovic et al. (2010) have shown that plakoglobin can regulate cell motility by regulating Fibronectin and Rho-dependent Src signaling. This study also demonstrated that plakoglobin

expression resulted in increased levels of Fibronectin mRNA without increasing expression from the Fibronectin promoter. Using Actinomycin D to inhibit transcription, the authors were able to demonstrate that plakoglobin expression led to the increased stability of Fibronectin mRNA, suggesting that in addition to its role in regulating gene expression at the level of transcription, plakoglobin may also regulate gene expression post-transcriptionally. However, how plakoglobin does so remains unclear.

Finally, a recent report demonstrated that plakoglobin regulates the expression of the desmosomal cadherin desmocollin 2 in keratinocytes through interactions with LEF-1 (Tokonzaba et al., 2013). The plakoglobin-mediated activation of the desmocollin-2 gene (*DSC2*) promoter was dependent on a functional LEF-1 binding site. Overall, these studies suggest that plakoglobin regulates gene expression at the transcriptional, and potentially at post-transcriptional levels.

1.13. Preliminary work and hypotheses

The focus of our lab is characterizing, at the molecular level, the mechanisms by which plakoglobin suppresses tumorigenesis and metastasis. We have developed two experimental model systems using squamous and breast carcinoma cell lines with no or very low plakoglobin expression and various degrees of transformation/invasiveness to specifically assess the growth/metastasis inhibitory activities of plakoglobin. Using a combination of molecular and cell biological approaches, including proteomics and transcriptome analysis, we

compared the protein and mRNA profiles of plakoglobin-deficient and plakoglobin-expressing cell lines and their *in vitro* migration and invasiveness. These analyses led to the identification of several growth regulatory genes that were differentially expressed in plakoglobin-expressing transfectants compared to their plakoglobin-deficient parental cells.

Comparison of the proteomic profiles of the plakoglobin-null SCC9 cells and their plakoglobin-expressing transfectants (SCC9-PG-WT) allowed us to identify several tumor/metastasis regulating proteins, which were differentially expressed in SCC9-PG-WT transfectants relative to parental SCC9 cells. Further RNA microarray experiments were performed to determine whether changes in protein levels were associated with changes in gene expression. To determine whether the subcellular distribution of plakoglobin had an effect on gene expression, we also compared the RNA profiles of SCC9 and SCC9-PG-WT cells with those of SCC9 cells expressing plakoglobin exclusively in the nucleus (SCC9-PG-NLS) or in the cytoplasm (SCC9-PG-NES). From these experiments, we identified three subsets of genes that were differentially expressed based on plakoglobin expression and its subcellular distribution: those whose differential expression required exclusively cytoplasmic plakoglobin, those whose differential expression required nuclear plakoglobin, and those whose differential expression required the ability of plakoglobin to shuttle between the nucleus and the cytoplasm. Based on the results of these

experiments and analysis of the expression patterns of plakoglobin-target genes in relation to plakoglobin subcellular distribution, we proposed that plakoglobin can regulate gene expression by three concurrent mechanisms (Figure 1-6).

The first of these mechanisms involves the action of plakoglobin in the cytoplasm, where it would sequester a protein involved in the regulation of gene expression. In this case, plakoglobin would prevent an inhibitor of a tumor suppressor gene or a promoter of an oncogenic gene from entering the nucleus and affecting gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-WT and SCC9-PG-NES cells and were opposite to SCC9-PG-NLS cells would be considered part of this group.

The second mechanism involves nuclear localized plakoglobin, which would directly associate with a nuclear factor and regulate gene expression. In this case, plakoglobin would interact with a transcriptional activator and promote gene expression, or conversely, it would interact with a transcriptional repressor and silence gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-WT and SCC9-PG-NLS cells and were opposite to SCC9-PG-NES cells would be considered part of this group.

The vast majority of plakoglobin target genes, however, belonged to the third group of genes: those whose differential expression depended on the ability of plakoglobin to shuttle between the nucleus and the cytoplasm. In

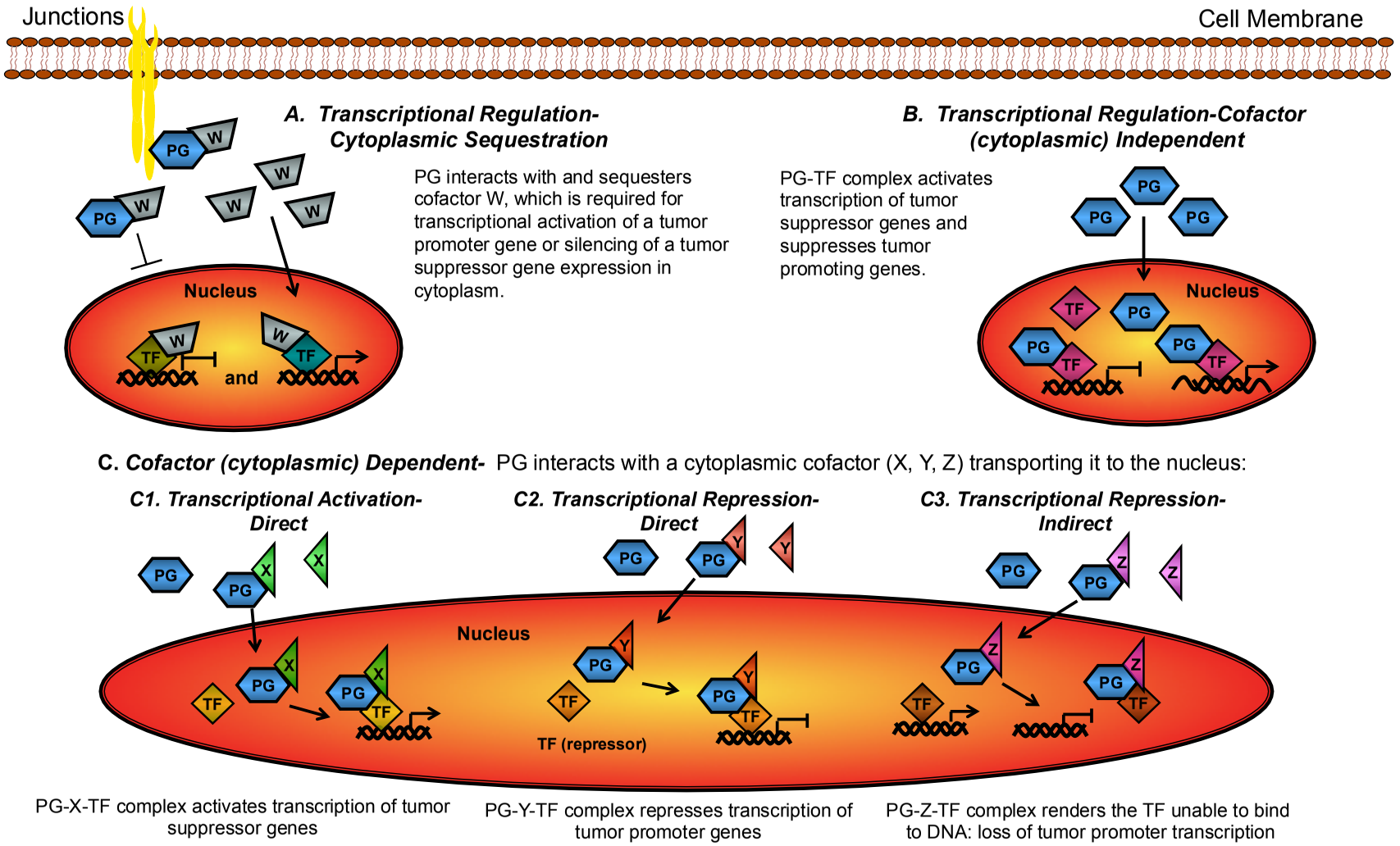

Figure 1-6. A potential model for regulation of gene expression by plakoglobin. Three concurrent mechanisms by which plakoglobin may regulate gene expression are proposed. (A) Cytoplasmic Sequestration: plakoglobin sequesters a factor in the cytoplasm which, in the nucleus, suppresses the expression of a tumor suppressor gene or activates the expression of an oncogene. (B) Cytoplasmic Cofactor Independent: plakoglobin-transcription factor complexes promote the expression of tumor suppressor genes and repress the expression of oncogenes. (C) Cytoplasmic Cofactor Dependent: plakoglobin interacts with a cytoplasmic cofactor and this complex moves into the nucleus where it activates tumor suppressor gene expression or represses oncogenic gene expression. PG, plakoglobin; TF, transcription factor.

this case, plakoglobin would interact with some cytoplasmic cofactor, translocate into the nucleus, and regulate gene expression. Plakoglobin target genes whose expression patterns were similar in SCC9-PG-NES and SCC9-PG-NLS cells and were opposite to SCC9-PG-WT cells would be considered part of this group.

Based on these initial results, **we hypothesized that plakoglobin regulates tumorigenesis and metastasis by interacting with and altering the levels, localization and/or function of various growth/metastasis regulating proteins or by interacting with transcription factors that regulate the expression of genes involved in tumorigenesis and metastasis.**

CHAPTER TWO: MATERIALS AND METHODS

2.1. Cell culture and conditions

All tissue culture reagents were purchased from Invitrogen (Burlington, ON, Canada) and all cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Growth media and supplement specifications for culturing of each cell line are presented in Table 2-1.

2.2. Plakoglobin shRNA transfection

Scrambled shRNA (TR30013) and human plakoglobin shRNA (combination of GI348173-6) plasmids were obtained from OriGene (Rockville, MD, USA) and used to transfect MCF-7 cells according to the manufacturer's protocol. Puromycin-resistant stable cell lines expressing the scrambled or plakoglobin shRNAs (shPG) were isolated and the decreased expression of plakoglobin was verified by western blot. Single-cell isolated clones were obtained by limiting dilution.

2.3. Generation of plakoglobin expressing MDA-231 cells

The construct containing the full-length PG cDNA (pBK-CMV-PG) has been described (Parker et al., 1998). MDA-231 cells were transfected with 4 mg of either pBK-CMV or pBK-CMV-PG using LipofectAMINE reagent (Life Technologies, Inc., CA, USA; Lam et al., 2012) according to the manufacturer's protocol. G418-resistant colonies were selected and screened for plakoglobin expression using immunofluorescence and immunoblot assays. Positive colonies expressing plakoglobin were subcloned by limiting dilution to obtain single-cell isolated clones.

2.4. p53 knock down

p53 siRNA (sc-29435) was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and used to transfect SCC9-PG cells. Transfection experiments were performed using Lipofectamine 2000 reagent following the manufacturer's instructions. Knock down was assayed by western blot 48 hours following transfection. For luciferase assays, both p53 siRNA and luciferase reporter constructs were simultaneously transfected into SCC9-PG cells and luciferase activity was measured 48 hours post transfection, as described.

2.5. Cloning of SCC9 mutant p53 and transfection into H1299 cells

The mutant p53 from SCC9 cells was cloned from cDNA by PCR and ligated into the pBK-CMV vector at KpnI and SacI sites, respectively. Primers used for the cloning reaction were CAGTggtaccATGGAGGAG-CCGCAGTCAGATCCT (forward, starting at p53 ORF codon 1) and AGCTgagctcTCAGTCTGAGTCAGGCCCTTCTGT (reverse, ending at p53 ORF codon 394). Sequence accuracy was confirmed by DNA sequencing. The p53 constructs were then transfected into H1299 cells using calcium phosphate as previously described (Wu et al., 2011). Fourty-eight hours after transfection, cells were processed for subcellular fractionation, western blot, immunofluorescence and chromatin immunoprecipitation.

2.6. Construction of SATB1-luciferase reporter constructs

The SATB1 gene promoter was cloned from SCC9 genomic DNA by PCR and ligated into the pBV-Luc vector at KpnI and SacI sites,

respectively. The primer sequences used for the cloning reaction were CAGTggtaccGCCA-GGGCGACTCTAGAG (forward, starting at base pair 14 in the *SATB1* gene) and AGCTgagctcCACTTCAAAACTTGACAGCACATA (reverse, ending at base pair 1222 in the *SATB1* gene). The plasmid was then used for transfection (see below; Chapter Five).

2.7. RNA isolation and RT-PCR

RNA was isolated from 150 mm confluent cell cultures using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Following isolation, RNA was pre-treated with RNase-free DNaseI and reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). Polymerase chain reaction (PCR) was performed (Fermentas, Burlington, ON, Canada) on the amplified cDNA. Primer sequences used for are outlined in Table 2-2. RT-PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. qRT-PCR was performed using PerfeCta SYBR Green FastMix reagent (Quanta Biosciences) as per the manufacturer's instructions.

2.8. Microarray expression analysis

Total RNA isolated from SCC9 and SCC9-PG cells was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its integrity evaluated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) according to the

manufacturer's protocols. RNA samples with RNA Integrity Numbers (RIN) greater than 7.0 were used in this study. The RNA was subjected to linear amplification and Cy3 labeling and hybridization to Agilent Whole Human Genome Arrays using Agilent kits (One Color Low RNA Input Linear Amplification Kit Plus, One Color RNA Spike-In Kit and Gene Expression Hybridization Kit) according to the manufacturer's recommended protocols. The arrays were scanned using an Agilent Scanner, the data extracted and the quality evaluated using Feature Extraction Software 9.5 (Agilent). The data was normalized and analyzed using GeneSpring GX 7.3.1 (Agilent).

2.9. Antibodies

A list of antibodies and their respective dilutions in specific assays is presented in Table 2-3.

2.10. Preparation of total cell extracts and western blotting

Confluent 150 mm culture dishes were washed twice 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. Protein determination was done using Bradford (Pierce) assays according to the manufacturer's instructions. Twenty-five micrograms of total cellular protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, processed for immunoblotting and developed by standard ECL (Perkin Elmer, Woodbridge, Canada) procedures.

2.11. Immunoprecipitation

Immunoprecipitation experiments were performed using one of two different protocols.

For characterization of plakoglobin-Nm23 interactions (Chapter Three), confluent cultures (100 mm) were washed twice (on ice) with cold PBS containing 1mM NaF, Na_3VO_4 and $CaCl_2$ and extracted for 20 minutes with a modified cytoskeleton extraction buffer (Pasdar and Nelson, 1988a; 10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail). Cells were removed from the plates and centrifuged at 20,000 rpm for 10 minutes. The resulting supernatant (soluble fraction) was separated from the pellet (insoluble), which was solubilized in SDS immunoprecipitation buffer (1% SDS, 10 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM DTT and PMSF) and boiled for 15 minutes. The SDS was diluted to 0.1% with immunoprecipitation buffer. The soluble and insoluble fractions were split equally and processed for immunoprecipitation.

To examine the interactions between plakoglobin and p53 (Chapter Four), confluent cultures (150 mm) were washed twice (on ice) with cold PBS containing 1mM NaF, Na_3VO_4 and $CaCl_2$ and extracted for 15 minutes with a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail) at 4°C. Cells were

removed from the plates and centrifuged at 20,000 rpm for 10 minutes. The resulting supernatant was divided into equal aliquots and processed for immunoprecipitation (see below).

Antibodies and 40 μl protein A Sepharose CL-4B beads (Pierce, Nepean, Canada) were then added to each respective extract, and incubated overnight on a rocker-rotator at 4°C. To ensure complete depletion, samples were centrifuged briefly and the resulting supernatants were processed for another round of immunoprecipitation for 3 hours. Beads from the two immunoprecipitations were combined, washed three times with RIPA buffer and immune complexes separated by solubilization in SDS sample buffer. Equivalent amounts of total cellular proteins immunoprecipitated from each cell line were loaded onto SDS polyacrylamide gels and processed for western blot as described above.

For immunoprecipitation of subcellular fractions (Chapter Three), cells were separated into nuclear and cytoplasmic fractions as previously described (Kim et al., 2009). Briefly, cells were lysed with cytoplasmic extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail) while rotating on a rocker-rotator at 4°C for 15 minutes. The cells were then centrifuged at 14,000 rpm at 4°C for 5 minutes and the resulting supernatant (cytoplasmic fraction) was collected. The pellet was resuspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT,

0.2% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail) and incubated at room temperature for 10 minutes, after which it was centrifuged at 14,000 rpm at 4°C for 5 minutes. The resultant supernatant (nuclear fraction) was removed from the pellet (cytoskeleton) and the purity of each fraction was assessed by immunoblotting with antibodies to tubulin and lamin, respectively, prior to immunoprecipitation. Equal volumes of cytoplasmic and nuclear fractions corresponding to equal cell numbers were processed for immunoprecipitation and western blot.

2.12. Immunofluorescence analysis

For colocalization between Nm23 and cadherins/catenins (Chapter Three), cells were plated on glass coverslips and grown to confluence, after which they were rinsed twice with cold PBS, extracted with cytoskeleton extraction (CSK; Pasdar and Nelson, 1988b) 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 µg/ml DNase and RNase) for 10 minutes and fixed on ice with 1.75% formaldehyde for 15 minutes. Alternatively, cells were fixed with formaldehyde first and then permeabilized with CSK buffer.

For characterization of p53 subcellular distribution in H1299-p53 transfected cells (Chapter Four), H1299 cells were plated on glass coverslips and transfected with the SCC9 mutant p53 as described above. Fourty-eight hours following transfection, the cells were rinsed twice with

cold PBS (on ice), and fixed/extracted with ice-cold methanol for 5 minutes.

Coverslips were then blocked for 1 hour with 4.0% goat serum and 50 mM NH_4Cl_4 in PBS containing 0.2% BSA (PBS-BSA) and processed for indirect immunofluorescence. One hour primary antibody incubation at room temperature was followed by 20 minutes incubation with fluorochrome-conjugated species-specific secondary antibodies. All antibodies were diluted in PBS-BSA. Nuclei were counterstained for 5 min with DAPI (1:2000 in PBS). Coverslips were mounted in elvanol containing 0.2% (w/v) paraphenylene diamine (PPD) and viewed using a 63X objective of an LSM510 META (Zeiss) laser scanning confocal microscope.

2.13. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (Peng and Jahroudi, 2003). Confluent 150 mm cultures were trypsinized and $2x10^7$ cells pelleted by centrifugation at 3,500 rpm for 10 minutes. The cell pellets were then resuspended in growth media to which formaldehyde (Fisher) was added to a final concentration of 1% and incubated at room temperature for 10 minutes. To stop fixation, glycine was added to a final concentration of 125 mM. The cell suspension was then centrifuged at 3,500 rpm at 4°C for 10 minutes. The resulting cell pellets were then washed twice with PBS containing 1 µg/ml aprotinin and leupeptin and 1 mM PMSF, after which

they were resuspended in cell lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.49 mM PMSF) and incubated on ice for 15 minutes. NP-40 was then added (final concentration of 0.6%) after which the samples were vortexed for 10 seconds at high speed and subsequently centrifuged at 13,000 rpm for 30 seconds. The resulting pellets were then resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8, 0.49 mM DTT and 0.02 µg/ml aprotinin and leupeptin) and left on ice for 10 minutes. The samples were then sonicated (Branson Sonifier 450) for 1 minute at 20% output for a total of four times.

The sonicated chromatin samples were then diluted ten-fold in chromatin dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8, 167 mM NaCl) after which 50 µl was removed (Input). Fourty µl Protein A/G Agarose beads (Calbiochem) were added and the samples were pre-cleaned on a rocker-rotator at 4°C for 2 hours. Following incubation, the samples were centrifuged briefly and the resulting supernatant (pre-cleaned chromatin) was split into equal aliquots and processed for immunoprecipitation. Each aliquot was incubated with 5 µg antibodies and 40 µl pre-cleaned (by overnight incubation with 4 µg Salmon Sperm DNA and BSA) Protein A/G Agarose beads overnight at 4°C on a rocker-rotator.

Following immunoprecipitation, the samples were centrifuged for 10 minutes at 2,000 rpm at 4°C, after which the resulting supernatants were

removed. The beads were then subjected to six 5 minute washes in each of the four following wash buffers: W1 (1% SDS, 1% Triton X-100, 2 mM Tris pH 8, 167 mM NaCl), W2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8, 500 mM NaCl), W3 (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 10 mM Tris pH 8, 1 mM EDTA) and W4 (10 mM Tris pH 8 and 1 mM EDTA). Following the washes, the protein-DNA complexes were eluted off the beads by incubation in elution buffer (1% SDS and 50 mM $NaHCO_3$) for 15 minutes at room temperature on a rocker-rotator. Following elution, 1 µg RNase and NaCl (final concentration 300 mM) were added to the samples, which were then incubated at 65°C for 4 hours. Next, Tris pH 6.8, EDTA (final concentrations of 40 mM and 10 mM, respectively) and 4 µg proteinase K were added to the samples, which were incubated at 45°C for 2 hours. The samples were then purified using a PCR Purification Kit (QIAGEN, Valencia, CA) and processed for PCR.

2.14. Nuclear Extraction

Confluent 150 mm cell cultures were trypsinized and centrifuged at 3,500 rpm for 10 minutes. Following centrifugation, the cell pellets were washed with PBS containing 1 mM NaF, Na_3VO_4 and $CaCl_2$, resuspended in cytoplasmic extraction buffer (100 mM HEPES pH 7.9, 1 M KCl, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 15 minutes. Next, NP-40 was added to a final concentration of 0.6% and the samples were vortexed on high speed for 10 seconds and centrifuged at 20,000 rpm for 30 seconds. Following centrifugation, the supernatant

was removed and the pellet was resuspended in nuclear extraction buffer (100 mM HEPES pH 7.9, 4 M NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated at 4°C on a rocker-rotator for 25 minutes. Following this incubation, the samples were centrifuged for 5 minutes at 20,000 rpm (4°C) and the supernatant (nuclear extract) was stored.

2.15. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) experiments were performed as previously described (Schreiber et al., 1989; Wang et al., 2004). Briefly, a double-stranded nucleotide corresponding to the p53 consensus sequence in the promoter of the 14-3-3σ (*SFN*) gene (Hermeking et al., 1997; Cai et al., 2009) was radioactively labeled with use of ^{32}P-ATP (adenosine 5'-triphosphate; Perkin Elmer). Nuclear extracts (5 mg) were incubated with oligonucleotide probes (15,000 cpm) on ice for 10 minutes in EMSA reaction buffer (50 mM HEPES pH 7.9, 250 mM KCl, 25 mM $MgCl_2$, 5 mM EDTA, 5% glycerol and 1 mg poly (dI-dC) (Sigma)). When antibodies were added, nuclear extracts were incubated with 1 µg of each antibody in the EMSA reaction buffer for 20 minutes on ice. The oligonucleotide probes were then added to the nuclear extract-antibody mixtures for 10 minutes on ice. Complexes were resolved on 5% non-denaturing polyacrylamide gels and exposed to film overnight.

2.16. Luciferase reporter assay

Confluent 35 mm cultures were transfected with 4 μg of various luciferase reporter plasmids. Regulation of p53 transcriptional activity was determined through the use of reporter constructs downstream of either the wild-type p53-binding sequence within the 14-3-3σ (*SFN*) gene or a consensus p53 sequence, mutants of these sequences, or control vectors (Chapter Four; Table 2-4; Kern et al., 1992; Hermeking et al., 1997; Addgene plasmids 16515, 16516, 16539, 16442 and 16443, which were a kind gift of Dr. Bert Vogelstein), together with 1 μg of a plasmid encoding β-galactosidase. To assess activity from the *NME1* promoter, cells were transfected with a reporter plasmid downstream of the *NME1* promoter (Qu et al., 2008; a kind gift of Dr. Shimian Qu; Chapter Five). SATB1 promoter activity was analyzed by using a reporter construct downstream of the full SATB1 promoter (Lei et al., 2010; Chapter Five). Fourty-eight hours post-transfection, luciferase and β-galactosidase activities were measured. Each experiment was repeated at least 3 times and the mean with standard deviation was calculated. Statistical analysis was performed using a Student's t-test.

2.17. Cell growth and proliferation assays

To measure growth, $5x10^4$ cells for each cell line were plated in triplicate in a 24-well plate. At 3, 5 and 7 days after plating, cultures were trypsinized and the cells were counted. Cell proliferation was assessed by performing BrdU incorporation experiments. For each cell line, $5x10^4$ cells

were plated on glass coverslips and allowed to proliferate for 6 days at which times they were incubated with BrdU (100 μM; Sigma B-5002) for 24 hours. To detect BrdU-labeled cells, coverslips were first prefixed by the addition of 3.7% formaldehyde directly to the culture media at a 1:1 ratio (volume). Coverslips were then rinsed, fixed with 3.7% formaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes. Coverslips were then washed with PBS and incubated in 2N HCl for 1 hour at room temperature followed by two 5-minute washes with 100 mM sodium borate (pH 8.5). Subsequently, coverslips were processed for immunofluorescence analysis using a mouse monoclonal anti-BrdU antibody (Table 2-3) as described above.

2.18. Transwell cell migration and invasion assays

For cell migration assays, $2x10^5$ cells were resuspended in 0.5 ml serum-free media containing 0.1% BSA and plated in the upper chamber of transwells (3 μm pore, 6.5 μm diameter; BD Biosciences, MD, USA). Normal media containing 10% FBS (0.75 ml) was added to the lower chamber. Cultures were incubated at 37°C for 12 or 48 hours to allow cell migration. The inserts were then removed from the chambers, gently submerged in PBS to remove the unattached cells and then fixed and stained using Diff Quick (IHC World, MD, USA). Following staining, membranes were cut, mounted on slides using permount (Fisher, Canada), viewed under an inverted microscope using a 20X objective and photographed. The migrated cells on the underside of the membrane were

counted in five random fields for each transwell filter from the photographs.

Matrigel invasion assays were performed according to the manufacturer's protocol (BD Bioscience). For each cell line, $5x10^5$ cells in 0.6 ml serum-free media containing 0.1% BSA were plated in the top compartment of Matrigel-coated invasion chambers (8 μm pore membrane). Fibroblast conditioned media (0.75 ml) was added to the bottom chambers and plates were incubated overnight at 37°C in 5% CO_2. Forty-eight hours later, the membranes were recovered, fixed, stained with Diff Quick, viewed under an inverted microscope using a 20X objective and photographed. The invaded cells were counted in five random fields for each membrane.

Each assay was repeated 3 independent times. The numbers of migrated/invaded cells were calculated using the ImageJ Cell Counter program and averaged.

Table 2-1. Growth conditions for various cell lines used.

Cell Line	Origin	Growth Media	Supplements	Selection
A431	Vulva	DMEM	10% FBS, 1% antibiotics	-
H1299	Lung	MEM	10% FBS, 1% antibiotics	-
MCF-10-2A	Breast	DMEM/F12	5% Horse Serum, 20 ng/ml EGF, 10 ng/ml insulin, 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, 50 U/ml penicillin, 50 mg/ml streptomycin and 1% antibiotics	-
MCF-7	Breast	MEM	10% FBS, 1% antibiotics	-
MCF-7-shPG	Breast	MEM	10% FBS, 1% antibiotics	Puromycin, 0.5 µg/ml
MDA-231	Breast	RPMI	10% FBS, 1% L-glutamine, Non-Essential Amino Acids, Sodium Pyruvate, antibiotics	-
MDA-231-PG	Breast	RPMI	10% FBS, 1% L-glutamine, Non-Essential Amino Acids, Sodium Pyruvate, antibiotics	Geneticin, 500 µg/ml
MDCK	Kidney	DMEM	10% FBS, 1% antibiotics	-
PC3	Prostate	F12K	10% FBS, 1% antibiotics	-
SCC9	Tongue	MEM	10% FBS, 1% antibiotics	-
SCC9-PG	Tongue	MEM	10% FBS, 1% antibiotics	Geneticin, 200 µg/ml
SCC9-PG-Flag-WT	Tongue	MEM	10% FBS, 1% antibiotics	Geneticin, 200 µg/ml
SCC9-PG-Flag-ΔN122	Tongue	MEM	10% FBS, 1% antibiotics	Geneticin, 200 µg/ml
SW620	Colon	Leibovitz's/L15	10% FBS, 1% L-glutamine, antibiotics	-

Table 2-2. Primer sequences and PCR conditions for reverse transcribed genes.

Gene	Primers	Fragment Size (bp)	Annealing	Reference
RT-PCR				
14-3-3σ	Sense: 5'-GTGTGTCCCCAGAGCCATGG-3' Antisense: 5'-ACCTTCTCCCGGTACTCACG-3'	279	60°C	Bhatia et al., 2003
NME1	Sense: 5'-CGCAGTTCAAACCTAAGCAGCAGCTGG-3' Antisense: 5'-AGATCCAGTTCTGAGCACAGCTCG-3'	483	60°C	Ayabe et al., 2004
NME2	Sense: 5'-TGACCTGAAAGACCGACCAT-3' Antisense: 5'-GAATGATGTTCCTGCCAACC-3'	193	55°C	Syed et al., 2005
SATB1	Sense: 5'- TGCAAAGGTTGCAGCAACCAAAAGC-3' Antisense: 5'- AACATGGATAATGTGGGGCGGCCT-3'	156	60°C	Han et al., 2008
GAPDH	Sense: 5'-GAAGGTGAAGGTCGGAGTC-3' Antisense: 5'-GAAGATGGTGATGGGATTTC-3'	220	60°C	Nakanishi et al., 2006
ChIP				
14-3-3σ	Sense: 5'-CATGAAAGGCGCCGTGGAGAA-3' Antisense: 5'-GCTGATGTCCATGGCCTCCTGG-3'	474	58.4°C	Pulukuri and Rao, 2006
MYC	Sense: 5'-GGGATCGCGCTGAGTATAAAA-3' Antisense: 5'-GAAGCCCCCTATTCGCTCC-3'	173	55°C	-
NME1	Sense: 5'-CAACTGTGAGCGTACCTTCAT-3' Antisense: 5'-AACAAGGCGGAATCCTTTCTG-3'	102	53.6°C	-
SATB1	Sense: 5'-GATCATTTGAACGAGGCAACTCA-3' Antisense: 5'-CCTGCATTTTTGCACCTGTACT-3'	157	53.6°C	-
VWF	Sense: 5'-GCTTGTGGCCAAGACCTTCATCTT-3' Antisense: 5'-AACAACACAGCTTCCTGATCCAGC-3'	200	58.4°C	-

For all primers, pre-denaturation was done at 95°C for 5 minutes. This was followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds, and extension at 72°C for 45 seconds.

Table 2-3. Antibodies and their respective dilutions in specific assays.

	Species	Assay				Source
		WB	IF	IP	ChIP	
1° Antibodies						
14-3-3σ (5D7)	Mouse	1:500	-	-	-	Santa Cruz, sc-100638
α-catenin	Mouse	-	1:100	1:100		Zymed, #18-0225
α-catenin	Rabbit	-	-	1:100		Sigma, C-2081
β-Actin	Mouse	1:2000	-	-	-	Sigma, A-5441
β-catenin**	Mouse	1:500	-	1:500	1:100	Sigma, C-7207
BrdU	Mouse	-	1:300	-	-	Sigma, B-5002
BRMS1	Mouse	1:200	-	-	-	Santa Cruz, sc-101219
c-Abl	Rabbit	1:1000	-	-	-	Santa Cruz, sc-131
Claudin-1	Mouse	1:500	-	-	-	Santa Cruz, sc-137121
Control IgG	Goat	-	-	1:500	1:2000	Sigma, M-5899
E-cadherin	Mouse	1:500	1:100	1:100	-	Transduction Laboratories, 610182
E-cadherin (3G8)	Mouse	-	Neat	1:20	-	Warren Gallin, U of A
ErbB2	Rabbit	1:1000	-	-	-	Upstate, 06-562
Flag	Mouse	1:1000	1:100	1:100	-	Sigma, F-3165
Kiss1	Rabbit	1:500	-	-	-	Santa Cruz, sc-15400
Lamin B1	Rabbit	1:500		-	-	Santa Cruz, sc-20682
MMP3	Mouse	1:100	-	-	-	Calbiochem, Ab-1
N-cadherin	Mouse	1:1000	1:200	1:100	-	Sigma, C-1821
N-cadherin	Rabbit	1:1000	-	1:100	-	(Li et al., 1998) Sigma, C-3678
Nm23-H1*	Mouse	-	-	1:200	-	Santa Cruz, sc-465
Nm23-H2*	Goat	-	-	1:200	-	Santa Cruz, sc-14789
Nm23-H1/H2	Rabbit	1:500	-	-	-	Chemicon, CBL-446
Nm23-H1/H2/H3	Rabbit	-	1:100	-	-	Santa Cruz, FL-152
p53 (DO-1)**	Mouse	1:500	1:100	1:300	1:80	Santa Cruz, sc-126
p53 (FL-393)	Rabbit	1:500	-	1:500	1:80	Santa Cruz, sc-6243
Plakoglobin	Mouse	1:500	1:100	-	1:100	Transduction Laboratories, 610254
Plakoglobin**	Rabbit	-	-	1:500	-	Pasdar et al., 1995
SATB1	Rabbit	1:1000	-	-	-	Cell Signaling, L745
Snail	Rabbit	1:2000	-	-	-	Abcam, ab17732
Tubulin/E7	Mouse	1:300	-	-	-	DSHB***
2° Antibodies						
Alexa Fluor 546	Goat	-	1:750	-	-	Molecular Probes, A11029
Alexa Fluor 488	Goat	-	1:750	-	-	Molecular Probes, A11035
Anti-mouse HRP	Goat	1:5000	-	-	-	Sigma, 054H-8914
Anti-mouse HRP, Light Chain specific	Goat	1:5000	-	-	-	Jackson, 115-005-174
Anti-rabbit HRP	Goat	1:5000	-	-	-	Sigma, 054H-8918
Anti-rabbit HRP, Light Chain specific	Goat	1:5000	-	-	-	Jackson, 211-002-177

*A cocktail of Nm23-H1 and Nm23-H2 were used for immunoprecipitation
** These antibodies were used for EMSA at a concentration of 1(antibody): 4(lysate)
***Developmental Studies Hybridoma Bank, NCI, USA

Table 2-4. Sequences of p53-response elements used for luciferase reporter assays.

Response Element	Sequence	Number of Repeats	Reference
14-3-3σ WT-p53 RE	CCTGTAGCATTAGCCCAGACATGTCCCTACTCCGTACGGA GTAGGGACATGTCTGGGCTAATGCTACAGGGTAC	3	Hermeking et al., 1997
14-3-3σ MT-p53 RE	CCTGTAGAATTATCCCAGAAATTTCCCTACTCCGTAC GGAGTAGGGAAATTTCTGGGATAATTCTACAGGGTAC	3	Hermeking et al., 1997
Consensus WT-p53 RE	CCAGGCAAGTCCAGGCAGG	13	Kern et al., 1992
Consensus MT-p53 RE	CCTTAATGGACTTTAATGG	15	Kern et al., 1992

CHAPTER THREE: PLAKOGLOBIN INTERACTS WITH AND INCREASES THE LEVELS OF NM23

3.1. Introduction[1]

3.1.1. Rationale

The observed phenotypic transition upon plakoglobin expression in SCC9 cells suggested that plakoglobin may act as a tumor suppressor. Therefore, in order to elucidate the molecular mechanism by which plakoglobin may exert this function, our lab performed proteomic and transcription microarray experiments to identify potential genes and proteins that were differentially expressed in SCC9-PG cells compared to SCC9 cells. These experiments identified a number of growth/tumor promoters whose expression and protein levels were decreased and various growth/tumor suppressors whose expression and protein levels were increased in SCC9-PG cells. Among these differentially expressed genes and proteins, we identified the metastasis suppressors Nm23-H1 and Nm23-H2. Therefore, we began our studies by examining the relationship between plakoglobin expression and the levels and subcellular localization of Nm23.

3.1.2. Nm23

Nonmetastatic protein 23 (Nm23) proteins are a family of nucleoside diphosphate kinases (NDPK) that are expressed from bacteria to mammals (Tee et al., 2006). In humans, there are ten members of the Nm23 family (Nm23-H1-10, respectively), with Nm23-H1 and -H2 being

[1] A version of this chapter has been published in: Aktary Z, Chapman K, Lam L, Lo A, Ji C, Graham K, Cook L, Li L, Mackey JR, Pasdar M (2010). Plakoglobin interacts with and increases the protein levels of metastasis suppressor Nm23-H2 and regulates the expression of Nm23-H1. *Oncogene* **29**: 2118-2129.

the best studied and characterized (Thakur et al., 2011; Marino et al., 2012). Nm23-H1 was the first metastasis suppressor identified, where its decreased expression was observed in murine melanoma cells with increased metastatic potential (Steeg et al., 1988). Since its initial discovery, several studies have observed decreased levels of Nm23-H1 in invasive/metastatic breast, melanoma, colon and oral squamous cell carcinoma cell lines (MacDonald et al., 1993; Hartsough and Steeg, 1998; Steeg et al., 2003; Marino et al., 2012). Nm23-H2, which was subsequently identified as a gene with sequence homology (approximately 90%) to Nm23-H1 (Stahl et al., 1991), also has metastasis suppressor activity and its decreased expression has been observed in a variety of invasive/metastatic carcinoma cell lines, including squamous, breast, ovarian and prostate (Hartsough and Steeg 2000; Ouatas et al. 2003). Decreased levels of Nm23 proteins have also been correlated with increased metastasis in various human tumors, including breast, melanoma, prostate, gastric, hepatocellular, lung and oral squamous (Muller et al., 1998; Pacifico et al., 2005; Hsu et al., 2007; Guo et al., 2010; Dong et al., 2011; Andolfo et al., 2011).

Nm23 proteins interact with numerous intracellular partners and have a wide variety of cellular functions (Marino et al., 2012). Nm23-H1 itself has diverse biological functions including nucleoside diphosphate kinase (NDPK), protein histidine kinase and 3'-5' exonuclease activities, all of which may potentially contribute to its metastasis suppressor function

(Wagner et al., 1997; Lacombe et al., 2000; Fan et al., 2003; Steeg et al., 2008; Novak et al., 2011). In addition, both Nm23-H1 and -H2, appear to have DNA-binding ability (Postel et al., 1993, 2000; Ma et al., 2002; Postel, 2003; Cervoni et al., 2006; Thakur et al., 2009; Choudhuri et al., 2010). However, the exact mechanisms by which these Nm23 proteins suppress migration, invasion and metastasis remain unclear.

Several studies have shown that the exogenous expression of Nm23 in cells lacking its expression results not only in decreased migration and invasion, but also in decreased cell proliferation and inhibition of anchorage independent growth (Lee and Lee, 1999; Khan et al., 2001; Suzuki et al., 2004; Jung et al., 2006; McDermott et al., 2008). Furthermore, Nm23 proteins reduce telomerase activity (Kar et al., 2011), promote cell-cell adhesion (Bago et al., 2009), cell-cycle arrest and apoptosis (Choudhuri et al., 2010) and DNA-repair following U.V. and ionizing radiation (Zhang et al., 2011; Jarrett et al., 2012). These results suggest that Nm23 proteins may also suppress tumor formation in addition to metastasis.

3.1.3. Specific aim and summary of results

In this chapter, we investigated the effect of plakoglobin expression on Nm23 levels and localization. Our results show that plakoglobin expression led to the increased levels of Nm23-H1 mRNA and increased protein levels of both Nm23-H1 and -H2. We also show that upon plakoglobin expression, Nm23 interacted not only with plakoglobin, but

also with N-cadherin and α-catenin, and that these interactions occured at the sites of cell-cell contacts. We further confirmed these results in a number of non-epidermal epithelial cell lines. These results suggest that plakoglobin may assert part of its tumor suppressive activity through modulating the expression and subcellular localization of the metastasis suppressor Nm23, and that α-catenin acts as a bridge between plakoglobin and Nm23.

3.2. Results

3.2.1. Increased Nm23 levels and its membrane localization in SCC9-PG cells.

Plakoglobin expression in SCC9 cells (SCC9-PG) induced a mesenchymal to epidermoid phenotypic transition (Parker et al., 1998). To identify the underlying molecular mechanism for this phenotypic conversion, we examined the protein and mRNA profiles of SCC9 and SCC9-PG cells. Various tumor suppressors were identified as being increased upon plakoglobin expression, and among them were Nm23-H1 and -H2. Figure 3-1 provides evidence to confirm the results of our proteomic and microarray studies. Upon plakoglobin expression, the levels of both Nm23-H1 and -H2 protein were increased, although the levels of Nm23-H2 were markedly higher than Nm23-H1 (Figure 3-1A), which was in agreement with our proteomics results, where Nm23-H2 levels were increased nearly 5-fold in SCC9-PG cells (unpublished data). To examine plakoglobin's effect on Nm23 at the level of transcription, we performed

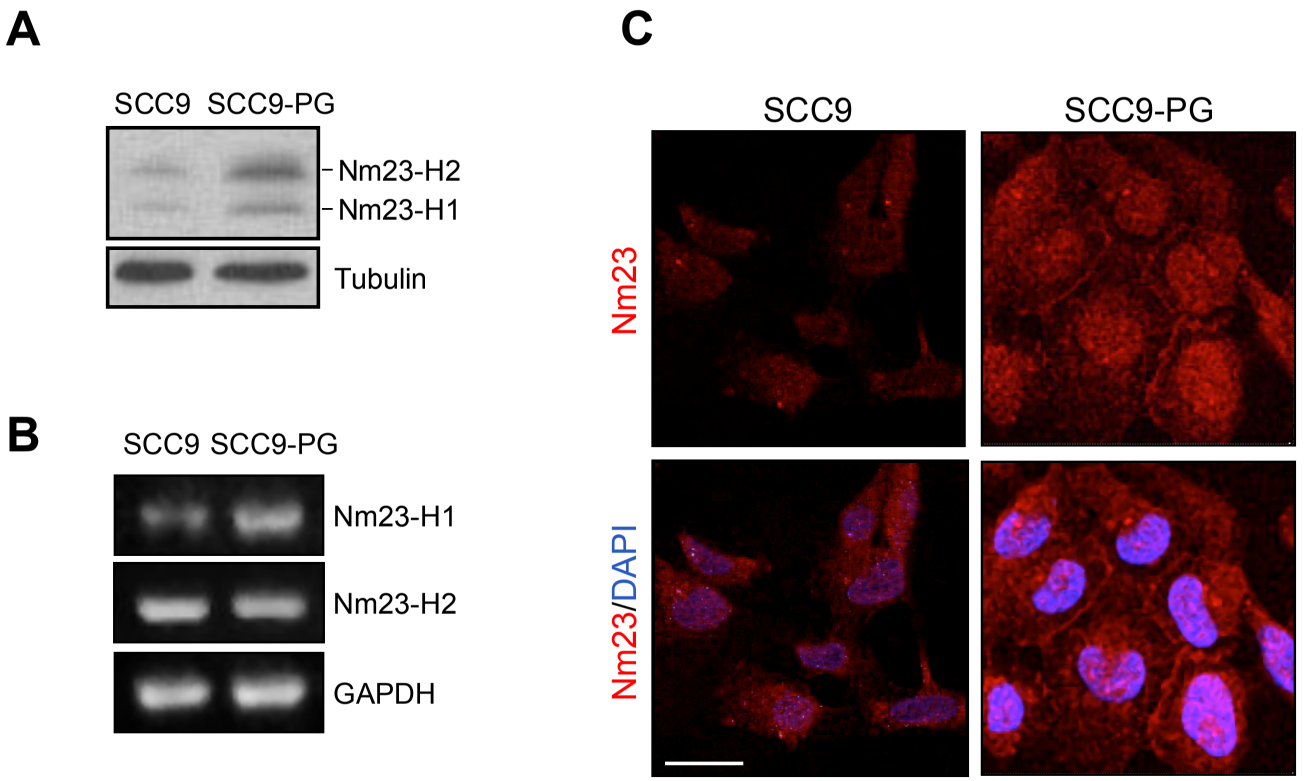

Figure 3-1. Plakoglobin expression results in increased Nm23 protein and mRNA levels and its localization to the areas of cell-cell contact. (A) Equal amounts of total cellular proteins from SCC9 and SCC9-PG cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **Nm23** and **tubulin**. **(B)** Total cellular RNA was isolated from SCC9 and SCC9-PG cells, reverse transcribed, and processed for PCR using primers specific to **Nm23-H1**, -**H2**, and **GADPH**. **(C)** SCC9 and SCC9-PG cells were grown on glass coverslips, methanol-acetone fixed and processed for confocal microscopy with **Nm23** antibodies (red). Nuclei were stained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

reverse-transcription experiments. Our data showed that while the levels of Nm23-H1 mRNA were increased in SCC9-PG cells, the levels of Nm23-H2 mRNA were unaltered (Figure 3-1B). These results were in agreement with our microarray data, which also revealed a two-fold increase in the level of Nm23-H1 mRNA, while -H2 levels remained unchanged.

3.2.2. Nm23 coprecipitates with plakoglobin and N-cadherin.

Figure 3-1C revealed that not only were Nm23 protein levels increased in SCC9-PG cells, but some of it also appeared localized to the cell-cell contact areas. This raised the question of whether the membrane distribution of Nm23 was due to its associations with cell-cell junctions. To address this possibility, soluble and insoluble (cytoskeleton-associated) fractions from SCC9 and SCC9-PG cell extracts were processed for coimmunoprecipitation with plakoglobin or N-cadherin antibodies followed by immunoblotting with Nm23 antibodies. Plakoglobin antibodies coprecipitated only Nm23-H2 from the soluble fraction, and both Nm23-H1 and -H2 from the insoluble fraction of SCC9-PG cells, although the amount of Nm23-H2 coprecipitated was notably higher than -H1 (Figure 3-2A, IP: PG). As expected, immunoprecipitation of plakoglobin yielded negative results for association with Nm23 in SCC9 cells. In SCC9 cells, N-cadherin antibodies coprecipitated very small amounts of Nm23-H1 and -H2, mostly in the insoluble fraction. In SCC9-PG cells,

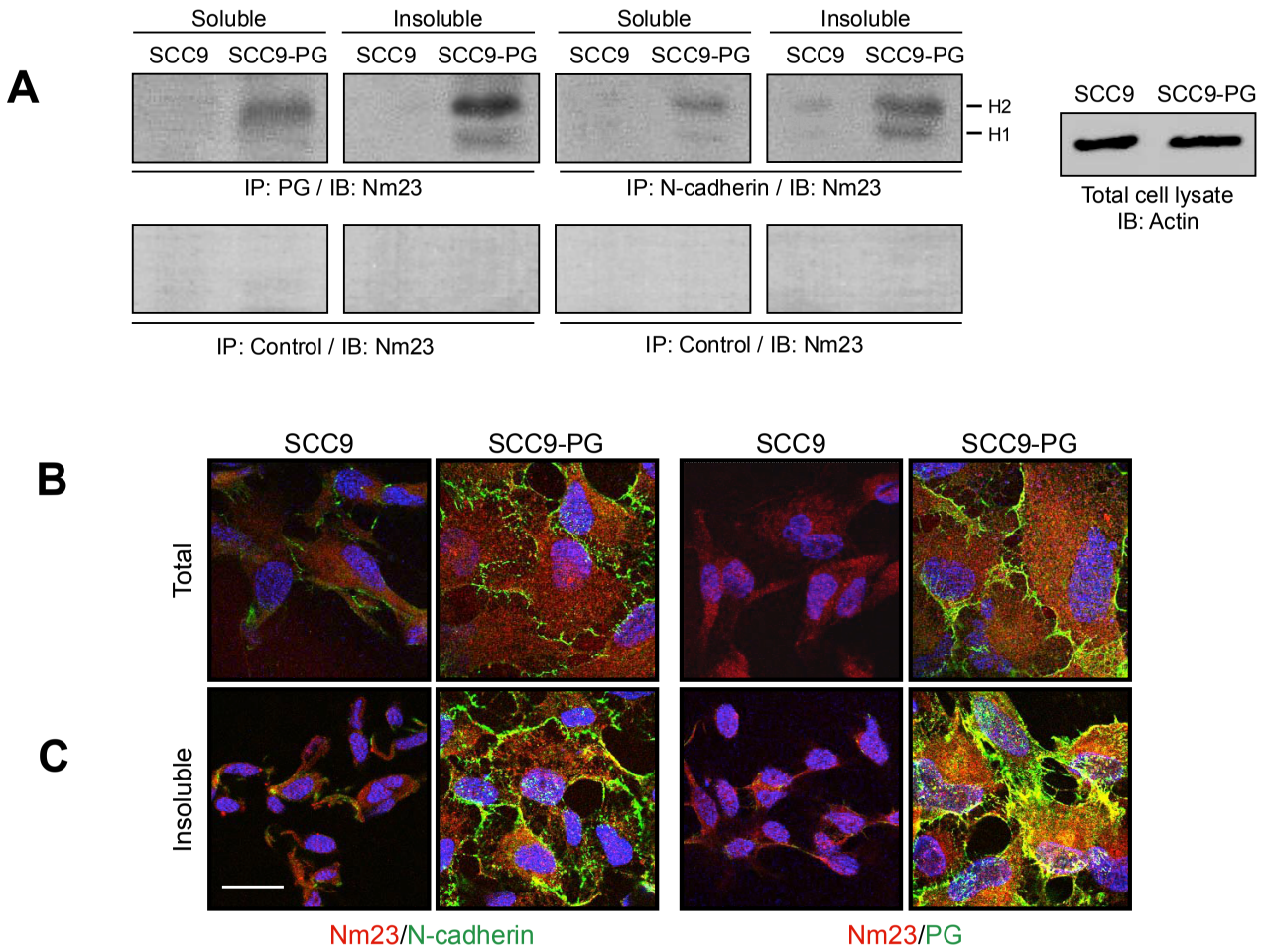

Figure 3-2. Nm23 interacts with plakoglobin and N-cadherin in SCC9-PG cells. (A) Soluble and insoluble fractions from SCC9 and SCC9-PG cell extracts were processed for immunoprecipitation using **N-cadherin**, **plakoglobin** and **control** antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **Nm23**. **(B)** SCC9 and SCC9-PG cells were formaldehyde fixed, CSK buffer extracted and stained with **Nm23** (red) and **N-cadherin** or **plakoglobin** (green) antibodies to visualize the total distribution of proteins. Nuclei were counterstained with DAPI (blue). **(C)** To visualize the cytoskeleton-associated (insoluble) proteins, cells were first extracted with CSK buffer and then fixed using formaldehyde before they were processed for confocal microscopy as described in (**B**). PG, plakoglobin. Bar, 20 μm.

D

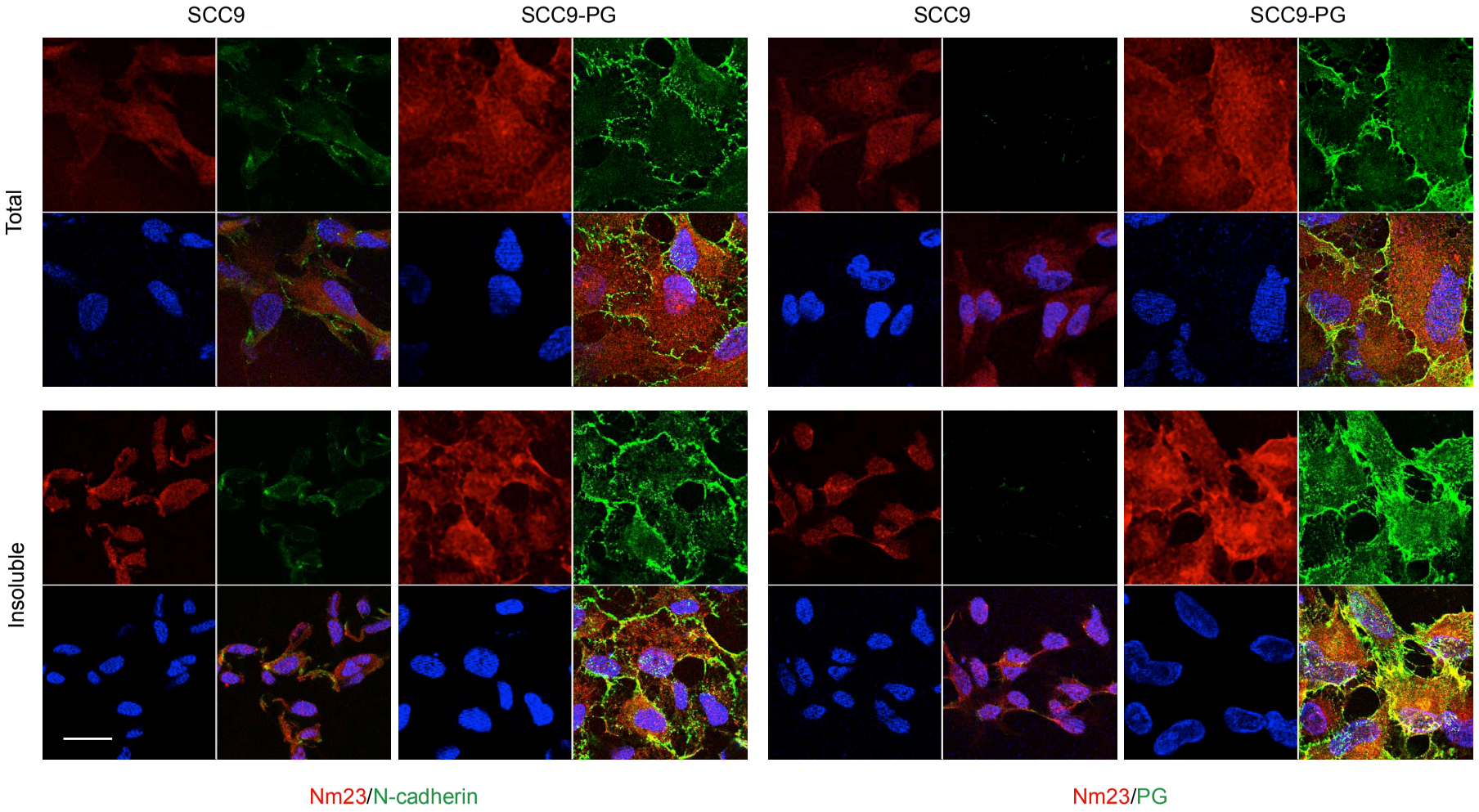

Figure 3-2 (continued). Nm23 colocalizes with plakoglobin and N-cadherin in SCC9-PG cells. (D) SCC9 and SCC9-PG cells were either first formaldehyde fixed and then CSK buffer extracted (Total) or first CSK buffer extracted and subsequently formaldehyde fixed (Insoluble) and stained with **Nm23** (red) and **N-cadherin** or **plakoglobin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

plakoglobin expression increased the amount of Nm23 coprecipitated with N-cadherin (Figure 3-2A, IP: N-cadherin). As with plakoglobin, more Nm23-H2 than -H1 was coprecipitated with N-cadherin antibodies. Nm23 associations with plakoglobin and N-cadherin were further confirmed by reciprocal coimmunoprecipitations in which cell extracts were immunoprecipitated with Nm23 antibodies and blotted for plakoglobin and N-cadherin (Figure 3-3B).

3.2.3. Nm23 colocalizes at the membrane with plakoglobin and N-cadherin.

We further confirmed the results of our coimmunoprecipitation studies with confocal microscopy using Nm23-H1/H2, plakoglobin and N-cadherin antibodies in conjunction with two different extraction/fixation protocols. In order to visualize the entire cellular pool of proteins, cells were first fixed using formaldehyde and subsequently permeabilized using CSK extraction buffer. Alternatively, CSK extraction buffer was used first to permeabilize and extract the soluble pool of cellular proteins, followed by fixation with formaldehyde, allowing for the visualization of the cytoskeleton-associated pool of proteins (Pasdar and Nelson, 1988b; Pasdar et al., 1995), including those stabilized by association with the adhesive complexes.

Staining of fixed/permeabilized SCC9 cells with N-cadherin and Nm23-H1/H2 showed that Nm23 was distributed throughout the cell while N-cadherin was primarily detected at the membrane (Figure 3-2B, D, SCC9, Nm23/N-cadherin). Under similar conditions, SCC9-PG cells showed

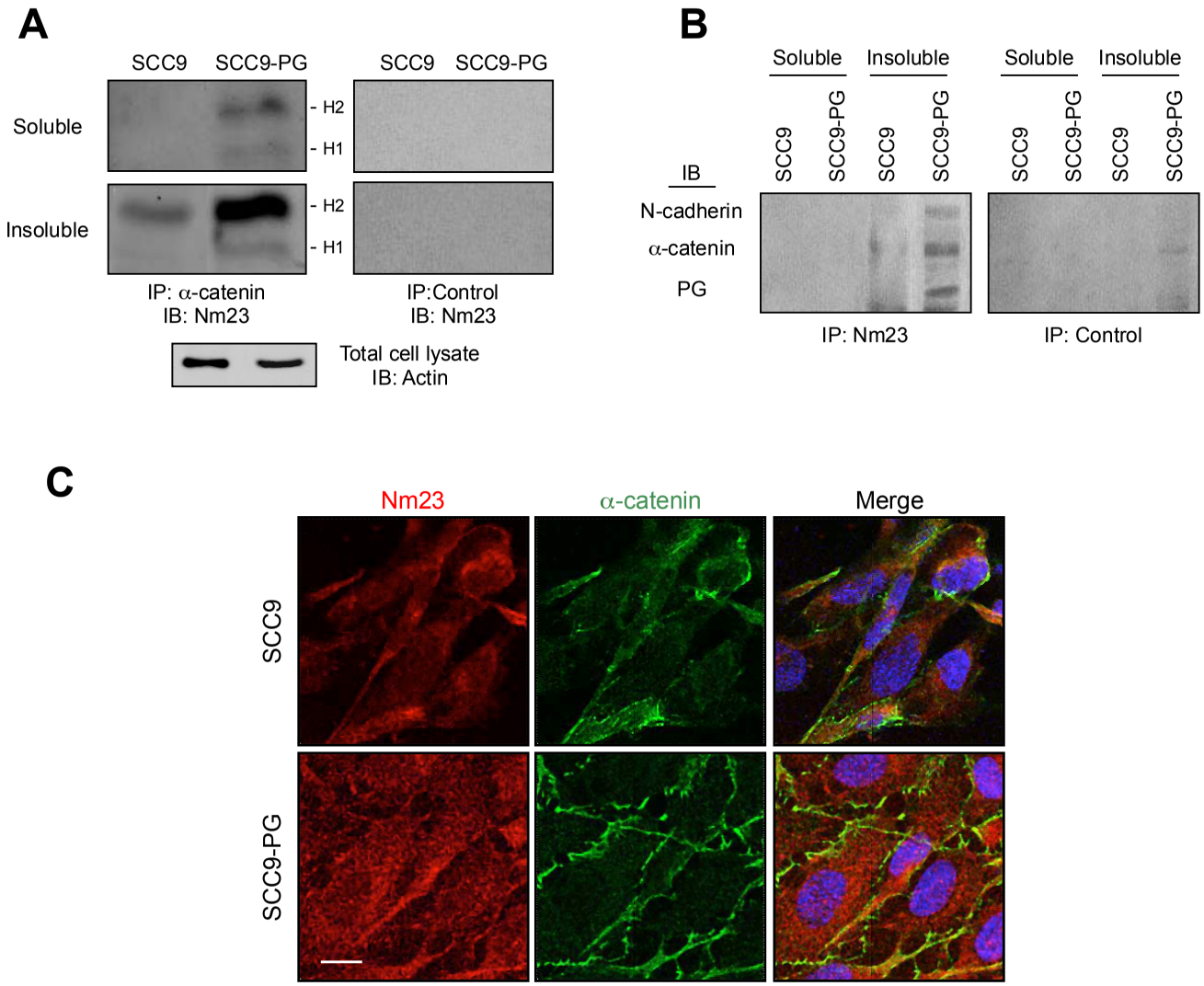

Figure 3-3. Nm23 interacts with α-catenin. (A) Soluble and insoluble fractions from SCC9 and SCC9-PG cells were processed for sequential immunoprecipitation and immunoblotting using **α-catenin** and **Nm23** antibodies, respectively. **(B)** Soluble and insoluble fractions from SCC9 and SCC9-PG cells were processed for sequential immunoprecipitation and immunoblotting using **Nm23** followed by **N-cadherin**, **α-catenin** and **plakoglobin** antibodies. **(C)** SCC9 and SCC9-PG cells were formaldehyde fixed, CSK buffer extracted and processed for confocal microscopy using **Nm23** (red) and **α-catenin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

overlapping distributions of Nm23-H1/H2 and N-cadherin (Figure 3-2B and D, SCC9-PG, Nm23/N-cadherin), as well as Nm23 and plakoglobin (Figure 3-2B, D, SCC9-PG, Nm23/PG).

In SCC9 cells that were extracted before fixation, most of the staining for both Nm23-H1/H2 and N-cadherin, which was observed when these cells were fixed before extraction, was removed (Figure 3-2C, D). Under these conditions, SCC9-PG cells showed colocalization between Nm23-H1/H2 and N-cadherin, as well as Nm23-H1/H2 and plakoglobin; furthermore, these codistributions were primarily at the membrane (Figure 3-2C, D, SCC9-PG). Thus, Nm23 colocalized with the cytoskeleton-associated pool of plakoglobin and N-cadherin.

3.2.4. Nm23 interacts with α-catenin.

Increased levels of Nm23-H1 and -H2 associated with the insoluble N-cadherin in SCC9-PG cells and its membrane codistribution with both plakoglobin and N-cadherin in these cells suggested that plakoglobin expression may have led to the association of Nm23 with the stable cadherin-catenin complexes at the adherens junction. To this end, we examined whether α-catenin, which mediates N-cadherin-plakoglobin interactions with the actin cytoskeleton, also associated with Nm23-H1 and -H2. Figure 3-3A shows that in SCC9 cells only Nm23-H2 was associated with α-catenin, mainly in the insoluble fraction (Figure 3-3A, SCC9). In contrast, in SCC9-PG cells, α-catenin antibodies coprecipitated Nm23-H2 and to a lesser extent -H1 from both the soluble and insoluble

fractions (Figure 3-3A, SCC9-PG). Reciprocal coimmunoprecipitation with Nm23 antibodies also detected α-catenin in the insoluble fraction of both SCC9 and SCC9-PG cells, with significantly higher levels in the latter (Figure 3-3B). These observations were further verified by immunofluorescence assays, which clearly showed α-catenin codistribution with Nm23-H1/H2 in SCC9-PG cells, while this colocalization was barely detectable in SCC9 cells (Figure 3-3C). Collectively, these results suggested that plakoglobin expression stabilized Nm23-H1 and -H2 interactions with cadherin-catenin complexes at the membrane.

3.2.5. The N-terminal domain of plakoglobin is necessary for interaction with Nm23.

So far, our data suggested that Nm23-H1 and -H2, plakoglobin, α-catenin and N-cadherin may be present in the same complex. We then asked whether α-catenin could be a bridge between Nm23 and plakoglobin-N-cadherin complexes. To clarify this point, we assessed the interactions between Nm23, plakoglobin and N-cadherin in SCC9 cells expressing Flag-tagged wild-type plakoglobin or a mutant plakoglobin with a deletion in the N-terminal α-catenin binding domain (PG-ΔN123; Li et al., 2007a; Kolligs et al., 2000). Soluble and insoluble cell extracts from PG-Flag and PG-ΔN123 transfectants were processed for sequential coimmunoprecipitation and immunoblotting with Flag and Nm23 antibodies respectively.

Plakoglobin coprecipitated Nm23-H2 from the insoluble fractions of PG-Flag cells but not the PG-ΔN123 cells in which plakoglobin is unable to bind α-catenin (Figure 3-4A, IP: Flag). No interaction between Nm23 and plakoglobin was detected in the soluble fraction. Since plakoglobin expression (SCC9-PG cells) increased the amount of Nm23 coprecipitated with N-cadherin (Figure 3-2A), we examined the effects of deleting the N-terminal domain of plakoglobin on the association between Nm23 and N-cadherin. N-cadherin was also found to coprecipitate Nm23 in PG-Flag cells and primarily in the insoluble fraction (Figure 3-4A, IP: N-cadherin). Immunofluorescence experiments confirmed the coimmunoprecipitation findings. Plakoglobin colocalized with Nm23-H1/H2 in PG-Flag cells, however, in -ΔN123 cells, plakoglobin/Nm23 colocalization was lost (Figure 3-4B, C, Nm23/Flag). Colocalization between Nm23-H1/H2 and N-cadherin was observed in PG-Flag cells, whereas their codistribution was reduced in the -ΔN123 transfectants (Figure 3-4B, C, Nm23/N-cadherin).

3.2.6. Nm23-plakoglobin interaction is dependent on α-catenin.

Upon showing that loss of the N-terminal domain of plakoglobin resulted in the loss of its association with Nm23-H1 and -H2, we set out to determine whether α-catenin was necessary for this interaction. To do so, we used PC3 cells, a prostate carcinoma cell line which lacks α-catenin, while expressing E-cadherin, plakoglobin and Nm23 (Morton et al., 1993; Daniel and Reynolds, 1995; Igawa et al., 1994). We first confirmed, by Western blot, that PC3 cells lacked α-catenin, and expressed E-cadherin,

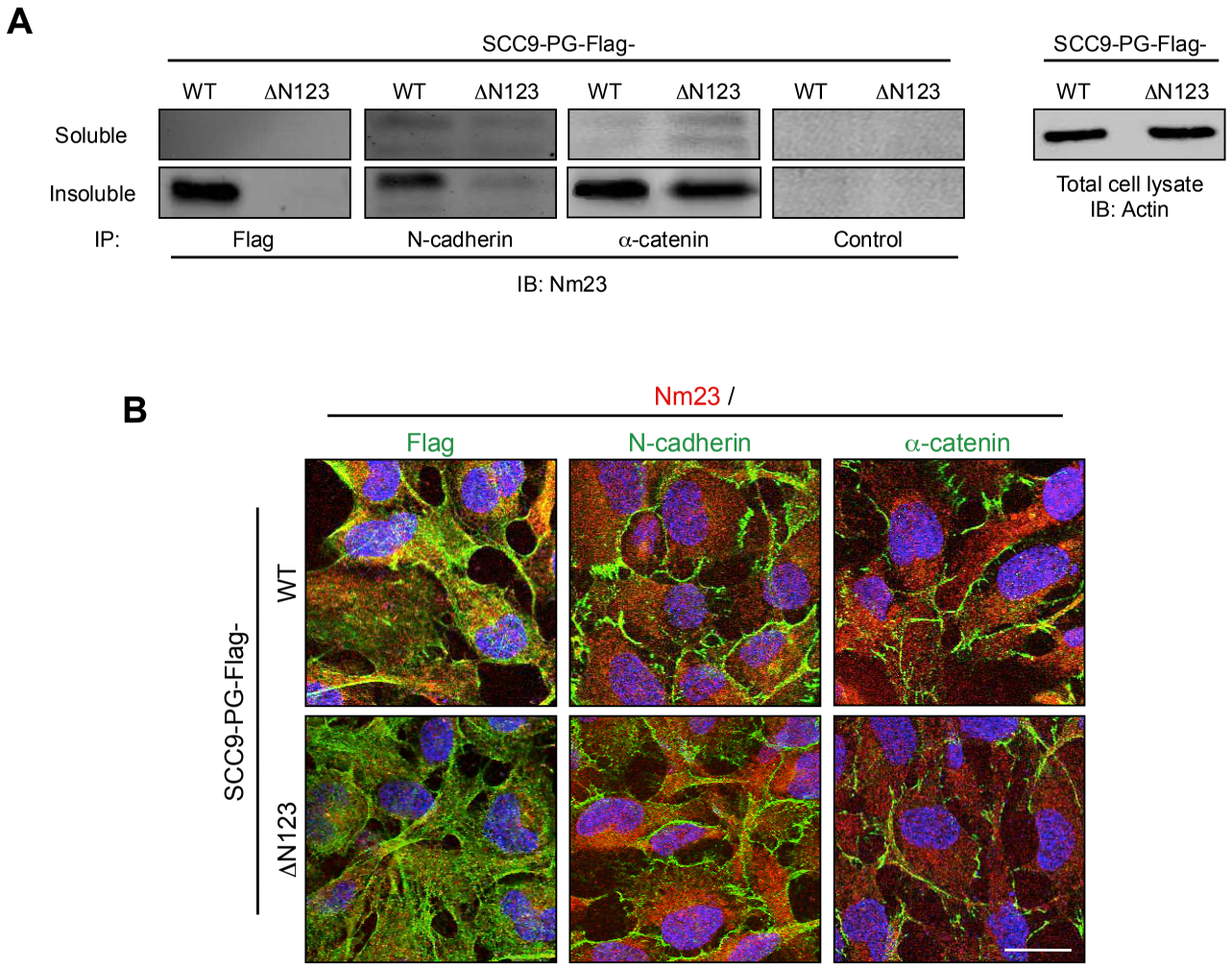

Figure 3-4. The N-terminal domain of plakoglobin is necessary for interaction with Nm23. (A) The soluble and insoluble fractions from SCC9-PG-Flag, and -ΔN123 cell extracts were processed for immunoprecipitation using **Flag**, **N-cadherin and α-catenin** antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **Nm23**. **(B)** SCC9-PG-Flag and -ΔN123 cells were formaldehyde fixed, extracted with CSK buffer and processed for confocal microscopy using **Nm23** (red) and **Flag, N-cadherin or α-catenin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

C

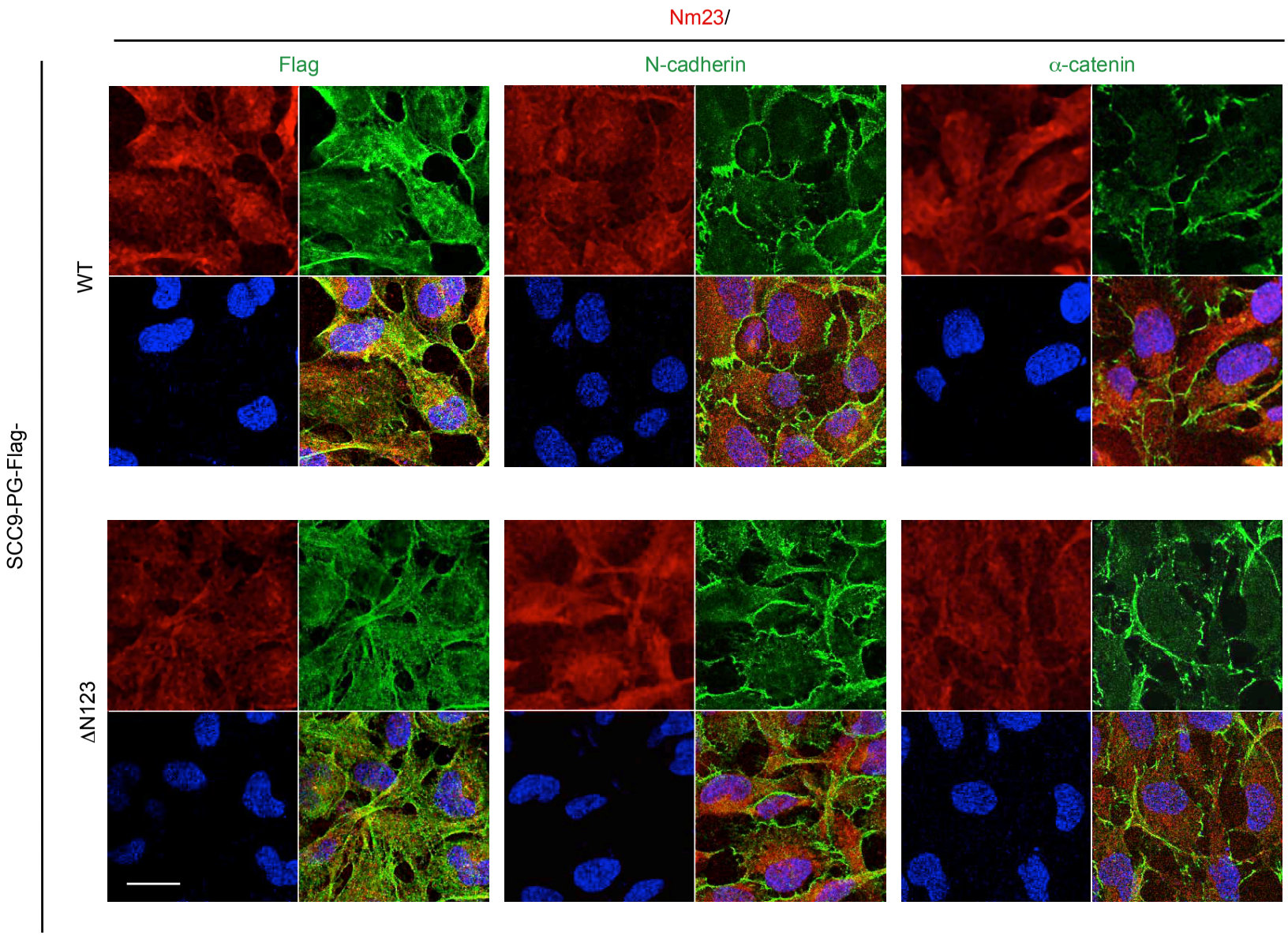

Figure 3-4 continued. The N-terminal domain of plakoglobin is necessary for interaction with Nm23. (C) SCC9-PG-Flag and -ΔN123 cells were formaldehyde fixed, extracted with CSK buffer and processed for confocal microscopy using **Nm23** (red) and **Flag, N-cadherin or a-catenin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

plakoglobin, and Nm23 (Figure 3-5A). Next, we performed coimmunoprecipitation experiments using plakoglobin and α-catenin antibodies, and showed that Nm23-H1 and -H2 were coprecipitated by neither of these antibodies in neither the soluble nor insoluble fractions (Figure 3-5B). Furthermore, the supernatants from the soluble and insoluble α-catenin and plakoglobin immunoprecipitates were processed for immunoblotting with Nm23. In these supernatants, Nm23-H1 and -H2 were detected in the soluble but not the insoluble fractions. Reciprocal coimmunoprecipitation experiments confirmed our findings, showing that plakoglobin was not coprecipitated with Nm23-H1 and -H2, but rather was present in the supernatants from both soluble and insoluble Nm23 immunoprecipitates (Figure 3-5C).

To further confirm the coimmunoprecipitation study results, PC3 cells were processed for immunofluorescence with anti-Nm23-H1/H2, plakoglobin and α-catenin antibodies. Consistent with the lack of α-catenin expression, there was no detectable staining for this protein in these cells. Furthermore, in these cultures, Nm23-H1/H2 staining was detected throughout the cells but was notably absent from the peripheries (Figure 3-5D, Nm23/α-catenin). In contrast, plakoglobin's localization was primarily peripheral and membrane associated. Consistent with their distinct localizations, no detectable Nm23/plakoglobin codistribution was observed in these cells (Figure 3-5D, Nm23/PG).

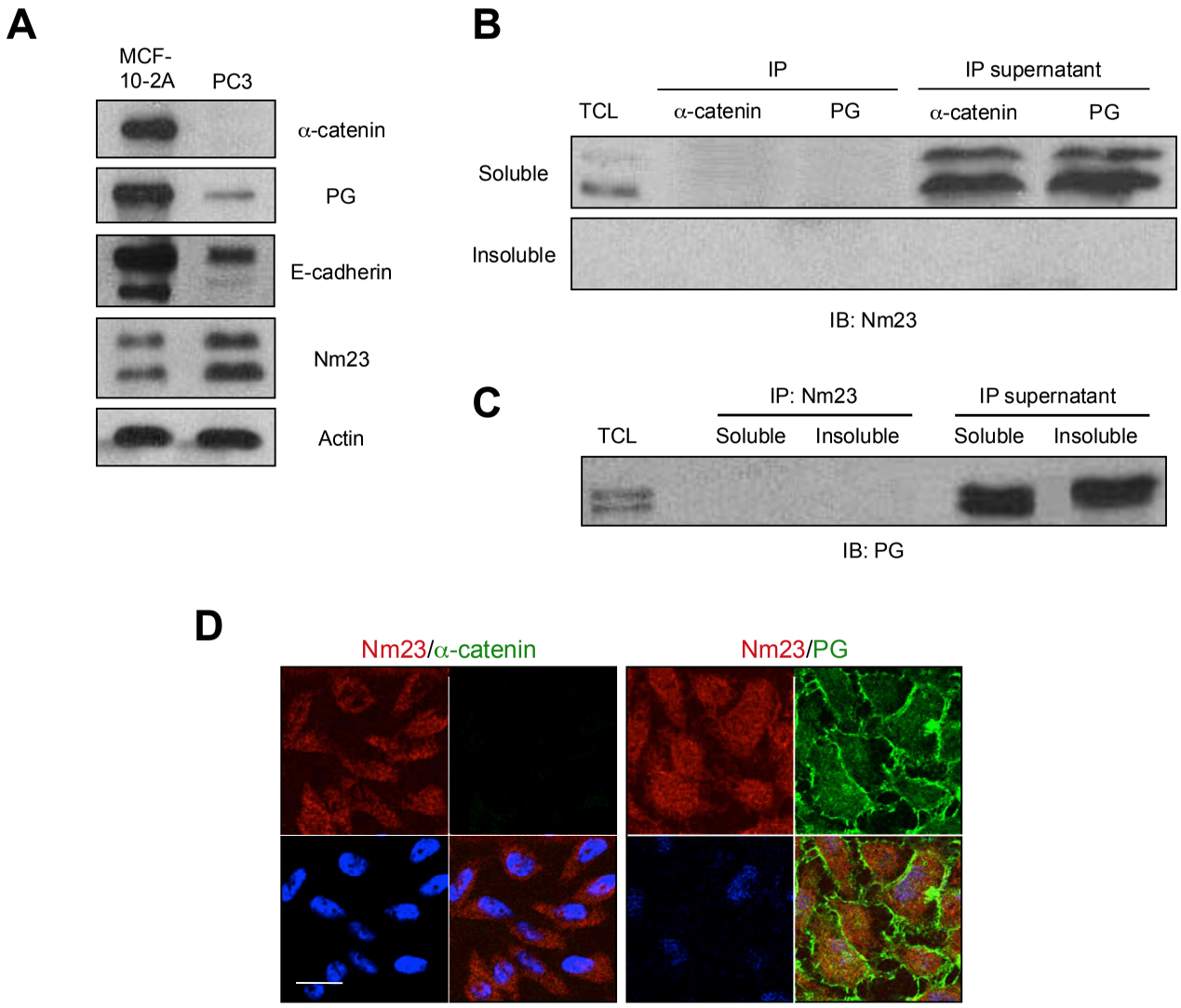

Figure 3-5. α-catenin is necessary for the interaction between plakoglobin and Nm23. (A) Thirty μg of total cellular proteins from PC3 and MCF-10-2A cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **α-catenin, plakoglobin, E-cadherin, Nm23 and actin. (B-C)** Soluble and insoluble fractions from PC3 cell extracts were processed for immunoprecipitation using **plakoglobin** and **α-catenin (B)** or Nm23 **(C)** antibodies. The immune complexes, as well as the supernatants from the immunoprecipitations were resolved by SDS-PAGE and blotted with **Nm23 (B)** or **plakoglobin (C)** antibodies. TCL, total cell lysate. **(D)** PC3 cells were formaldehyde fixed, extracted with CSK buffer and processed for confocal microscopy using **Nm23** (red) and **α-catenin** or **plakoglobin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

3.2.7. Nm23 interaction with plakoglobin, cadherin and α-catenin is not cell line specific.

To confirm that Nm23's interactions with the cadherin-catenin complex were not N-cadherin or cell line specific, we examined Nm23's associations with plakoglobin, α-catenin and E-cadherin in several non-epidermal cell lines. Soluble and insoluble fractions from the E-cadherin and plakoglobin expressing MCF-10-2A, MCF-7, SW620 and MDCK cells were processed for coimmunoprecipitation and confocal microscopy using plakoglobin, E-cadherin, α-catenin and Nm23 antibodies. Nm23-H1 and -H2 coprecipitated with plakoglobin and α-catenin in both the soluble and insoluble pools, whereas they were associated with E-cadherin only in the insoluble pool (Figure 3-6A). Reciprocal coimmunoprecipitation using Nm23 antibodies further supported these results, where plakoglobin, E-cadherin, and α-catenin coprecipitated with Nm23-H1 and -H2 from insoluble fractions of all cell lines (Figure 3-6B). Immunofluorescence experiments also showed distinct colocalization between Nm23-H1/H2 and plakoglobin, E-cadherin, and α-catenin in all cell lines (Figure 3-6C). These observations showed that Nm23-H1 and -H2 interacted with both N- and E-cadherin and furthermore, its associations with plakoglobin, α-catenin and cadherins were not cell specific.

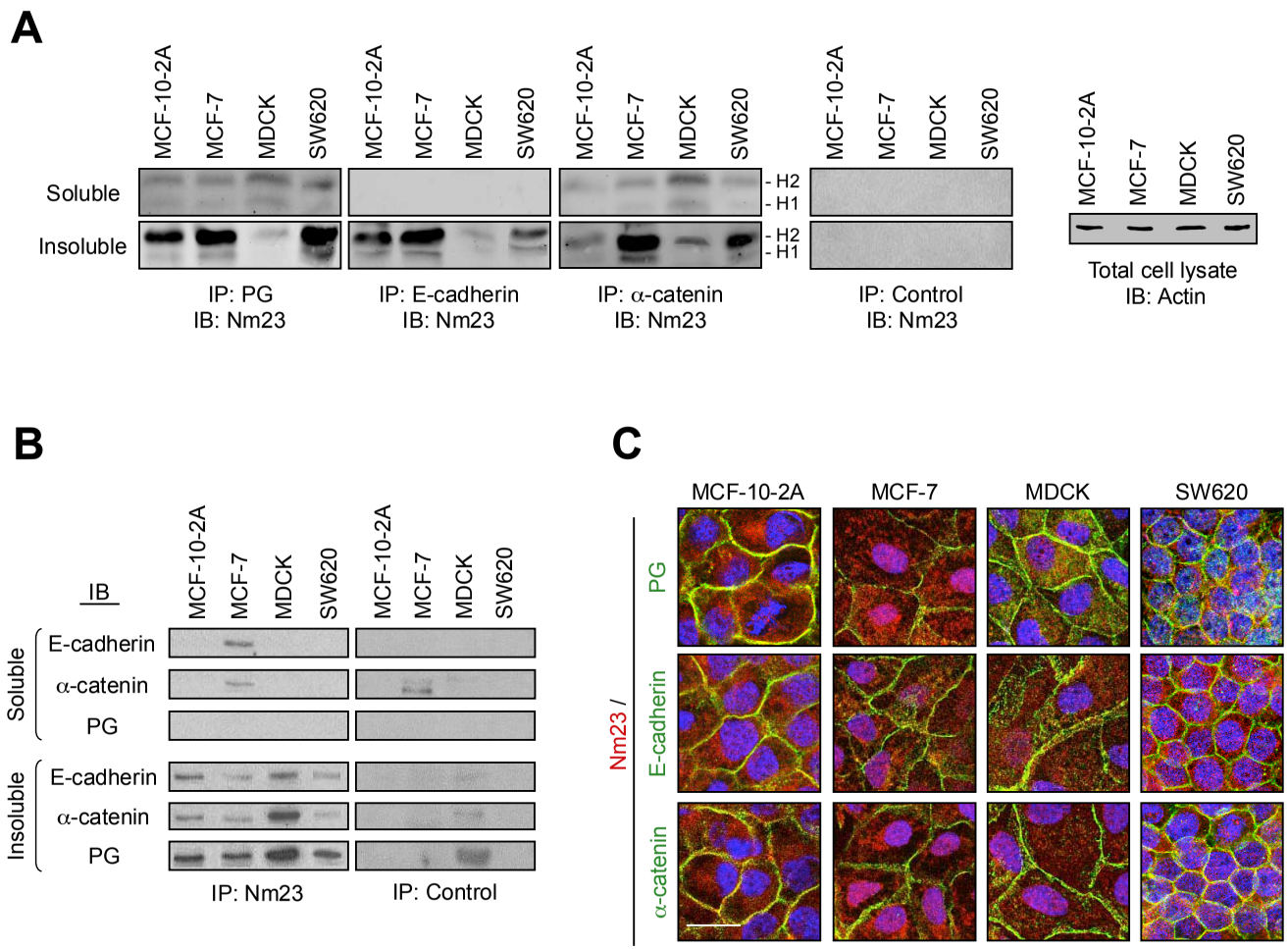

Figure 3-6. Nm23 interacts with plakoglobin, E-cadherin and α-catenin in various epithelial cell lines. (A) Soluble and insoluble fractions from MCF-10-2A, MCF-7, MDCK, and SW620 cell extracts were processed for immunoprecipitation using **plakoglobin**, **E-cadherin** and **α-catenin** antibodies. The immune complexes were resolved by SDS-PAGE and blotted with **Nm23** antibodies. **(B)** Soluble and insoluble fractions from MCF-10-2A, MCF-7, MDCK, and SW620 cell extracts were processed for immunoprecipitation using **Nm23** antibodies. The immune complexes were resolved by SDS-PAGE and blotted with **E-cadherin, α-catenin and plakoglobin** antibodies. **(C)** MCF-10-2A, MCF-7, MDCK, and SW620 cells were CSK buffer extracted, formaldehyde fixed, and processed for confocal microscopy using **Nm23** (red) and **plakoglobin**, **E-cadherin**, or **α-catenin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

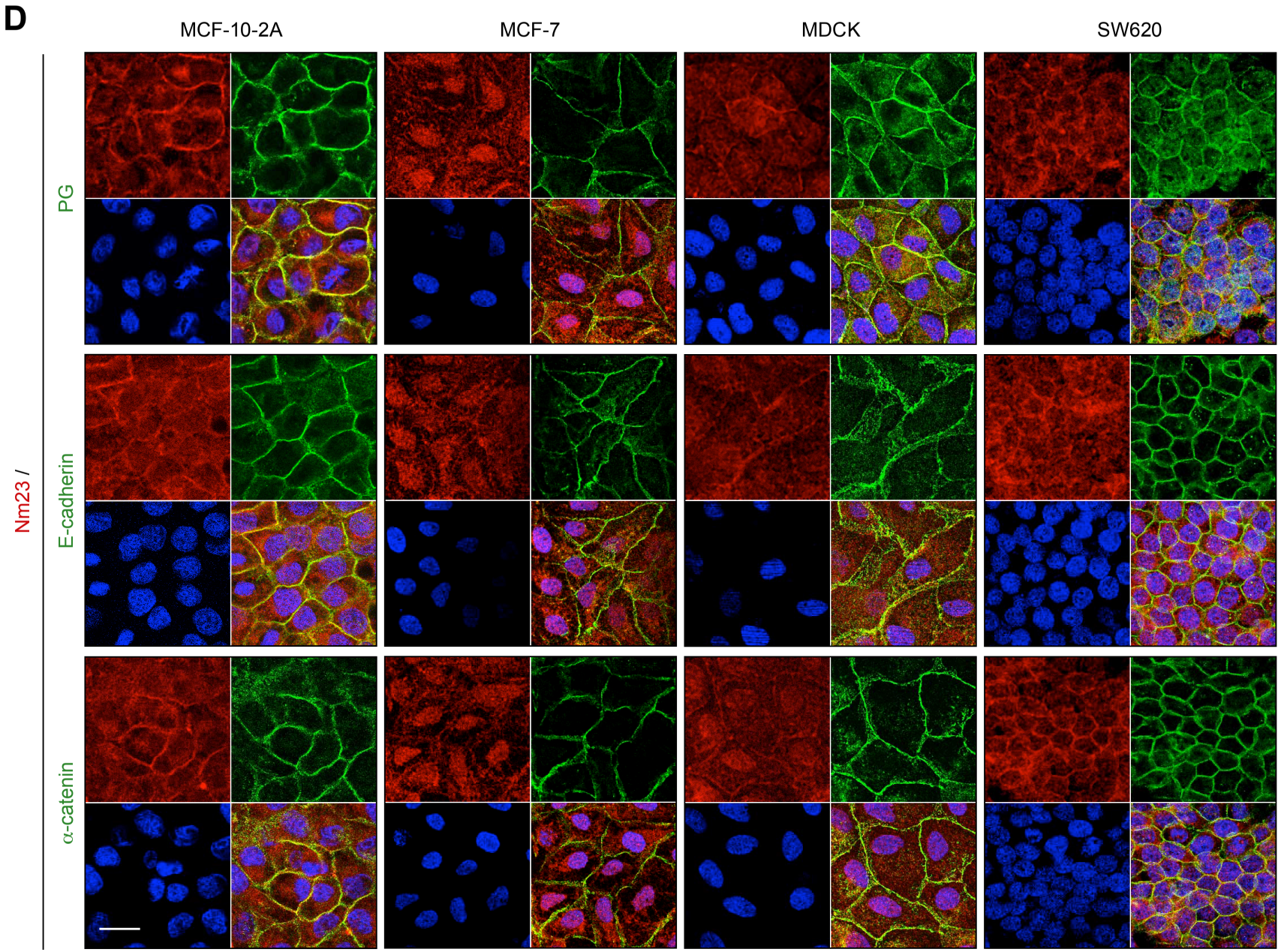

Figure 3-6 (continued). Nm23 colocalizes with plakoglobin, E-cadherin and α-catenin in various epithelial cell lines. (D) MCF-10-2A, MCF-7, MDCK, and SW620 cells were CSK buffer extracted, formaldehyde fixed, and processed for confocal microscopy using **Nm23** (red) and **plakoglobin**, **E-cadherin**, or **α-catenin** (green) antibodies. Nuclei were counterstained with DAPI (blue). PG, plakoglobin. Bar, 20 μm.

3.3. Discussion

We identified Nm23 as a protein differentially expressed between plakoglobin-deficient SCC9 cells and their plakoglobin-expressing transfectants. Further analysis showed that plakoglobin expression led to the membrane localization of Nm23-H1/H2 and its interactions with plakoglobin, N-cadherin and α-catenin. Plakoglobin's interaction with Nm23-H1 and -H2 required the first 123 amino acids in the N-terminal domain of plakoglobin, which mediates its interaction with α-catenin, and furthermore, in cells lacking α-catenin, interactions between plakoglobin and Nm23-H1 and -H2 were lost. Finally, we showed that the interactions between Nm23 and plakoglobin, cadherin, and α-catenin are not cell line specific.

Our proteomic and microarray analyses identified several tumor suppressors whose levels were increased upon expression of plakoglobin in SCC9 cells. We chose to focus on Nm23 for several reasons, the first of which being that it in a variety of different cancers, both a decrease in Nm23 and cadherin-mediated adhesion is observed while the genes encoding these proteins remain unaltered (Chen et al., 2005; Che et al., 2006). Previous studies have also shown that Nm23 is both targeted by and regulates c-myc, which was the first identified target of the Wnt/β-catenin signaling network (He et al., 1998; Schuldiner et al., 2002; Arnaud-Dabernat et al., 2004). Additionally, we have shown that the levels and subcellular localization of plakoglobin can modulate the amount of β-

catenin and its signaling function in a cell context-dependent manner (Li et al., 2007a).

Our results lend support to the observation made during our proteomic studies, which identified Nm23-H2 as being increased nearly five-fold in SCC9-PG cells as compared to parental SCC9 cells. Western blot analysis revealed that whereas the levels of both Nm23-H1 and -H2 were significantly increased upon plakoglobin expression, it was the levels of Nm23-H2 that were more notably increased. Examination of the effect of plakoglobin expression on the amount of Nm23-H1 and -H2 mRNA by RT-PCR showed increased levels of Nm23-H1 mRNA only, consistent with the microarray analysis, which revealed significantly increased *NME1* (Nm23-H1) but not *NME2* (Nm23-H2) expression in SCC9-PG cells. These results together suggest that plakoglobin may regulate *NME1* at the level of transcription, and increases the levels of both Nm23-H1 but more so Nm23-H2 protein. Plakoglobin may increase Nm23-H2 protein levels by increasing its stability through their interactions, by inhibiting Nm23-H2's degradation, or by other post-translational mechanisms. Additionally, the higher levels of Nm23-H1 transcripts that resulted in increased protein levels (albeit not as notable as Nm23-H2) could promote interactions between Nm23-H1 and -H2, resulting in its increased stability. Whether plakoglobin acts directly as a transcriptional regulator of Nm23-H1 expression or alters the activity of other transcription factors is examined in Chapter Five of this thesis. Furthermore, why plakoglobin regulates

Nm23-H1 (*NME1*) gene expression and -H2 protein levels remains unclear.

We observed Nm23-H1/H2 at the membrane in SCC9-PG cells, which was concurrent with a mesenchymal to epidermoid transition and the formation of stable junctional complexes and decided to further investigate whether Nm23-H1/H2 localized to junctional complexes, taking into account prior studies describing its membrane localization (Palacios et al., 2002; Che et al., 2006). Immunofluorescence analysis revealed that Nm23-H1/H2 colocalized with the total, as well as the cytoskeleton-associated pool of plakoglobin and N-cadherin in SCC9-PG cells.

Reciprocal coimmunoprecipitation studies corroborated our immunofluorescence results and further, allowed us to distinguish between Nm23-H1 and -H2, and their respective interactions with the junctional components, which could not be differentiated by microscopy because the immunofluorescence antibodies recognized both Nm23-H1 and -H2. More specifically, greater amounts of Nm23-H2 coprecipitated with plakoglobin in insoluble fractions than Nm23-H1, while only Nm23-H2 coprecipitated with plakoglobin in the soluble fraction of these cells, suggesting that Nm23-H2-plakoglobin interactions may not require or precede Nm23-H1 associations. While Nm23-H1 and (primarily) -H2 also interacted with both pools of N-cadherin in SCC9-PG cells, these interactions were significantly increased in the insoluble pool. That Nm23 interacted with plakoglobin and N-cadherin, and primarily in the cytoskeleton associated pool of proteins,

suggested that plakoglobin may play a role in recruiting Nm23-H2, and to a lesser extent, Nm23-H1, to the cadherin-catenin complex at the adherens junction. Since, at this junction, α-catenin mediates the link between the cadherin-catenin complex and the actin cytoskeleton, we sought to determine whether Nm23-H1/H2 and α-catenin interact. We found that they did interact, that the primary interaction was between Nm23-H2 and α-catenin, and that these interactions were independent of plakoglobin. Furthermore, we identified the N-terminal domain of plakoglobin, which is essential for its interaction with α-catenin (Sacco et al., 1995), as being necessary for its interaction with Nm23.

Recent studies have shown the existence of two distinct cellular pools of α-catenin: one, composed of α-catenin monomers, that associates with the β-catenin-E-cadherin complex at the membrane, and another, composed of α-catenin dimers, that associates with the actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005). These distinct pools of α-catenin may help to explain why so much more Nm23-H2 is associated with the junctional components in the cytoskeleton-associated pool: it may be possible that one pool of α-catenin associates with both Nm23-H1 and -H2, while the other pool associates primarily with Nm23-H2.

Coimmunoprecipitation experiments in the α-catenin deficient PC3 cell line were performed to further characterize the role that α-catenin plays in mediating the interactions between plakoglobin and Nm23-H1 and -H2. The results of these experiments confirmed that α-catenin is a critical

component of the plakoglobin-Nm23-H1/H2 complex, as Nm23-H1 and -H2 did not coprecipitate with plakoglobin antibodies in these cells. Furthermore, all of Nm23 in PC3 cells was detected in the immunoprecipitates supernatants from the soluble fractions only. Consistent with these results, Nm23-H1 and -H2 were found in total cell extracts from the soluble fraction of PC3 cells, but not the insoluble fraction. These findings suggest that in cells that lack α-catenin, Nm23-H1 and -H2 do not interact with any junctional components that are cytoskeleton-associated, and as a result remain exclusively in soluble fractions. Reciprocal coimmunoprecipitation experiments using Nm23 antibodies further confirmed the absence of plakoglobin in Nm23 immunoprecipitates. In these cells, plakoglobin was detected in the immunoprecipitates supernatants after Nm23 was removed. That Nm23-H1/H2 and plakoglobin were not associated in PC3 cells also was detected by confocal microscopy, which clearly showed the absence of their membrane codistribution in these cells. Finally, examination of additional cell lines expressing E-cadherin showed that Nm23-H1 and -H2 interact with both N- and E-cadherin and its associations with cadherins and plakoglobin is neither tissue nor species specific.

Plakoglobin has often been associated with tumor suppressor activity, although the mechanisms behind this activity remain unclear (Simcha et al., 1996; Parker et al., 1998; Pantel et al., 1998; Winn et al., 2002; Rieger-Christ et al., 2005). More recently, downregulation of plakoglobin

also was shown to increase the risk of pulmonary metastasis in soft tissue sarcomas (Kanazawa et al., 2008), further supporting the idea that plakoglobin may act to suppress metastasis in addition to tumor formation. Similarly, a number of studies have shown that in addition to its well-documented role as a metastasis suppressor, Nm23-H1 and -H2 have growth inhibitory activities (Lee et al., 2009; Jin et al., 2009). Our results suggest that plakoglobin's tumor/metastasis suppressor activity may be mediated through the modulation of the levels, stability and subcellular localization of Nm23. In particular, plakoglobin, at least in the context of squamous cell oral carcinomas, exerts its effects more notably on Nm23-H2. This result is supported by a previous study, which found that Nm23-H2 plays a critical role as a metastasis/tumor suppressor in oral carcinomas (Miyazaki et al., 1999). Collectively, these observations suggest that plakoglobin and Nm23 may exert (at least part of) their anti-tumor/metastasis activities in conjunction with one another. To this point, no studies have been performed which have examined the (lack of) expression of both Nm23 and plakoglobin in tumors.

In conclusion, although the exact roles that Nm23 plays in tumorigenesis and metastasis remain unclear, our results and those of others indicate that it is the interactions of Nm23 with other cellular proteins that determine what type of a role it will play in these processes (Kim et al., 2009). Here, we identified plakoglobin as a novel Nm23 interacting partner, an observation that also provides a potential

mechanism for the often-suggested role of plakoglobin as a tumor suppressor. The larger implication of this novel observation is the role of plakoglobin as a potential modulator of growth regulating proteins whose expressions are compromised or altered during tumor progression and metastasis.

CHAPTER FOUR: PLAKOGLOBIN INTERACTS WITH P53 AND REGULATES THE EXPRESSION OF 14-3-3σ

4.1. Introduction[1]

4.1.1. Rationale

During the microarray experiments, we identified numerous tumor and metastasis suppressor genes that were upregulated and oncogenes that were downregulated in SCC9-PG cells (compared to SCC9 cells). Intriguingly, we noticed that several p53-target genes (including the tumor suppressor 14-3-3σ) were differentially expressed in SCC9-PG cells, which suggested that perhaps plakoglobin regulates gene expression in conjunction with p53. To address this possibility, we examined whether plakoglobin and p53 interact and if they associate with the same target gene promoters.

4.1.2. p53

The p53 tumor suppressor is regarded as one of the most important tumor suppressors and plays essential roles in the regulation of cell proliferation, senescence, survival, apoptosis and metabolism (Levine and Oren, 2009; Maddocks and Vousden, 2011; Mirzayans et al., 2012). As a tumor suppressor, the most documented role of p53 is that of a transcription factor, regulating the expression of genes involved in cell-cycle control, apoptosis, tumorigenesis and metastasis (Harris and Levine, 2005; Junttila and Evan, 2009; Menendez et al., 2009; Meek, 2009; Cicalese et al., 2009; Goh et al., 2011). In addition to its role in regulating gene expression, recent studies have demonstrated that p53 has non-

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genomic functions in the cytoplasm, where it interacts with pro- and anti-apoptotic proteins and promotes apoptosis by inducing mitochondrial outer membrane permeabilization (Mihara et al., 2003; Green and Kroemer, 2009; Vaseva and Moll, 2009; Brady and Attardi, 2010; Golubovskaya and Cance, 2011; Lindenboim et al., 2011; Stegh, 2012).

Inactivating mutations of p53 occur in half of all tumors, whereas in the remaining tumors, mutations in other components of the p53 pathway account for its functional inactivation (Junttila and Evan, 2009; Menendez et al., 2009; Goh et al., 2011). Furthermore, some mutations in p53, known as the "gain-of-function" mutations, endow this tumor suppressor with oncogenic activities that lead to the increased expression of tumor and metastasis promoting genes (O'Farrell et al., 2004; Tepper et al., 2005; Brosh and Rotter, 2009; Oren and Rotter, 2010; Muller et al., 2012).

Normally, p53 protein levels are kept under tight control, with the steady-state levels of the protein being quite low. Various studies have suggested that p53 has a half-life of roughly 20-30 minutes in non-stressed cells (Moll and Petrenko, 2003; Agrawal et al., 2006). The levels of p53 are regulated by Hdm2, an E3 ubiquitin ligase, which under normal conditions interacts with and ubiquitinates p53 (Collavin et al., 2010; Wang and Jiang, 2012; Pei et al., 2012). Following ubiquitination, p53 is exported out of the nucleus, where it is degraded via cytoplasmic proteasomes (Figure 4-1A), although various studies have shown that it can be also be degraded in the nucleus by nuclear proteasomes (Boehme and Blattner,

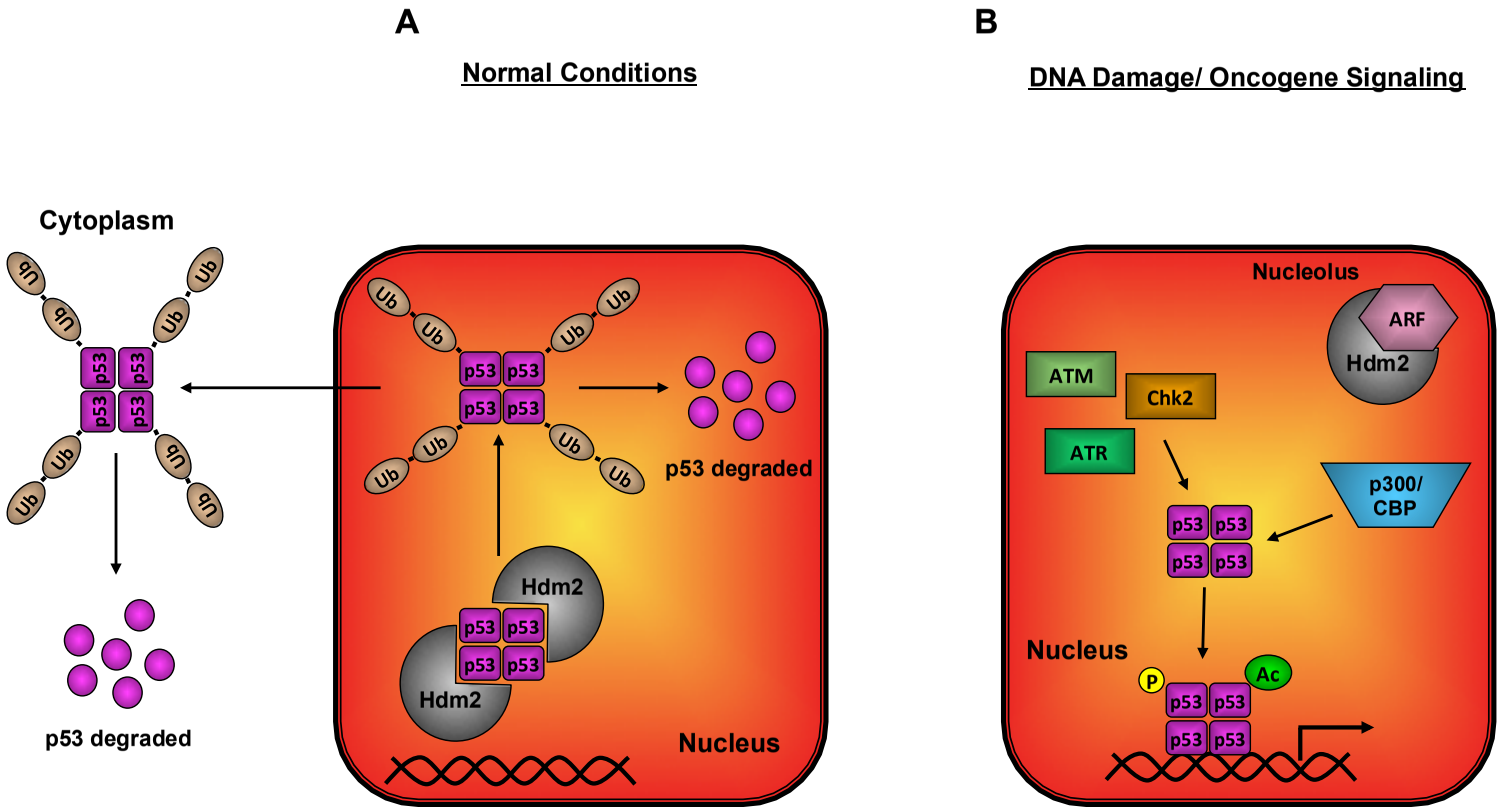

Figure 4-1. The p53 tumor suppressor pathway. (A) Under normal conditions, p53 levels are kept low. This is accomplished by the activity of Hdm2, which interacts with p53 and leads to its ubiquitination and degradation by either nuclear or cytoplasmic proteasomes. **(B)** Upon DNA damage, hypoxia, oncogene signaling, etc., p53 levels are stabilized and rapidly increase. Upon DNA damage, numerous kinases (ATM, ATR, Chk2 etc.) are activated and phosphorylate both p53 and Hdm2. As a result, the two proteins can no longer interact and p53 is not degraded. The interaction between p53 and Hdm2 is further inhibited by the tumor suppressor ARF, which interacts with and sequesters Hdm2 in the nucleolus. The remaining p53 can also be acetylated (Ac) by p300/CBP histone acetyltransferases, which leads to the recruitment of p53 to the promoters of genes involved in DNA damage, cell-cycle arrest and apoptosis.

2009; Collavin et al., 2010). When cells are stressed (e.g. exposed to UV or ionizing radiation, hypoxic environment or oncogenic insults, etc.), the levels of p53 are dramatically increased. Under these conditions, Hdm2-mediated degradation of p53 is blocked, mainly through the actions of the tumor suppressor ARF, which sequesters Hdm2 in the nucleolus and liberates p53 (Figure 4-1B). Furthermore, under these conditions, various kinases (e.g. ATM, Chk2, etc.) become activated and phosphorylate p53, which inhibits its interaction with Hdm2. The stable p53 is then able to regulate the expression of its target genes and promote cell-cycle arrest and apoptosis (Boehme and Blattner, 2009; Collavin et al., 2010; Wang and Jiang, 2012).

p53 protein stability is also regulated by several other post-translational modifications, including phosphorylation, acetylation and sumoylation (Boehme and Blattner, 2009; Collavin et al., 2010), and by its interactions with different cytoplasmic and nuclear proteins, which also alter its activity and function (Junttila and Evan, 2009; Menendez et al., 2009; Boehme and Blattner, 2009; Collavin et al., 2010; Goh et al., 2011). One such interacting protein that is known to activate the transcriptional activity of p53 is the tumor suppressor 14-3-3σ (Yang et al., 2003; Lee and Lozano, 2006).

4.1.3. 14-3-3 proteins

The 14-3-3 family of proteins are abundant acidic polypeptides that are found in all eukaryotic organisms. Currently seven 14-3-3 isoforms have

been identified, which can form homo- and heterodimers (Sluchanko and Gusev, 2012). These proteins have a wide variety of cellular functions, ranging from cell survival and apoptosis to cell cycle control, and are known to interact with a vast array of cellular proteins, including transcription factors, cytoskeletal proteins, biosynthetic enzymes and signaling molecules (Obsilova et al., 2008; Morrison, 2008; Van Heusden, 2009; Sluchanko and Gusev, 2012).

14-3-3σ (also called stratifin, encoded by the *SFN* gene) was originally characterized as a human mammary epithelial-specific (HME1) marker that was downregulated in mammary carcinoma cells (Prasad et al., 1992) and is the only 14-3-3 isoform induced by p53 upon DNA damage (Lodygin and Hermeking, 2006; Lee and Lozano, 2006). In accordance, 14-3-3σ has a well-documented tumor suppressor activity through its negative regulation of the cell cycle and positive regulation of p53 transcriptional activity. In addition, 14-3-3σ downregulation is observed in a variety of solid tumors including breast, squamous cell, lung, liver, ovarian and prostate cancer and this downregulation has been associated with increased tumor metastasis (Yang et al., 2003; Lodygin and Hermeking, 2006; Lee and Lozano, 2006).

4.1.4. Specific aim and summary of results

In this chapter, we have identified plakoglobin as a novel p53-interacting protein and examined the effect of plakoglobin expression on the levels of the p53 target gene 14-3-3σ. We show that plakoglobin

expression resulted in the induction of 14-3-3σ mRNA and protein. Furthermore, we show that plakoglobin interacted with p53 in squamous (SCC9-PG, A431) and mammary (MCF-10-2A, MCF-7) epithelial cell lines, that plakoglobin and p53 both associated with the 14-3-3σ gene (*SFN*) promoter, and that plakoglobin promoted p53 transcriptional activity. Our results show that plakoglobin interacts with p53 and suggest that together, plakoglobin and p53 control the expression of tumor/metastasis regulating genes, a function which also may account, in part, for plakoglobin's often-described tumor suppressor activity (Simcha et al., 1996; Pantel et al., 1998; Charpentier et al., 2000; Winn et al., 2002; Reiger-Christ et al., 2005; Yin et al., 2005; Kanazawa et al., 2008; Narkio-Makela et al., 2009; Todorovic et al., 2010; Aktary et al., 2010).

4.2. Results

4.2.1. 14-3-3σ levels are induced in SCC9-PG cells.

Our microarray experiments showed that the levels of several p53 target genes were altered in SCC9-PG cells compared to SCC9 cells. This result, while intriguing, was surprising since it has been reported that in SCC9 cells, p53 carries a mutation in its DNA-binding domain (Jung et al., 1992). Interestingly, *SFN*, the gene encoding the tumor suppressor 14-3-3σ, was upregulated 30-fold in SCC9-PG cells and was chosen for further investigation. To confirm the results of the microarray experiment, we began by performing RT-PCR and qRT-PCR experiments using mRNA from SCC9 and SCC9-PG cells and observed that while 14-3-3σ mRNA

was essentially undetectable in SCC9 cells, its levels were significantly upregulated in SCC9-PG cells (Figure 4-2B, C). Subsequent Western blot experiments verified that the expression of 14-3-3σ mRNA was accompanied by significant amounts of its protein in SCC9-PG cells, which was undetectable in SCC9 cells (Figure 4-2A). We repeated these experiments using different isolated clones of independent SCC9-PG transfectants and observed similar results with upregulation of both 14-3-3σ mRNA and protein levels (data not shown).

4.2.2. Plakoglobin interacts with p53 in SCC9-PG cells.

The results from Figure 4-2A-C showed that plakoglobin expression resulted in the induction of 14-3-3σ mRNA and protein, which suggested that plakoglobin may regulate the expression of 14-3-3σ. Since 14-3-3σ is a well-known target of p53 (Obsilova et al., 2008; Morrison, 2008; Van Heusden, 2009), we set out to determine whether plakoglobin and p53 interacted by performing reciprocal coimmunoprecipitation experiments using plakoglobin and p53 antibodies. Plakoglobin antibodies coprecipitated p53 in SCC9-PG cells and as expected, no interaction was observed in SCC9 cells due to their lack of endogenous plakoglobin expression (Figure 4-2D, IP: PG/IB: PG and p53). Reciprocal coimmunoprecipitation experiments using p53 antibodies coprecipitated plakoglobin from SCC9-PG cells but not SCC9 cells (Figure 4-2D, IP: p53/IB: PG and p53). Since plakoglobin and β-catenin have common interacting partners (Peifer et al., 1992; Zhurinsky et al., 2000b; Stemmler,

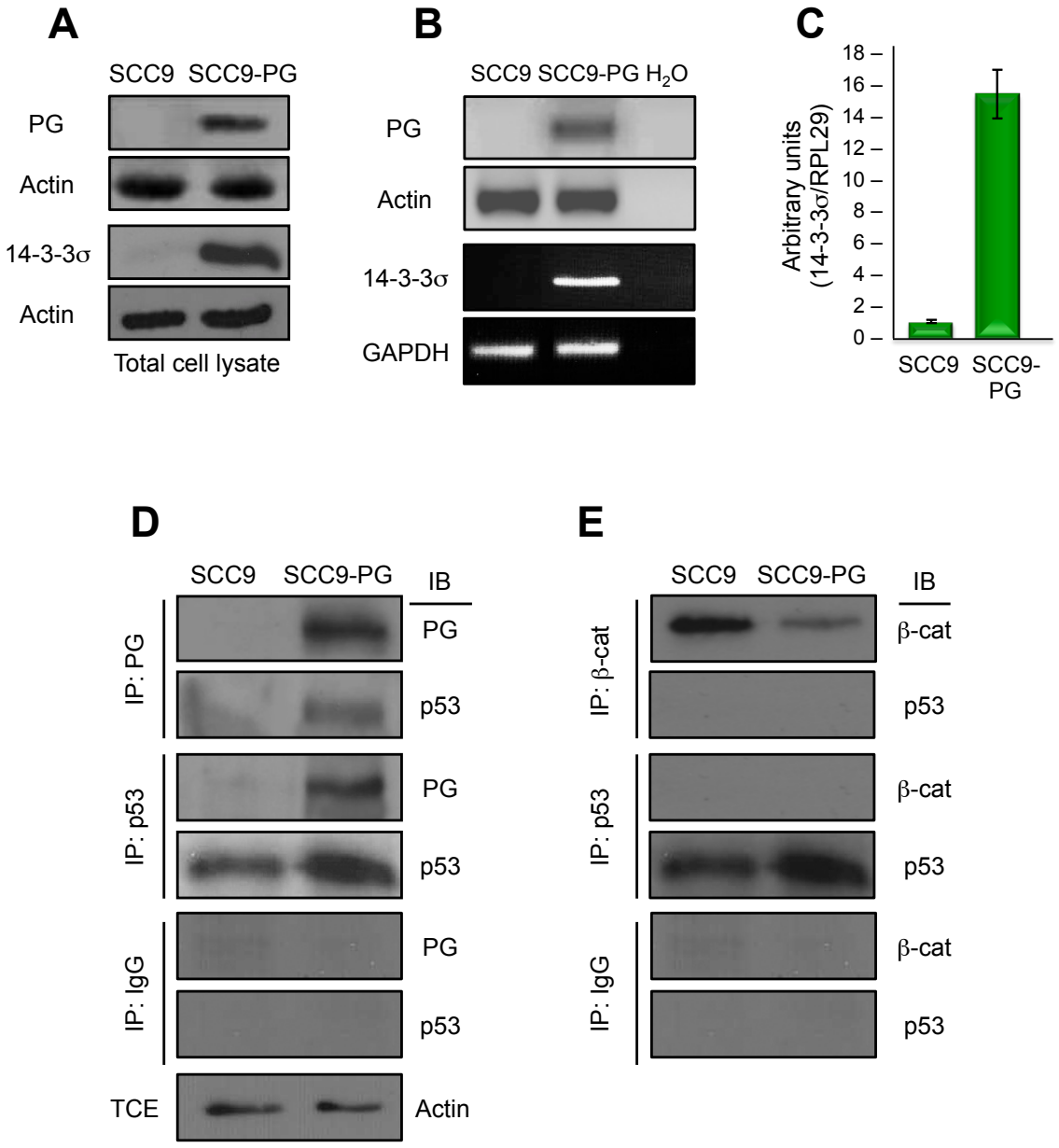

Figure 4-2. Plakoglobin interacts with p53 and its expression results in induction of 14-3-3σ mRNA and protein levels. (A) Equal amounts of total cellular proteins from SCC9 and SCC9-PG cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **plakoglobin**, **14-3-3σ** and **Actin**. **(B)** Total cellular RNA was isolated from SCC9 and SCC9-PG cells, reverse transcribed and processed for PCR using primers specific to **plakoglobin**, **Actin**, **14-3-3σ** and **GAPDH**. **(C)** Total cellular RNA was isolated from SCC9 and SCC9-PG cells, reverse transcribed and processed for quantitative PCR using primers specific to **14-3-3σ** and the ribosomal protein **RPL29**. The levels of 14-3-3σ mRNA were first normalized to the amount of RPL29 in each cell line and then to SCC9 cells. **(D-E)** SCC9 and SCC9-PG cell extracts were processed for immunoprecipitation using **(D) plakoglobin** or **p53** or **preimmune** or **(E) β-catenin** or **p53** or **preimmune** antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **(D**) **plakoglobin** and **p53** or **(E) β-catenin** and **p53**. β-cat, β-catenin; PG, plakoglobin.

2008), we also examined β-catenin-p53 interactions in SCC9 and SCC9-PG cells by reciprocal coimmunoprecipitation followed by immunoblotting using β-catenin and p53 antibodies. These experiments demonstrated that β-catenin did not interact with p53 (Figure 4-2E) and that the plakoglobin-p53 interaction is specific to these two proteins.

4.2.3. Plakoglobin and p53 interaction is not cell line specific.

To confirm that the observed plakoglobin-p53 interaction is not specific to SCC9-PG transfectants, we performed coimmunoprecipitation experiments using MCF-10-2A, a normal mammary epithelial cell line, MCF-7, a mammary carcinoma cell line, and A431, a vulvar carcinoma cell line, which all express plakoglobin and p53 (Setzer et al., 2004; Li et al., 2005; Kwok et al., 1994; Lam et al., 2009; Figure 4-3). We first confirmed that these cell lines expressed 14-3-3σ by Western blot analysis (Figure 4-3A, TCL). Next, reciprocal coimmunoprecipitation experiments using plakoglobin (Figure 4-3B), p53 (Figure 4-3C) and preimmune (Figure 4-3D) antibodies followed by immunoblotting demonstrated that plakoglobin and p53 were coprecipitated in non-epidermoid as well as epidermoid cell lines by plakoglobin and p53 but not preimmune antibodies. We also observed plakoglobin-p53 interactions in human and mouse fibroblast cell lines, supporting that these interactions were not cell type specific (data not shown). Finally, reciprocal coimmunoprecipitation experiments using β-catenin and p53 antibodies in the non-epidermoid cell lines showed that

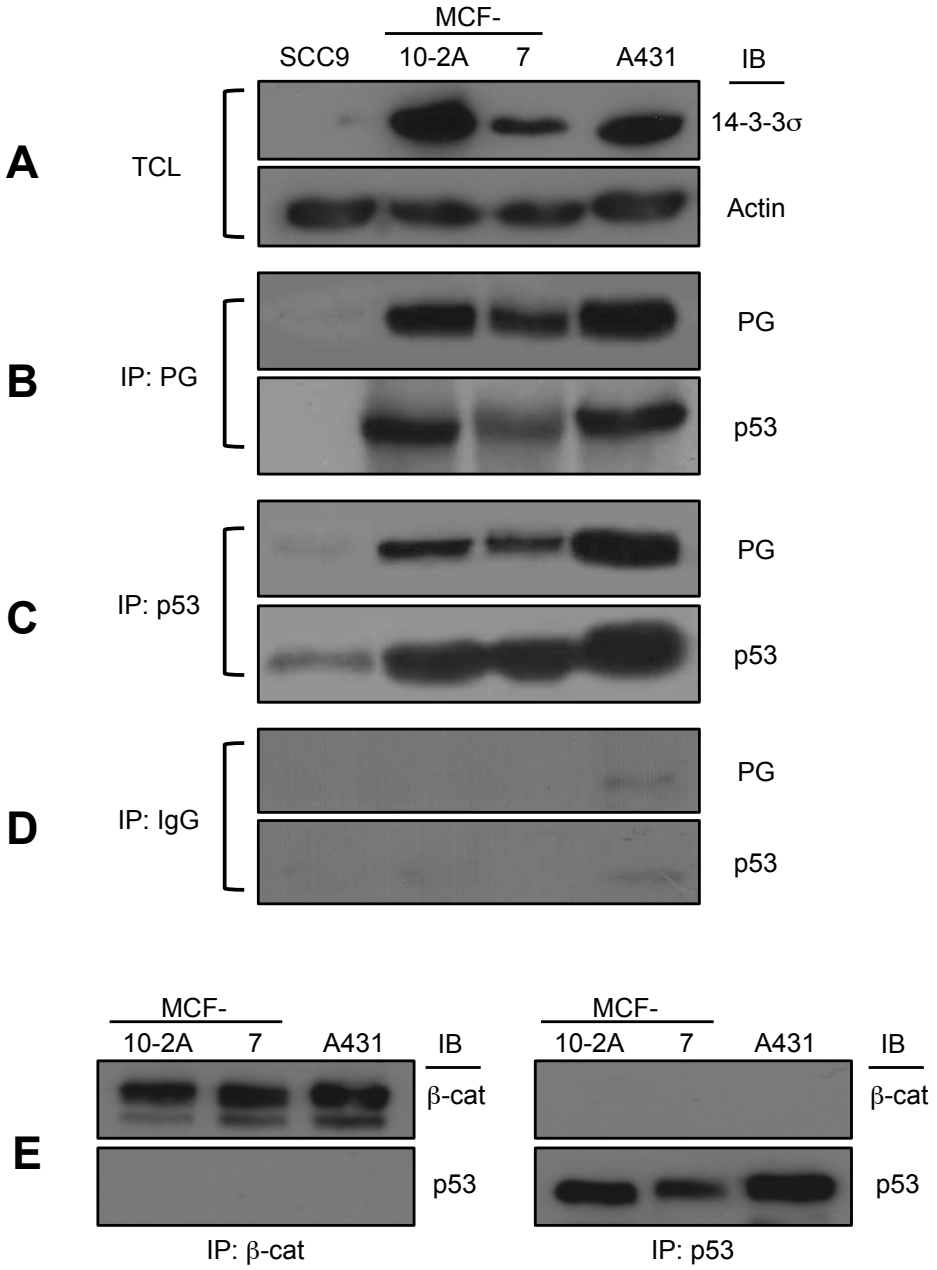

Figure 4-3. Plakoglobin interacts with p53 in different epithelial cell lines. (A) Equal amounts of total cellular proteins from SCC9, MCF-10-2A, MCF-7 and A431 cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **14-3-3σ** and **Actin**. **(B-D)** SCC9, MCF-10-2A, MCF-7 and A431 cell extracts were processed for immunoprecipitation using **(B) plakoglobin**, **(C) p53** or **(D) preimmune** antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **plakoglobin** and **p53**. **(E)** SCC9, MCF-10-2A, MCF-7 and A431 cell extracts were processed for reciprocal immunoprecipitation using **β-catenin** and **p53** antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **β-catenin** and **p53**. PG, plakoglobin. TCL, total cell lysate.

these two proteins did not interact, further demonstrating the specificity of the plakoglobin-p53 interaction (Figure 4-3E).

4.2.4. Plakoglobin and p53 interact in both the cytoplasm and nucleus.

Figures 1 and 2 demonstrated that plakoglobin interacted with p53, however, whether the interaction occurs in a specific subcellular compartment remained unclear. Since p53 functions as a transcription factor in the nucleus, we examined whether these two proteins interacted in the nucleus. To that end, we performed subcellular fractionation experiments in SCC9, SCC9-PG, A431, MCF-10-2A and MCF-7 cell lines and obtained distinct cytoplasmic and nuclear fractions that were processed for immunoprecipitation with p53 antibodies followed by Western blot with plakoglobin and p53 antibodies (Figure 4-4A, B). The results of these experiments confirmed the presence of p53 in both the cytoplasmic and nuclear fractions of all cell lines and the presence of plakoglobin in all cell lines except SCC9 (Figure 4-4A, B, IP: p53/IB: PG and p53). Furthermore, plakoglobin was coprecipitated with p53 in both the nuclear and cytoplasmic pools of protein in all cell lines except SCC9 (Figure 4-4A, B, IP: p53/IB: PG).

4.2.5. Plakoglobin and p53 associate with the 14-3-3σ gene promoter.

Taken together, the results so far showed that plakoglobin expression resulted in induction of 14-3-3σ mRNA and protein expression and that plakoglobin and p53 interacted in the nucleus as well as in the cytoplasm.

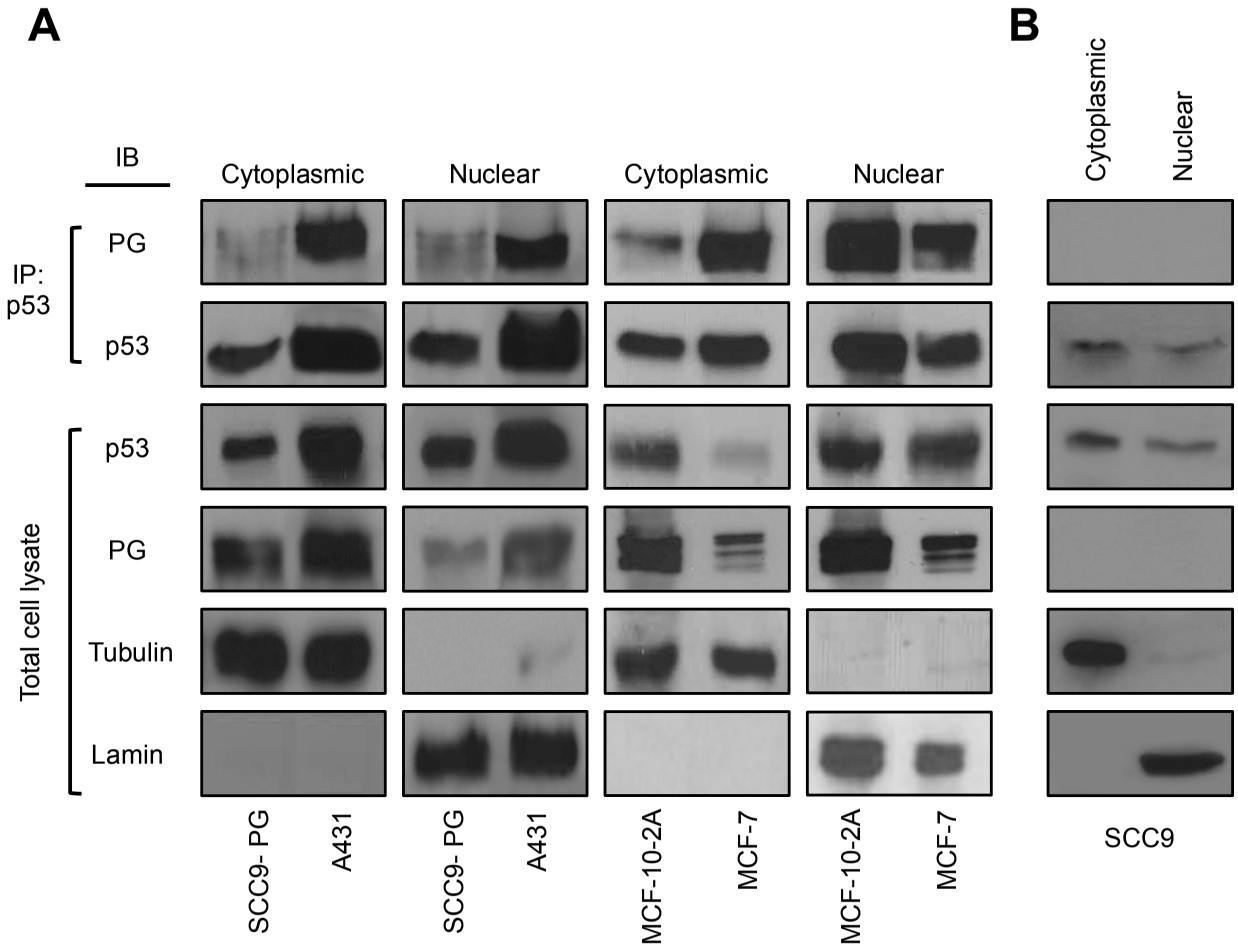

Figure 4-4. Plakoglobin and p53 interact in both the cytoplasm and nucleus. Cytoplasmic and nuclear extracts from **(A)** SCC9-PG, A431, MCF-10-2A, MCF-7 and **(B)** SCC9 cells were processed for immunoprecipitation using p53 antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to **p53** and **plakoglobin**. The purity of each fraction was assessed by immunoblotting with antibodies to **lamin** and **tubulin**. PG, plakoglobin.

These results suggested that plakoglobin and p53 may coordinately regulate gene expression. To examine this possibility, we performed chromatin immunoprecipitation (ChIP) experiments using extracts from SCC9 and SCC9-PG cells. We immunoprecipitated the chromatin with plakoglobin and p53 antibodies, respectively, and isolated the DNA associated with each protein. Subsequent PCR experiments using primers to detect the 14-3-3σ (*SFN*) promoter (Table 2-2) showed that both plakoglobin and p53 associated with the 14-3-3σ (*SFN*) promoter in SCC9-PG cells only (Figure 4-5A, SCC9 and SCC9-PG). ChIP with control IgG antibodies produced negative results.

Since we observed the plakoglobin-p53 interaction in MCF-10-2A, MCF-7 and A431 cells, we performed the ChIP experiments using chromatin from these cell lines. The results of these experiments were in agreement with the ChIP experiments from SCC9-PG cells: both plakoglobin and p53 associated with the 14-3-3σ promoter in these cell lines (Figure 4-5A, MCF-10-2A, MCF-7 and A431).

In addition, we performed ChIP experiments using β-catenin antibodies and chromatin from SCC9, SCC9-PG, MCF-10-2A and SW620 cells. The colon carcinoma cell line SW620 was used because it expresses p53 and transcriptionally active β-catenin (Lamy et al., 2010; El-Bahrawy et al., 2004; Li et al., 2007a). In agreement with the coimmunoprecipitation data, we did not observe an association between the 14-3-3σ (*SFN*) gene promoter and β-catenin in any cell line (Figure 4-

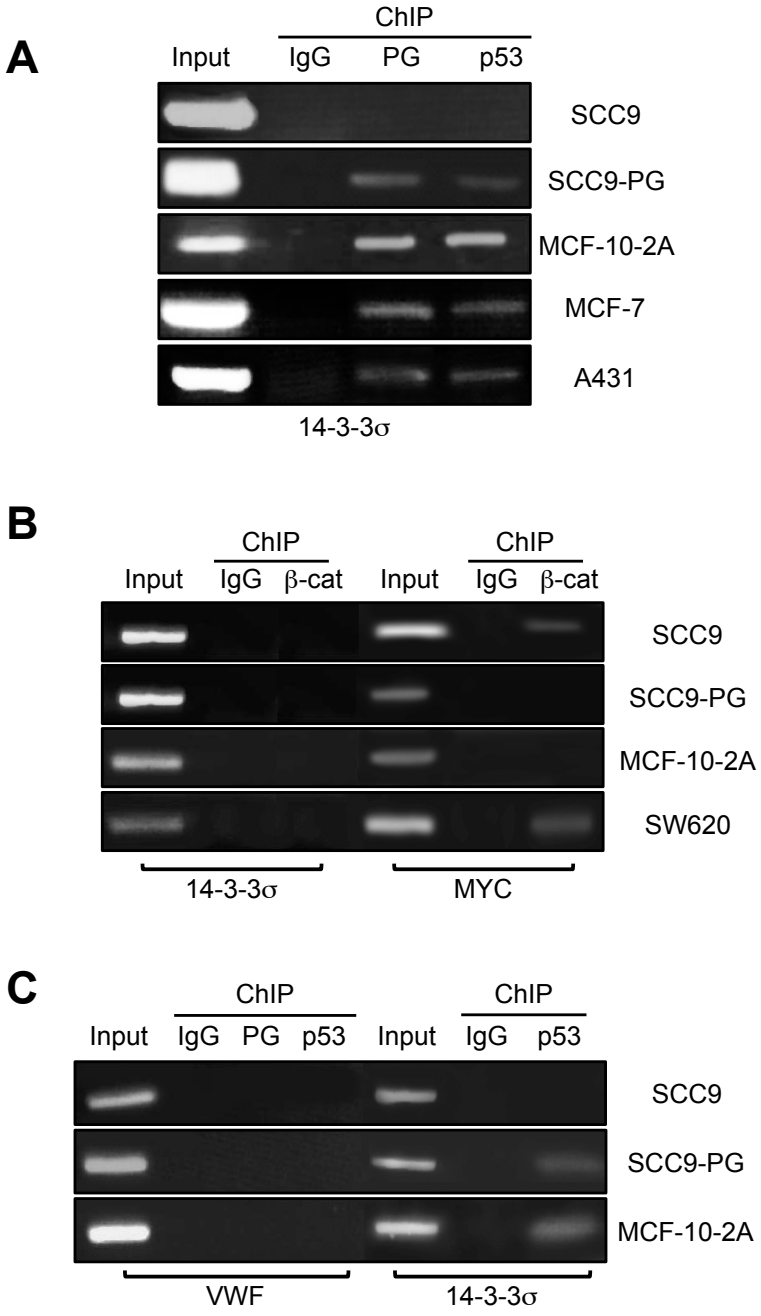

Figure 4-5. Plakoglobin and p53 associate with the 14-3-3σ gene promoter. SCC9, SCC9-PG, MCF-10-2A, MCF-7, A431 and SW620 cells were formaldehyde fixed and processed for chromatin immunoprecipitation. Following sonication, extracts were immunoprecipitated using control **IgG**, **plakoglobin**, **p53** or **β-catenin** antibodies. Following extensive washes, immunoprecipitated DNA was separated from the immune complexes and purified using standard DNA purification protocols. The purified DNA was then processed for PCR using **14-3-3σ (A) 14-3-3σ** and **MYC (B)** or **VWF** and **14-3-3σ (C)** primers. As positive control, total cellular DNA (Input) was amplified using the same primers. β-cat, β-catenin; PG, plakoglobin.

5B). As a positive control, we examined if β-catenin was associated with the *MYC* gene promoter, which is a well-known β-catenin target gene (He et al., 1998). β-catenin was associated with the *MYC* promoter in SCC9 and SW620 cells, but not in SCC9-PG and MCF-10-2A cells. Finally, plakoglobin and p53 ChIP samples were processed for PCR using primers to the *VWF* gene (negative control), and no amplification was observed, whereas the same p53 ChIP sample clearly amplified the 14-3-3σ (*SFN*) promoter in both SCC9-PG and MCF-10-2A cells (Figure 4-5C).

4.2.6. Plakoglobin binds the p53-consensus sequence in the 14-3-3σ promoter.

Since plakoglobin interacted with p53 and regulated the 14-3-3σ gene, we hypothesized that plakoglobin may bind to the p53 consensus sequence in the 14-3-3σ gene promoter, potentially through its interaction with p53. To verify this, we first performed electrophoretic mobility shift assays (EMSA) using MCF-10-2A nuclear extracts and a radioactively labeled probe that corresponded to the p53 consensus sequence (GTAGCATTAGCCCAGACATGTCC) in the 14-3-3σ gene promoter (Hermeking et al., 1997; Cai et al., 2009). MCF-10-2A cells were first used for these experiments because they express endogenous plakoglobin and wild-type p53 (Li et al., 2005; Lam et al., 2009). The results showed the formation of a distinct complex (Figure 4-6A, Lane 2) that was inhibited by the addition of a specific oligonucleotide competitor (unlabeled probe in 50-fold excess; Figure 4-6A, Lane 6) but not by a non-specific

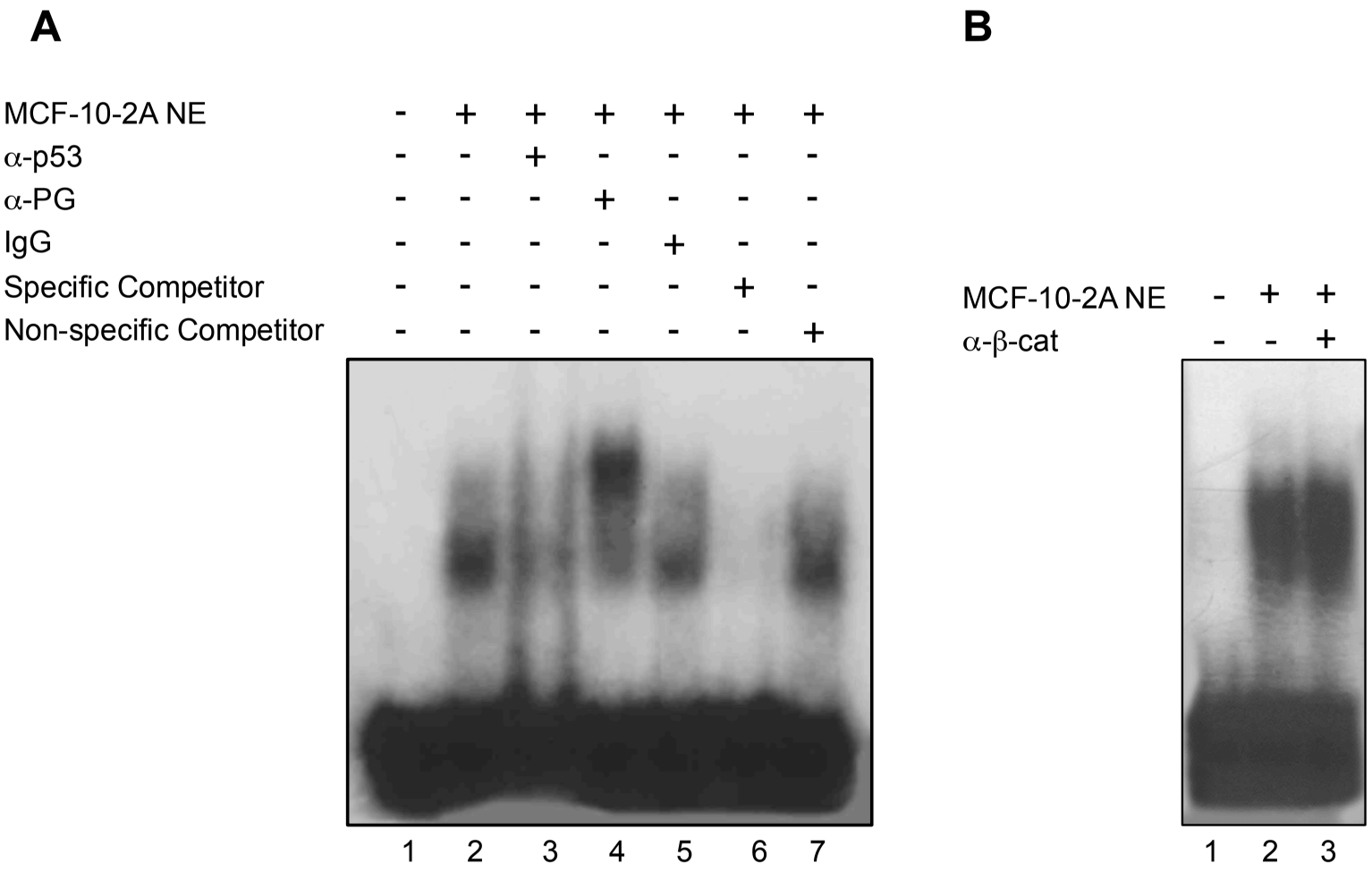

Figure 4-6. Plakoglobin and p53 bind to the p53 consensus sequence in the 14-3-3σ gene promoter in MCF-10-2A cells. Nuclear extracts from MCF-10-2A cells were incubated in the presence of radioactively labeled double stranded oligonucleotide probes corresponding to the p53 consensus sequence in the 14-3-3σ gene promoter. To confirm the binding of **(A) plakoglobin** and **p53** or **(B) β-catenin** to the probe, antibodies corresponding to each protein were added to the reaction mixtures, which were then run on a 5% non-denaturing polyacrylamide gel and processed for autoradiography. NE, Nuclear Extract. PG, plakoglobin.

oligonucleotide (corresponding to the *NFY* gene; Figure 4-6A, Lane 7). The addition of p53 antibodies to the reaction mixture resulted in a reduction in specific DNA-protein complex formation, as demonstrated by a decrease in signal intensity (Figure 4-6A, Lane 3). When plakoglobin antibodies were added to the reaction mixture, a supershift was observed (Figure 4-6A, Lane 4), whereas the addition of IgG to the reaction mixtures had no effect on the band shift (Figure 4-6A, Lane 5). In contrast to plakoglobin antibodies, when β-catenin antibodies were added to the reaction mixtures, no effect was observed (Figure 4-6B, Lane 3).

Similarly, in Figure 4-7, EMSA experiments using nuclear extracts from SCC9 and SCC9-PG cells and the same radioactively labeled probe resulted in the formation of a distinct complex (Figure 4-7, Lane 2) that was inhibited by the addition of a specific competitor but not by a non-specific oligonucleotide (Figure 4-7, Lanes 7 and 8). When plakoglobin antibodies were added to the reaction mixtures, a supershift was observed in SCC9-PG but not in SCC9 cells (Figure 4-7, Lane 3). The addition of p53 antibodies to the reaction mixtures containing the SCC9-PG, but not SCC9 nuclear extracts resulted in a reduction in specific DNA-protein complex formation (Figure 4-7, Lane 4). The addition of β-catenin antibodies or IgG to the reaction mixtures had no effect on the band shift in either cell line (Figure 4-7, Lanes 5 and 6).

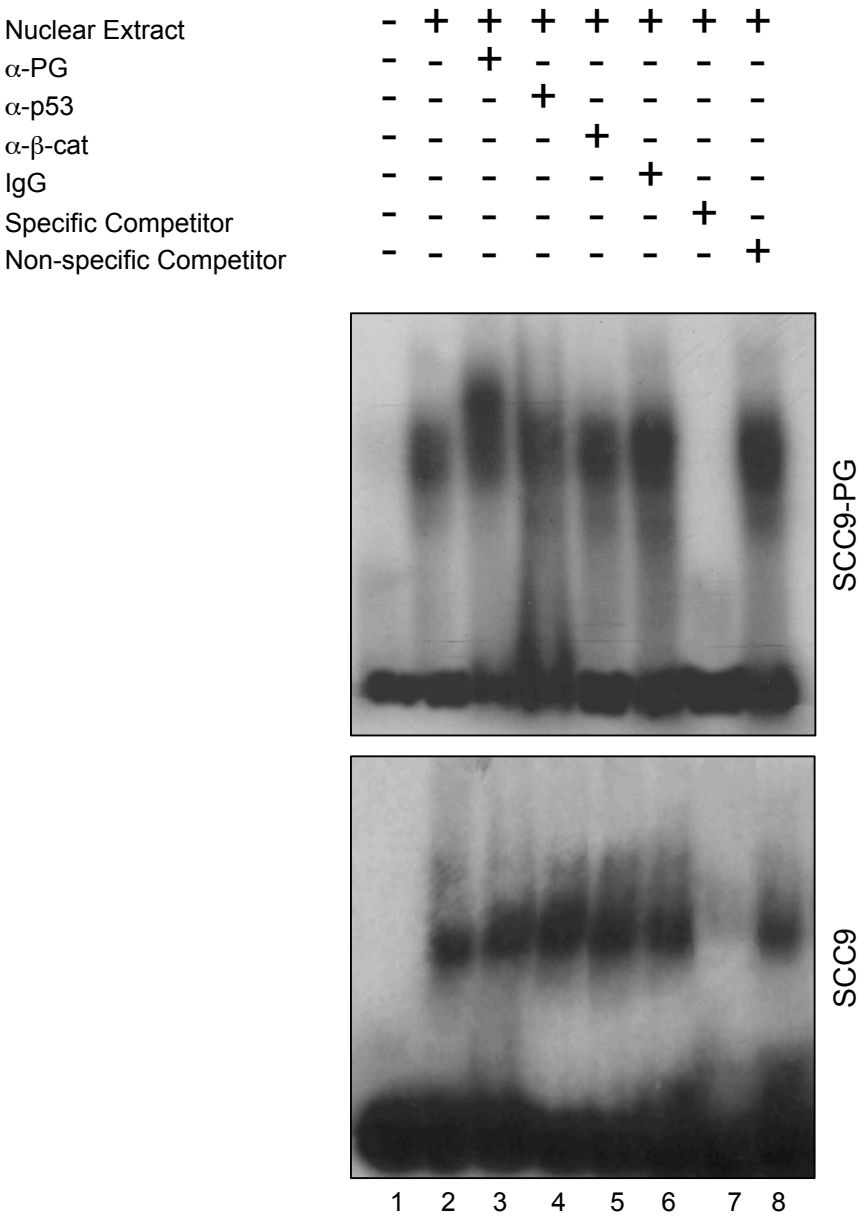

Figure 4-7. Plakoglobin and p53 bind to the p53 consensus sequence in the 14-3-3σ gene promoter in SCC9-PG cells. Nuclear extracts from SCC9 and SCC9-PG cells were incubated in the presence of radioactively labeled double stranded oligonucleotide probes corresponding to the p53 consensus sequence in the 14-3-3σ gene promoter. To confirm the binding of **plakoglobin**, **p53** or **β-catenin** to the probe, antibodies corresponding to each protein were added to the reaction mixtures, which were then run on a 5% non-denaturing polyacrylamide gel and processed for autoradiography. NE, Nuclear Extract. PG, plakoglobin.

4.2.7. Plakoglobin promotes p53 transcriptional activity.

The results from the ChIP experiments revealed that p53 associated with the 14-3-3σ gene promoter in SCC9-PG but not in SCC9 cells, suggesting that plakoglobin may play a role in regulating the transcriptional activity of p53. To investigate this further, we performed reporter gene assays, by transfecting SCC9 and SCC9-PG cells with constructs encoding the luciferase gene downstream of the wild type or mutant p53-binding sequence in the 14-3-3σ gene promoter (Hermeking et al., 1997; Table 2-4). SCC9 cells transfected with the control vector, wild-type p53 or mutant p53 containing plasmids showed minimal luciferase activity regardless of the plasmid (Figure 4-8A, SCC9). However, in SCC9-PG cells, while the luciferase activity of the control was similar to SCC9 cells, it was significantly increased when these cells were transfected with either the wild-type (2.2-fold) or mutant (2.9-fold) plasmids, respectively (Figure 4-8A, SCC9-PG). This unexpected result suggested that perhaps regulation of the 14-3-3σ gene in SCC9-PG cells is independent of p53, since luciferase activity was induced from the mutant p53 sequence, to which p53 should not bind. To examine this possibility, we knocked down p53 in SCC9-PG cells using siRNA and examined the effect on luciferase reporter activity. We observed that knock down of p53 resulted in decreased 14-3-3σ protein in SCC9-PG cells and in almost a complete loss of luciferase reporter activity from both the wild-type and mutant

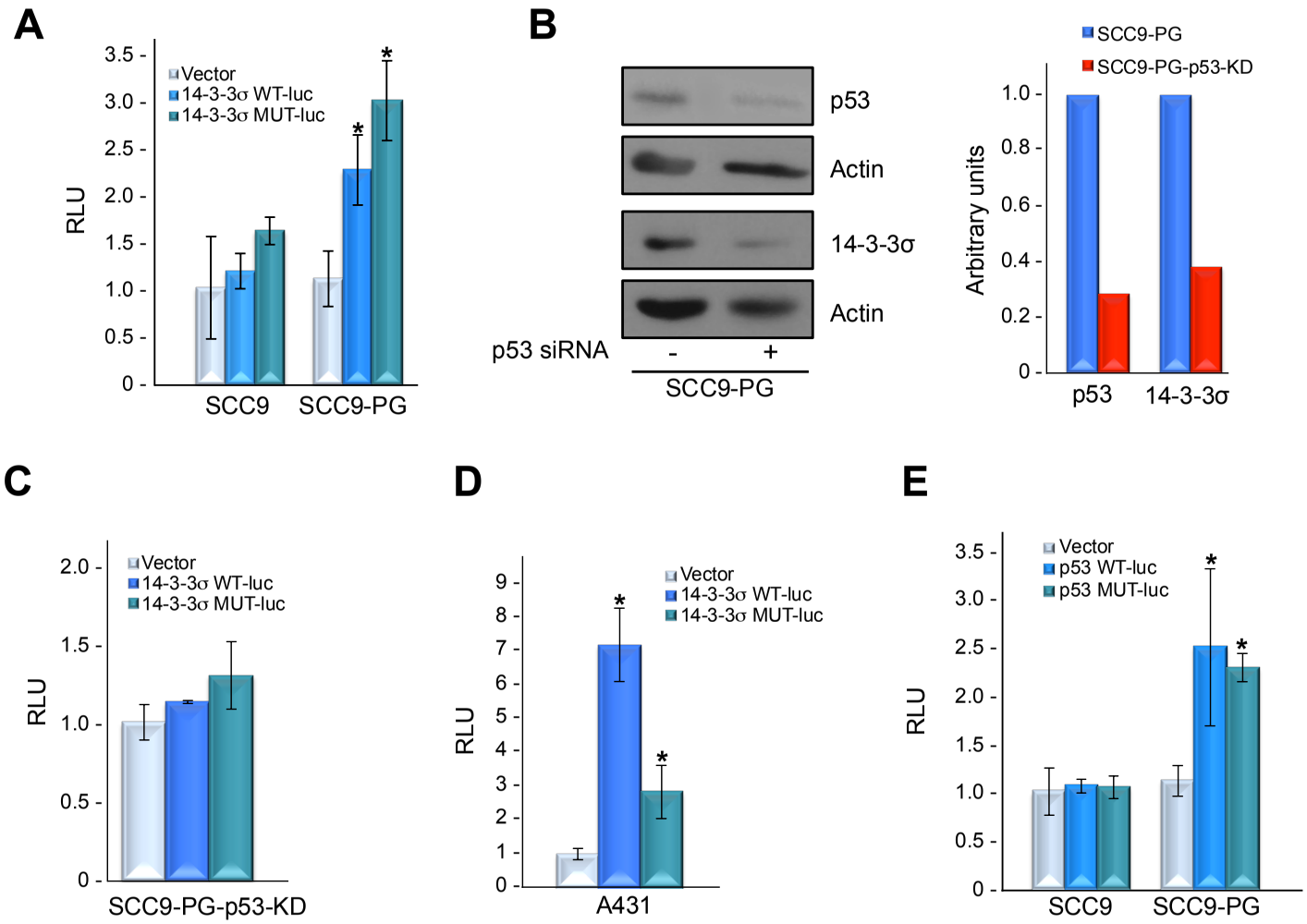

Figure 4-8. Plakoglobin regulates mutant p53 transcriptional activity. (A) SCC9 and SCC9-PG cells were transfected with luciferase reporter constructs under the control of the p53-binding sequence from the 14-3-3σ (*SFN*) gene. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector **(Vector)**, wild-type **(14-3-3σ WT-luc)** and mutant **(14-3-3σ MUT-luc)** p53-binding sequences were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. **(B)** Western blot analysis and quantitation of **p53** and **14-3-3σ** levels in SCC9-PG cells following p53 knock down using siRNA. **(C-D)** SCC9-PG-p53-KD **(C)** and A431 **(D)** cells were transfected with luciferase reporter constructs as described in **(A)**. **(E)** SCC9 and SCC9-PG cells were transfected with luciferase reporter constructs under the control of a consensus p53 binding sequence and luciferase activities from the vector **(Vector)**, wild-type **(consensus p53 WT-luc)** and mutant **(consensus p53 MUT-luc)** p53 binding sequences were determined as described in **(A)** (*$p < 0.01$, as compared to SCC9 or A431 cells transfected with the vector plasmid). PG, plakoglobin. RLU, Relative Light Units.

plasmids (Figure 4-8B, C), suggesting that mutant p53 could bind to both the wild type and mutant response element and was involved in regulating 14-3-3σ gene expression in SCC9-PG cells. That mutant p53 proteins have the potential to activate gene expression from both wild-type and mutant p53 response elements is not cell specific and has been suggested previously (Muller and Vousden, 2013 and references therein). To this end, we verified that the induction of luciferase activity from the mutant 14-3-3σ construct was not specific to SCC9-PG cells by performing the luciferase reporter assays in A431 cells, which express another p53 mutant (Kwok et al., 1994). The results of these assays also showed a significant increase in the luciferase activity from both the wild-type (~7-fold) and mutant (~3-fold) constructs (Figure 4-8D) in these cells.

To verify that plakoglobin's regulation of p53 transcriptional activity was not specific to the 14-3-3σ gene, we performed similar luciferase assays using luciferase constructs downstream of a wild-type and mutated consensus p53-binding sequence (Kern et al., 1992). The results showed that luciferase activity was induced from both the wild-type and mutant consensus p53 plasmids in SCC9-PG cells, whereas no induction was observed in SCC9 cells (Figure 4-8E).

We further performed the same experiments in MCF-7 cells, which express wild-type p53 (Li et al., 2005). We argued that since SCC9-PG and A431 cells express mutant p53, these mutant proteins may be able to induce luciferase activity from both the wild-type and mutant p53

promoters. On the other hand, activation from the mutant promoter would not be expected in MCF-7 cells with wild-type p53 expression. Additionally, to further confirm the role of plakoglobin in regulating the transcriptional activity of wild-type p53, we knocked down plakoglobin in MCF-7 cells using shRNA (Figure 4-9A) and assessed the effects on luciferase activity. When MCF-7 cells were transfected with the same constructs, we observed a significant induction of luciferase activity (nearly 300-fold) when the wild-type construct was transfected, whereas the control and mutant constructs showed no activity (Figure 4-9B). In MCF-7 cells, knock down of plakoglobin resulted in a significant (~21-fold) decrease in luciferase activity from the wild-type construct (Figure 4-9B, MCF-7-shPG), demonstrating that p53 transcriptional activity was enhanced in the presence of plakoglobin.

Luciferase assays using the reporter constructs downstream of the wild-type and mutated consensus p53-binding sequences demonstrated that luciferase activity was significantly induced only from the wild-type plasmid in MCF-7 cells (Figure 4-9C, MCF-7). Finally, knock down of plakoglobin in MCF-7 cells (MCF-7-shPG) resulted in significantly (~7-fold) decreased luciferase activity from the wild-type consensus p53 plasmid in these cells (Figure 4-9C, MCF-7-shPG).

4.3. Discussion

Our microarray studies identified several p53-target genes whose levels were altered upon plakoglobin expression in SCC9 cells. Among these

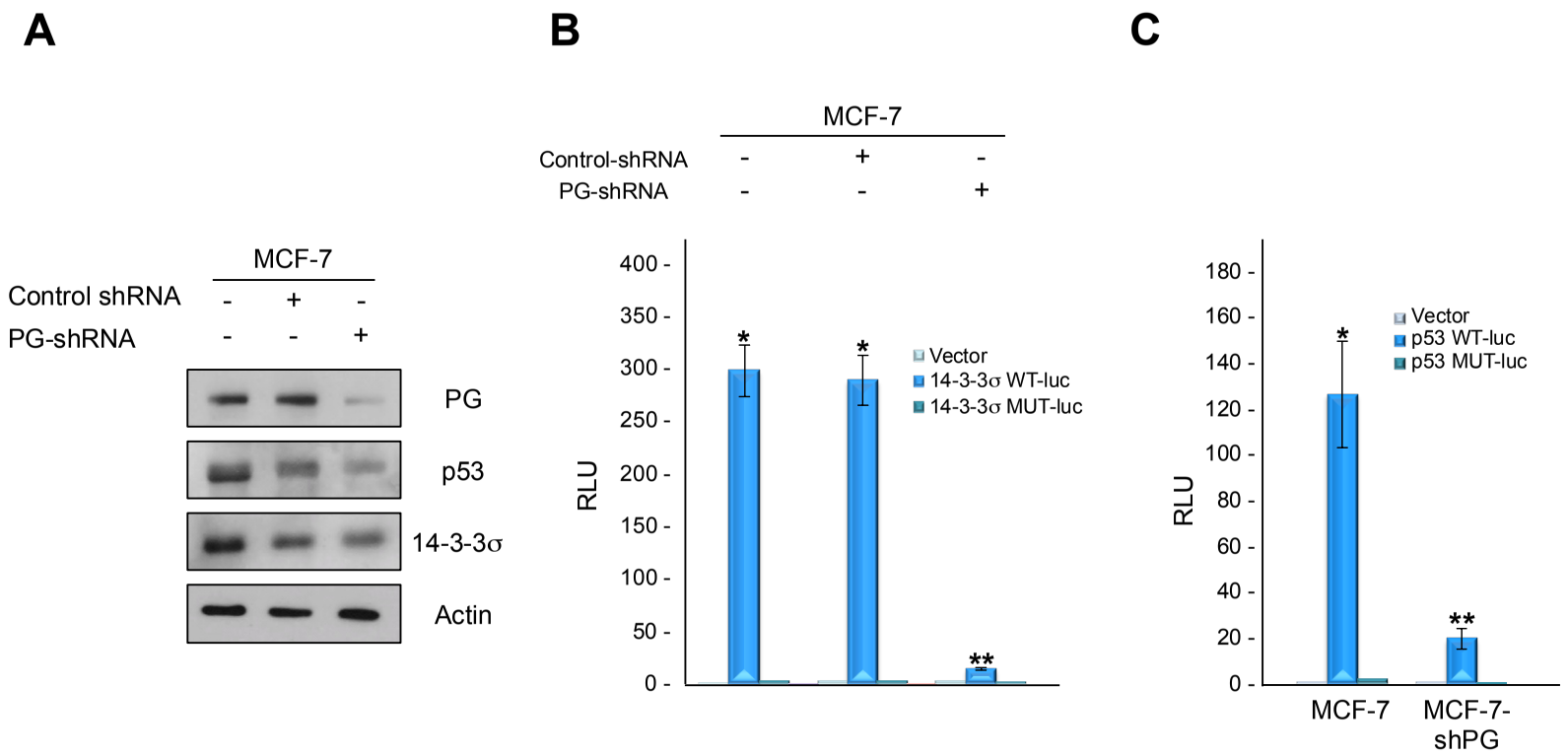

Figure 4-9. Plakoglobin regulates wild-type p53 transcriptional activity. (A) MCF-7 cells were transfected with plakoglobin-specific or scrambled control shRNA constructs. Total cellular extracts from all cell lines were subjected to Western blot analysis with antibodies to **plakoglobin**, **p53**, **14-3-3σ** and **Actin**. **(B)** MCF-7, MCF-7-shControl and MCF-7-shPG cells were transfected with luciferase reporter constructs under the control of the p53-binding sequence from the 14-3-3σ (*SFN*) gene. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector **(Vector)**, wild-type **(14-3-3σ WT-luc)** and mutant **(14-3-3σ MUT-luc)** p53-binding sequences were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. **(C)** MCF-7 and MCF-7-shPG cells were transfected with luciferase reporter constructs under the control of a consensus p53 binding sequence and luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector **(Vector)**, wild-type **(consensus p53 WT-luc)** and mutant **(consensus p53 MUT-luc)** p53 binding sequences were determined as described in **(A)** (*$p < 0.01$, as compared to MCF-7 cells transfected with the vector plasmid; **$p<0.01$, as compared to MCF-7 cells transfected with the WT plasmid). PG, plakoglobin. RLU, Relative Light Units.

genes was the tumor suppressor 14-3-3σ. We chose to focus on 14-3-3σ because a) its mRNA levels were increased over 30-fold in SCC9-PG cells, one of the most notable increases in any of the identified p53-target genes, b) it is a well-documented tumor and metastasis suppressor (Lodygin and Hermeking, 2006; Lee and Lozano, 2006; Yi et al., 2009), c) members of the 14-3-3 family are known to interact with a wide range of cellular partners and regulate several biological processes (Obsilova et al., 2008; Morrison, 2008; Van Heusden, 2009), d) 14-3-3σ itself has been shown to interact with plakophilin, a component of the desmosomal plaque, which also contains plakoglobin (Benzinger et al., 2005) and e) more recently it has been shown that various 14-3-3 proteins can regulate the Wnt pathway and β-catenin signaling (Li et al., 2008), functionally linking these proteins to catenin proteins. Furthermore, we and others have shown that plakoglobin also regulates β-catenin subcellular localization and in turn its transcriptional activity (Salomon et al., 1997; Klymkowsky et al., 1999; Zhurinsky et al., 2000a; Li et al., 2007a), thereby suggesting that both plakoglobin and 14-3-3σ act to regulate the Wnt signaling pathway in similar, albeit not identical ways.

The change in the expression of several p53-target genes, including 14-3-3σ, in SCC9-PG cells suggested that plakoglobin participated in p53-mediated regulation of gene expression. Coimmunoprecipitation experiments determined that plakoglobin and p53 interacted with one another in both the nuclear and cytoplasmic pool of proteins. It is well

documented that p53 interacting proteins play important roles in regulating its stability and function (Boehme and Blattner, 2009; Collavin et al., 2010). By identifying plakoglobin as a p53 interacting partner, we are, to the best of our knowledge, the first to show that a catenin protein interacts with p53. Although we did not observe an interaction between β-catenin and p53, a relationship between them exists whereby p53 regulates the stability of β-catenin, via the upregulation of the ubiquitin ligase Siah-1, which in turn degrades β-catenin. Furthermore, β-catenin overexpression has been shown to increase p53 levels via upregulation of p14/19 ARF, which sequesters Hdm2 and leads to increased p53 protein stability (Damalas et al., 1999; 2001; Harris and Levine, 2005).

The observation that a number of p53 target genes, including *SFN*, were upregulated in SCC9-PG cells and that plakoglobin and p53 interacted in both the cytoplasm and nucleus suggested that perhaps these proteins regulate gene expression concurrently. ChIP and EMSA experiments showed that plakoglobin and p53 were both associated with the 14-3-3σ gene promoter (Figures 4-5, 4-6, 4-7). These results suggest that plakoglobin and p53 are part of a transcriptional complex that regulates gene expression, which is novel when considering that reports linking plakoglobin to the regulation of gene expression are limited. Interestingly, previous studies implicating plakoglobin in the regulation of gene expression have shown that plakoglobin does so in conjunction with the TCF/LEF transcription factors (Simcha et al., 1999; Kolligs et al., 2000;

Zhurinsky et al., 2000a; Li et al., 2007a; Williamson et al., 2006). However, several of these studies have demonstrated that the plakoglobin-TCF complex is inefficient in binding to DNA (Simcha et al., 1999; Zhurinsky et al., 2000a; Li et al., 2007a; Kolligs et al., 2000) and suggest that plakoglobin's ability to regulate gene expression may have more to do with its modulation of the signaling activity of β-catenin than with its own independent function. More recently, it has been shown that in addition to regulating the signaling activity of β-catenin itself, plakoglobin is also capable of regulating β-catenin oncogenic signaling by interacting with and promoting the nuclear export of the transcription factor SOX4, which interacts with β-catenin and promotes its transcriptional activity (Sinner et al., 2007; Scharer et al., 2009; Lai et al., 2011). However, a more direct mechanism of plakoglobin-mediated regulation of gene expression has been documented, as it has been shown that plakoglobin, in conjunction with LEF-1, is a repressor of oncogenic Myc, and that the loss of this repression is observed in pemphigus vulgaris (Williamson et al., 2006).

The importance of our result lies in the fact that plakoglobin appears to be regulating gene expression through its association with non-TCF/LEF transcription factors, in this case, p53. Indeed, the 14-3-3σ gene promoter has no identified TCF/LEF binding sites and is not known to be a Wnt/β-catenin target gene. In accordance, we previously showed that overexpressed/high levels of plakoglobin, by modulating the signaling activity of β-catenin, regulated the expression of the *BCL2* gene in SCC9-

PG cells and this regulation did not involve TCF (Li et al., 2007a). This suggests that plakoglobin can regulate gene expression and more importantly tumorigenesis and metastasis independent of TCF. Similarly, a previous report showed that plakoglobin may regulate the expression of the *PML* gene independent of TCF/LEF (Shtutman et al., 2002). Interestingly, the *PML* gene has recently been shown to be a p53-target gene (de Stanchina et al., 2004), which further supports the notion that plakoglobin may regulate gene expression in conjunction with p53.

The experiments described in this study were all performed in the absence of cellular stressors such as staurosporine treatment or DNA damage. As such, it appears that plakoglobin and p53 regulate gene expression under steady state cellular conditions, implying that this activity is a basic function within cells. The disruption of this gene regulation function (as per the loss of plakoglobin expression in SCC9 cells) may contribute to tumorigenesis. In agreement, we observed plakoglobin-p53 interactions in various epithelial and fibroblast cell lines that we examined, suggesting that this interaction occurs in cell lines expressing both proteins (either endogenously or exogenously). Furthermore, we observed that plakoglobin and p53 interacted in the cytoplasm as well as the nucleus (Figure 4-4), which suggests that the two proteins may associate with one another in the cytoplasm and then translocate into the nucleus. In addition, p53 is known to play non-genomic functions in the cytoplasm (particularly at the mitochondria; Mihara et al., 2003; Vaseva and Moll,

2009; Lindenboim et al., 2011) and since plakoglobin associated with p53 in the cytoplasm, it is conceivable that plakoglobin may also play some role in the non-genomic functions of p53.

Previous studies have identified p53 as being mutated in the DNA binding domains in SCC9 and A431 cells (Jung et al., 1992; Kwok et al., 1994). Jung et al. (1992) showed that the *TP53* gene in SCC9 cells contains a 32-base pair deletion starting at codon 274 which results in a premature stop codon and a truncated protein, whereas Kwok et al. (1994) showed that p53 contains a point mutation (R273H) in its DNA binding domain in A431 cells. However, we observed a p53 protein in SCC9 cells that appeared approximately 50 kDa and that accumulated in the nucleus (Figure 4-4). This discrepancy is most likely the result of the heterogeneity of the original isolated SCC9 cell line. We addressed this possibility by sequencing and characterizing the *TP53* gene in our SCC9 cells. We observed not only the expected 32-base pair deletion, but also a number of single base pair deletions spanning nucleotides 906-1162 (Table 4-1). These deletions have eliminated the expected premature stop codon and generated a p53 protein slightly smaller than wild-type p53 in which the p53 protein sequence contains stretches of wild-type p53 amino acids interspersed with sequences unrelated to p53. We further characterized this mutant protein by expressing the p53 cDNA clone isolated from SCC9 cells in the p53-null H1299 cell line (Lin and Chang, 1996; Wu et al., 2011). Following its expression, the mutant p53 protein accumulated in

Table 4-1. p53 status in various cell lines used.

Cell Line	p53 Status	p53 Mutation	References
MCF-10-2A	Wild-type	-	Li et al., 2005
MCF-7	Wild-type	-	Li et al., 2005
SCC9	Mutant	A 32-bp deletion in DNA binding domain (starting at codon 274)	Jung et al., 1992
	Mutant	A 32-bp deletion in DNA binding domain (starting at codon 274) with further deletions at bp 906, 1039, 1062, 1076, 1083, 1090, 1098, 1120, 1121, 1152, 1153, and 1162	Current Study
A431	Mutant	R273H	Kwok et al., 1994

these cells, localized to both the cytoplasm and the nucleus and 14-3-3σ protein levels were increased. ChIP experiments showed that the mutant p53 protein was associated with the 14-3-3σ promoter (Figure 4-10). Collectively, these results suggested that this mutant p53 was capable of regulating 14-3-3σ (*SFN*) expression.

The ChIP results suggested that despite their p53 mutations, plakoglobin and the mutated p53 protein still associated with the 14-3-3σ gene promoter in SCC9-PG and A431 cells (Figure 4-5). This result, while unexpected, is not unparalleled, as a number of studies have shown that mutant p53 protein is capable of binding to its target gene sequences and regulating their expression (Pan and Haines, 2000; O'Farrell et al., 2004; Weisz et al., 2007; Chandrachud and Gal; 2009; Perez et al., 2010; Rasti et al., 2012). Since the mutant p53 did not associate with the 14-3-3σ promoter in the absence of plakoglobin (SCC9 cells), this suggests a role for plakoglobin in associating p53 with its target gene promoter(s). In agreement, luciferase reporter assays in SCC9 and SCC9-PG cells showed that the transcriptional activity of p53 was stimulated upon plakoglobin expression, as SCC9 cells showed minimal luciferase activity, whereas luciferase activity was significantly enhanced in SCC9-PG cells (Figure 4-8A, E). However, while we observed 2-3 fold increases in luciferase activity in SCC9-PG cells, the qRT-PCR results suggested a larger increase in 14-3-3σ gene expression in these same cells. This

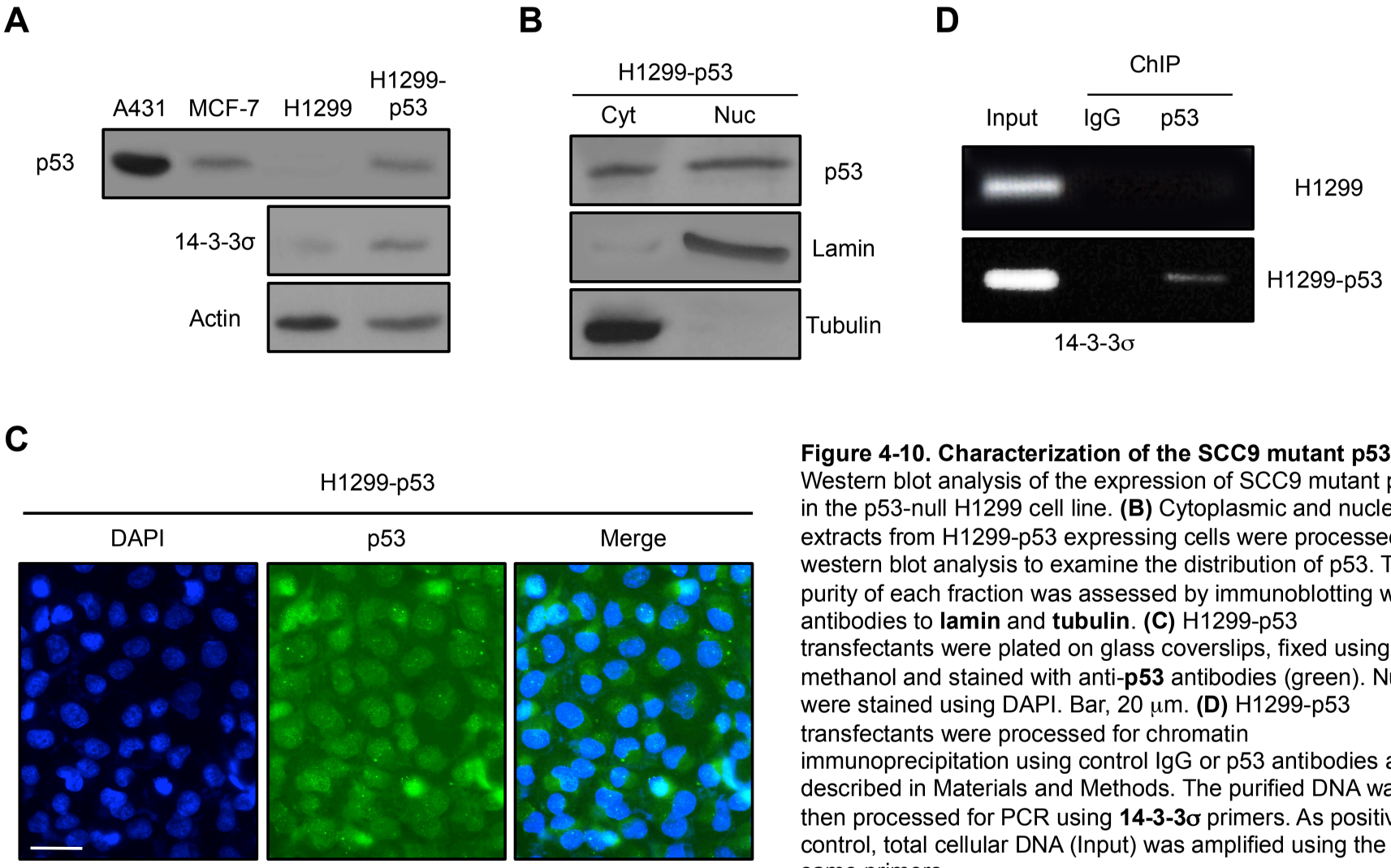

Figure 4-10. Characterization of the SCC9 mutant p53. (A) Western blot analysis of the expression of SCC9 mutant p53 in the p53-null H1299 cell line. **(B)** Cytoplasmic and nuclear extracts from H1299-p53 expressing cells were processed for western blot analysis to examine the distribution of p53. The purity of each fraction was assessed by immunoblotting with antibodies to **lamin** and **tubulin**. **(C)** H1299-p53 transfectants were plated on glass coverslips, fixed using methanol and stained with anti-**p53** antibodies (green). Nuclei were stained using DAPI. Bar, 20 μm. **(D)** H1299-p53 transfectants were processed for chromatin immunoprecipitation using control IgG or p53 antibodies as described in Materials and Methods. The purified DNA was then processed for PCR using **14-3-3σ** primers. As positive control, total cellular DNA (Input) was amplified using the same primers.

discrepancy may be explained by the involvement of other factors that partake in regulating 14-3-3σ gene expression (e.g. p63, p73, BRCA1; Danilov et al., 2011; Sang et al., 2006; Aprelikova et al., 2001). Similarly, while knock down of plakoglobin in MCF-7 cells resulted in a 21-fold decrease in luciferase activity from the 14-3-3σ promoter, 14-3-3σ protein levels were decreased by 2-fold in these same cells. This may be due once again to the involvement of other proteins that regulate 14-3-3σ expression. In addition, while MCF-7 shPG transfectants had decreased p53 levels, p53 protein was still present in these cells and therefore may have been able to promote 14-3-3σ expression.

Knock down of p53 in SCC9-PG cells also resulted in decreased luciferase activity from both the wild-type and mutant 14-3-3σ promoter constructs (Figure 4-8C), suggesting that the mutant p53 protein in these cells was directly involved in regulating 14-3-3σ gene expression. Furthermore, the decreased luciferase activity from the mutant promoter construct is further confirmation that the mutant p53 protein can promote gene expression from the mutant promoter. However, while knock down of p53 almost completely abrogated luciferase activity from the reporter constructs, minimal amounts of 14-3-3σ protein remained (Figure 4-8B). Collectively, our data suggest that p53 and plakoglobin are the primary regulators of 14-3-3σ expression although it is possible that other factors may also be involved.

The results from the luciferase assays suggested that in addition to wild-type p53-binding sequences, the mutant p53 protein in SCC9-PG cells could bind to and activate gene expression under the control of a mutant p53-binding sequence. However, the activation from mutant p53-binding sequences required the presence of plakoglobin, since minimal luciferase activity was observed in SCC9 cells. That a mutant p53 protein's function can be modified following the introduction of an interacting partner is not unprecedented. It has been previously shown that another p53 interacting protein, ANKRD11, can interact with and restore the normal tumor/metastasis suppressor function and transcriptional activity of a mutant p53 in breast cancer cells (Neilsen et al., 2008; Noll et al., 2012). Our data suggests that in the presence of plakoglobin, mutant p53, which otherwise would not associate with its target gene promoters, may be capable of regulating the expression of its target genes (anti-tumor/metastasis genes). Similar to ANKDR11, it is possible that plakoglobin, as an interacting partner of p53, may be able to alter the conformation of the mutant p53 protein, thus allowing it to bind to its target gene promoters. This is a novel and important result with potentially significant therapeutic implications, since p53 is inactivated in half of all tumors (Rahman-Roblick et al., 2007; Goh et al., 2011; Junttila and Evan, 2009). As such, the relationship between p53 and plakoglobin is one that requires further investigation and could potentially lead to the identification of plakoglobin as a useful marker in the diagnosis and

prognosis of cancer. The ability of plakoglobin to interact with both wild-type and mutant p53 and to activate the expression of tumor suppressor genes suggests that plakoglobin itself may be a useful target for therapeutic interventions in the treatment of tumors with mutated p53 protein.

When looking at wild-type p53 expressing cells (MCF-7), we showed that while luciferase activity was induced from the wild-type p53-binding sequence, no activity was observed from the mutant sequence (Figure 4-9B, C), demonstrating that wild-type p53 can only activate gene expression from wild-type p53-binding sequences. Interestingly, knock down of plakoglobin in MCF-7 cells resulted in significantly decreased luciferase activity, suggesting that plakoglobin normally plays a role in regulating the transcriptional activity of p53. Plakoglobin may also regulate the levels of p53, as we observed significantly higher p53 levels in SCC9-PG cells relative to SCC9 cells (Figure 4-2D). Furthermore, MCF-7-shPG transfectants had lower levels of p53 compared to parental MCF-7 cells (Figure 4-9A). These observations suggest that, as an interacting partner of p53, plakoglobin may be involved in p53 stability and that the increased p53 transcriptional activity in the presence of plakoglobin may be due, in part, to the increased amount of p53 protein in plakoglobin-expressing cells. However, plakoglobin most likely plays some other role in regulating p53 transcriptional activity, since the p53 in SCC9 cells, which is expressed to considerable amounts, did not associate with the 14-3-3σ

gene promoter (Figure 4-5A). Also, plakoglobin may play a role in regulating the subcellular distribution of p53 as was recently demonstrated for NPM (Lam et al., 2012), since there was considerably more p53 in the nuclear fractions of SCC9-PG cells compared to SCC9 cells (compare Figure 4-4, SCC9 and SCC9-PG, IB: p53).

While the tumor and metastasis suppressor activity of plakoglobin has remained unclear, new reports are beginning to shed light on this topic. We recently showed that plakoglobin expression resulted in the increased levels (mRNA and protein) and membrane localization of the metastasis suppressors Nm23-H1 and H2 and that plakoglobin interacted with Nm23 (Chapter Three; Aktary et al., 2010). Also, plakoglobin expression was shown to regulate cell motility through both cell-cell adhesion dependent and independent mechanisms (Yin et al., 2005). The formation of stable cell-cell junctional complexes is an intuitive way plakoglobin may regulate tumorigenesis and metastasis. However, plakoglobin may function as a tumor/metastasis suppressor independent of its adhesive function by modulating Rho, Fibronectin and Vitronectin-dependent Src signaling (Todorovic et al., 2010; Franzen et al. 2012), by acting as a transcriptional repressor of oncogenic Myc (Williamson et al., 2006) and by increasing the expression of metastasis suppressors such as Nm23 (Chapter Three; Aktary et al., 2010) and 14-3-3σ. These *in vitro* observations are supported by clinical studies that have shown decreased plakoglobin expression leads to tumorigenesis, increased risk of metastasis and poor

overall prognosis in various tumors (Pantel et al., 1998; Kanazawa et al., 2008; Narkio-Makela et al., 2009; Nozoe et al., 2009; Aktary and Pasdar, 2012, Holen et al., 2012).

Overall, this chapter demonstrates, for the first time, the role of plakoglobin in the regulation of gene expression in conjunction with p53. By interacting with p53 and associating with the promoter of the 14-3-3σ (*SFN*) gene, plakoglobin appears to be playing an active role in the regulation of gene expression. The larger implication of this work is that plakoglobin has the potential to interact with transcription factors and to regulate the expression of various genes, including those that are involved in tumorigenesis and metastasis.

CHAPTER FIVE: PLAKOGLOBIN REGULATES THE EXPRESSION OF SATB1 AND SUPPRESSES IN VITRO PROLIFERATION, MIGRATION AND INVASION

5.1. Introduction[1]

5.1.1. Rationale

One of the differentially expressed genes identified in our microarray experiments was *SATB1* (special AT-rich sequence binding protein 1). SATB1 is a global regulator of gene expression and an oncogene. We noted that *SATB1* expression was decreased 3-fold in SCC9-PG cells relative to parental SCC9 cells. This suggested that plakoglobin may play a role in regulating the *SATB1* gene and consequently have an effect on the expression of its target genes involved in tumorigenesis and metastasis. As such, we set out to characterize the effect of plakoglobin expression on the expression of *SATB1* and whether plakoglobin expression altered the *in vitro* proliferation, migration and invasion of various cancer cell lines.

5.1.2. SATB1

SATB1 was initially identified as a DNA-binding protein that was highly expressed in the thymus (Dickinson et al., 1992; de Belle et al., 1998). This protein had a high affinity for binding to base-unpairing regions (BURs), which are genomic DNA sequences with high unfolding potential, containing clusters of sequences (approximately 20-40 base pairs long) with a bias in G and C distribution (i.e. one DNA strand contains only A, T and C residues; Dickinson et al., 1992; Kohwi-Shigematsu et al., 1990, 2013; Bode et al., 1992). Importantly, since BUR sequences are thought to be found all throughout the genome and since SATB1 demonstrated a

[1] A version of this chapter has been submitted for publication to PLoS One.

specificity for these BUR sequences, it became evident that SATB1 could, through its interactions with different BUR sequences in different gene promoters, cause the looping of chromatin (Cai et al., 2003, 2006; Kumar et al., 2007; Kohwi-Shigematsu et al., 2013). These chromatin loops could, in turn, potentially result in the close physical proximity and coordinated regulation of genes that would otherwise remain silent. In addition to forming these chromatin loops, SATB1 was shown to recruit different chromatin remodeling enzymes to the gene loci close to the BURs and as a result, altered gene expression (Yasui et al., 2002; Kumar et al., 2005; Wen et al., 2005; Han et al., 2008).

SATB1 has been shown to promote tumorigenesis and metastasis in various tumor cell lines, including breast, lung, ovarian, colorectal, liver, laryngeal, glioma and melanoma (Han et al., 2008; Li et al., 2010; Zhao et al., 2010; Chen et al., 2011; Xiang et al., 2012; Tu et al., 2012; Nodin et al., 2012; Chu et al., 2012; Huang et al., 2013). Specifically, SATB1 has been shown to induce the expression of tumor and metastasis-promoting genes while suppressing the expression of metastasis suppressor genes (Han et al., 2008; Notani et al., 2010a; Tu et al., 2012).

5.1.3. Specific aim and summary of results

In this chapter, we examined the role of plakoglobin in regulating the expression of the *SATB1* gene and one of its targets, *NME1*. We show that plakoglobin, in coordination with p53, interacted with the *SATB1* promoter and downregulated its expression. The decreased levels of

SATB1 mRNA were accompanied by its decreased protein levels in squamous and mammary carcinoma cell lines expressing plakoglobin. Furthermore, plakoglobin expression led to an increase and a decrease in the protein levels of a subset of SATB1 repressed and activated target genes, respectively. Concurrent with these transcriptional changes, plakoglobin expression resulted in decreased cell growth and *in vitro* migration and invasion. Taken together, our data suggests that plakoglobin suppresses tumorigenesis and metastasis (at least *in vitro*) through the regulation of genes involved in these processes.

5.2. Results

5.2.1. Plakoglobin regulates *SATB1* expression.

To confirm that *SATB1* expression was decreased in SCC9 cells following plakoglobin expression, we first performed RT-PCR experiments and observed a notable decrease in SATB1 mRNA in SCC9-PG cells compared to SCC9 cells (Figure 5-1A, left). In agreement with this result, western blot analysis revealed that while SATB1 protein was expressed in SCC9 cells, its levels were significantly decreased and barely detectable in SCC9-PG cells (Figure 5-1A, right).

To determine whether plakoglobin regulates the *SATB1* gene, we performed chromatin immunoprecipitation (ChIP) experiments using plakoglobin antibodies and chromatin from SCC9 and SCC9-PG cells. The isolated DNA was then processed for PCR using primers specific to the *SATB1* promoter (Table 2-2). These experiments showed that plakoglobin

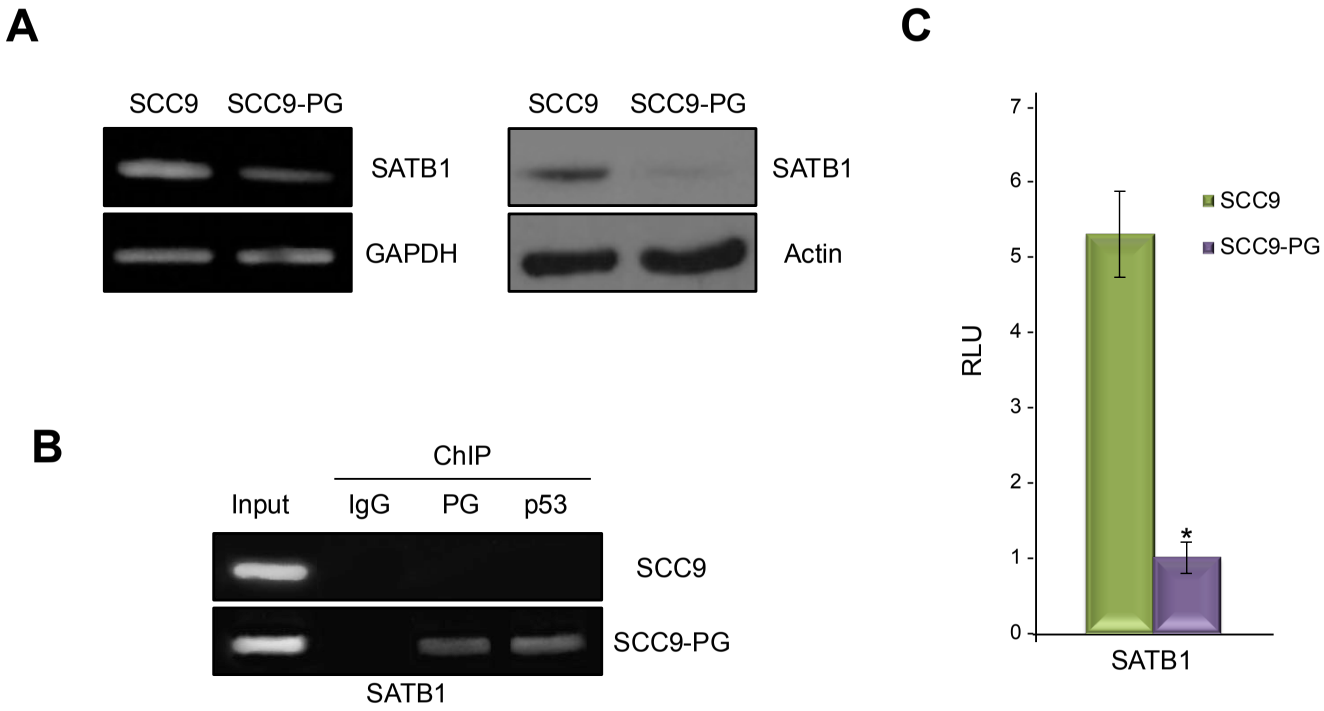

Figure 5-1. Plakoglobin associates with and suppresses the *SATB1* promoter in SCC9-PG cells. (A) (Left) Total cellular RNA was isolated from SCC9 and SCC9-PG cells, reverse transcribed and processed for PCR using primers specific to **SATB1** and **GAPDH**. (Right) Equal amounts of total cellular proteins from SCC9 and SCC9-PG cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **SATB1** and **Actin**. **(B)** SCC9 and SCC9-PG cells were formaldehyde fixed and processed for chromatin immunoprecipitation. Following sonication, extracts were immunoprecipitated using control **IgG**, **plakoglobin** and **p53** antibodies. Following extensive washes, immunoprecipitated DNA was separated from the immune complexes and purified using standard DNA purification protocols. The purified DNA was then processed for PCR using **SATB1** primers. As positive control, total cellular DNA (Input) was amplified using the same primers. **(C)** SCC9 and SCC9-PG cells were transfected with luciferase reporter constructs under the control of a 1.2 kb sequence of the *SATB1* gene promoter. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector and SATB1 reporter constructs were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. The *SATB1* promoter activity was normalized to the corresponding vector activity for each cell line and then normalized to SCC9 (*$p < 0.01$). PG, plakoglobin; RLU, Relative Light Units.

associated with the *SATB1* promoter in SCC9-PG cells, but not in SCC9 cells (Figure 5-1B). ChIP experiments using control IgG antibodies produced negative results. Since we have shown that plakoglobin interacts with and regulates gene expression in conjunction with p53 (Chapter Four; Aktary et al., 2013), we also performed the ChIP experiments using p53 antibodies, which showed the association of p53 with the *SATB1* promoter in SCC9-PG cells but not in SCC9 cells (Figure 5-1B).

The association of plakoglobin and p53 with the *SATB1* promoter and the decreased levels of SATB1 mRNA and protein in SCC9-PG cells suggested that plakoglobin and p53 function as negative regulators of the *SATB1* promoter. To test this hypothesis, luciferase reporter assays were conducted using luciferase reporter constructs downstream of a 1.2 kb *SATB1* promoter fragment (Li et al., 2010). Consistent with the role of plakoglobin in the negative regulation of the *SATB1* promoter, the luciferase activity of the reporter constructs was significantly decreased (over 5-fold) in SCC9-PG cells compared to SCC9 cells (Figure 5-1C).

5.2.2. Plakoglobin regulates *SATB1* in mammary epithelial cell lines.

In addition to SCC9 cells, we also examined the role of plakoglobin on *SATB1* expression in mammary epithelial cell lines, since it has been shown that SATB1 plays a major role in the regulation of breast cancer progression and metastasis (Han et al., 2008). As such, we set out to determine whether the results from SCC9-PG could be extended to breast cancer cell lines. To do so, we took two approaches: first, we knocked

down plakoglobin in MCF-7 cells (MCF-7-shPG), which express considerable levels of plakoglobin, and second, we expressed plakoglobin in MDA-231 cells (MDA-231-PG), which express very low levels of endogenous plakoglobin (Lam et al., 2012). RT-PCR and western blot experiments showed that knock down of plakoglobin in MCF-7 cells resulted in increased levels of both SATB1 mRNA and protein. In contrast, plakoglobin expression in MDA-231 cells resulted in a decrease in both SATB1 mRNA and protein, although SATB1 protein was still detectable in MDA-231-PG cells (Figure 5-2A).

ChIP experiments showed that similar to SCC9-PG cells, both plakoglobin and p53 associated with the *SATB1* promoter in MCF-7 cells. Furthermore, both proteins associated with the *SATB1* promoter in MDA-231-PG cells, but not MDA-231 cells (Figure 5-2B). To further demonstrate that plakoglobin and p53 negatively regulate the *SATB1* promoter, we performed the same luciferase assay experiments using the SATB1-luciferase reporter constructs in MCF-7, MCF-7-shPG, MDA-231 and MDA-231-PG cells. The results of these experiments were consistent with those from SCC9-PG cells: luciferase activity in MDA-231-PG cells was decreased (over 2-fold) compared to MDA-231 cells, whereas activity in MCF-7-shPG cells was induced (approximately 2-fold) compared to MCF-7 cells (Figure 5-2C). Taken together, the results from these experiments suggest that plakoglobin and p53 negatively regulate *SATB1* expression.

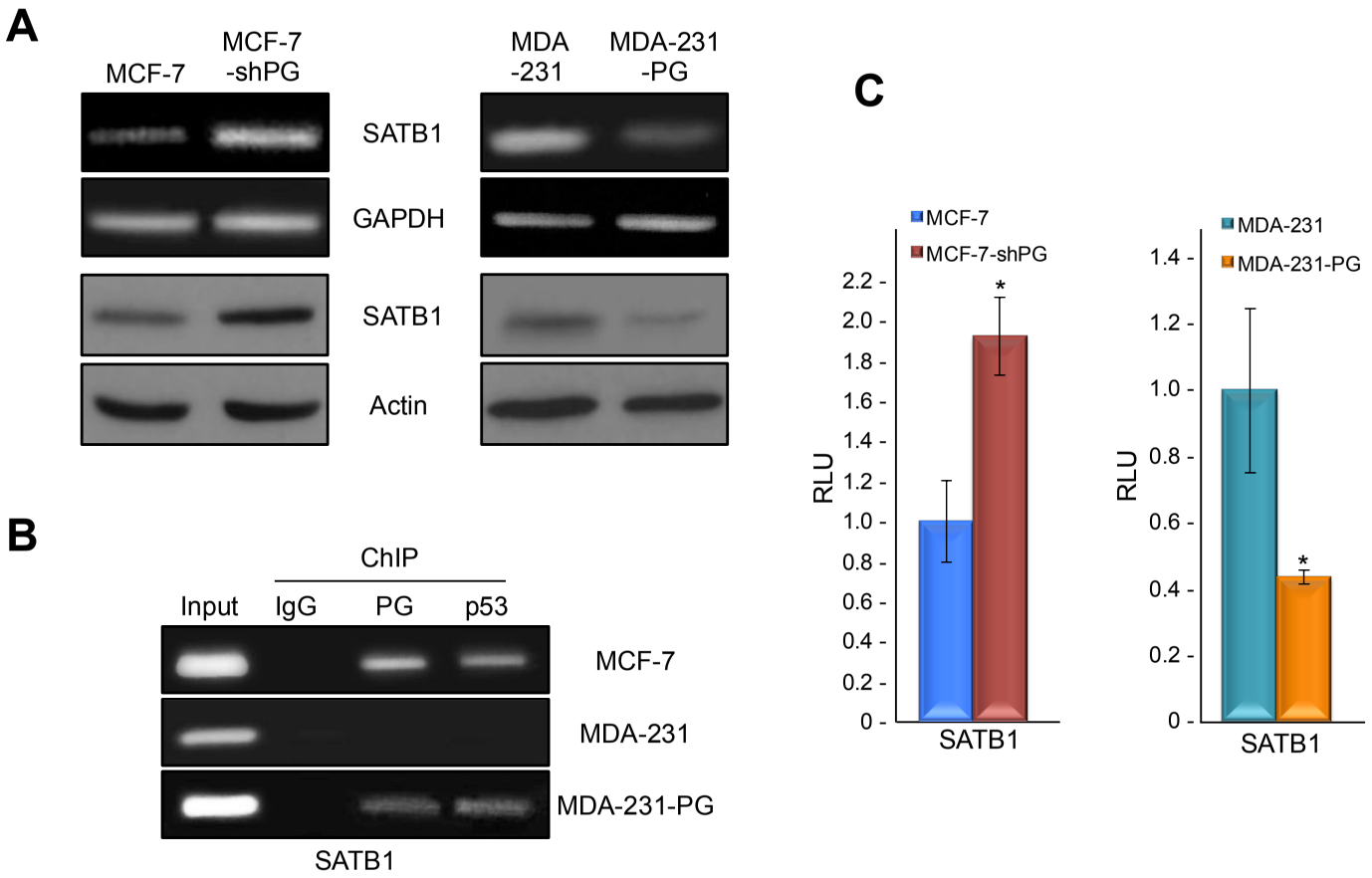

Figure 5-2. Plakoglobin suppresses *SATB1* in mammary epithelial cell lines. (A) (Top) Total cellular RNA was isolated from MCF-7, MCF-7-shPG, MDA-231 and MDA-231-PG cells, reverse transcribed and processed for PCR using primers specific to **SATB1** and **GAPDH**. (Bottom) Equal amounts of total cellular proteins from these cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **SATB1** and **Actin**. **(B)** MCF-7, MDA-231 and MDA-231-PG cells were formaldehyde fixed and processed for chromatin immunoprecipitation. Following sonication, extracts were immunoprecipitated using control **IgG**, **plakoglobin** and **p53** antibodies. Following extensive washes, immunoprecipitated DNA was separated from the immune complexes and purified using standard DNA purification protocols. The purified DNA was then processed for PCR using **SATB1** primers. As positive control, total cellular DNA (Input) was amplified using the same primers. **(C)** MCF-7, MCF-7-shPG, MDA-231 and MDA-231-PG cells were transfected with luciferase reporter constructs under the control of a 1.2 kb sequence of the *SATB1* gene promoter. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector and SATB1 reporter constructs were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. The *SATB1* promoter activity was normalized to the corresponding vector activity for each cell line and then normalized to MCF-7 or MDA-231, respectively (*$p < 0.01$). PG, plakoglobin; RLU, Relative Light Units.

5.2.3. Plakoglobin associates with and activates the *NME1* promoter.

It has been suggested that the metastasis suppressor Nm23-H1 is a potential target of SATB1 (Han et al., 2008). We previously identified the metastasis suppressors Nm23-H1 and -H2 as being differentially expressed in SCC9-PG cells and showed that plakoglobin expression resulted in increased Nm23-H1 and -H2 protein levels as well as increased Nm23-H1 (*NME1*), but not Nm23-H2 (*NME2*) gene expression (Chapter Three; Aktary et al., 2010). Therefore, we set out to determine if the increased levels of *NME1* in SCC9-PG cells were simply due to decreased SATB1 expression or whether plakoglobin actively promoted the expression of *NME1.* In order to do so, we performed ChIP experiments using plakoglobin antibodies and primers specific to the *NME1* promoter (Table 2-2). Plakoglobin associated with the *NME1* promoter in SCC9-PG cells, but not SCC9 cells (Figure 5-3A). Similar ChIP experiments were performed using p53 antibodies, which demonstrated that while p53 associated with the *NME1* promoter in SCC9-PG cells, this association was absent in SCC9 cells (Figure 5-3A). ChIP experiments using control IgG antibodies produced negative results.

To confirm the role of plakoglobin in the regulation of *NME1* expression, luciferase assays were done using luciferase reporter constructs downstream of a 2kb *NME1* promoter fragment (Qu et al., 2008). In these experiments, luciferase activity was induced approximately 6-fold in SCC9-PG cells compared to SCC9 cells (Figure 5-3B), demonstrating that

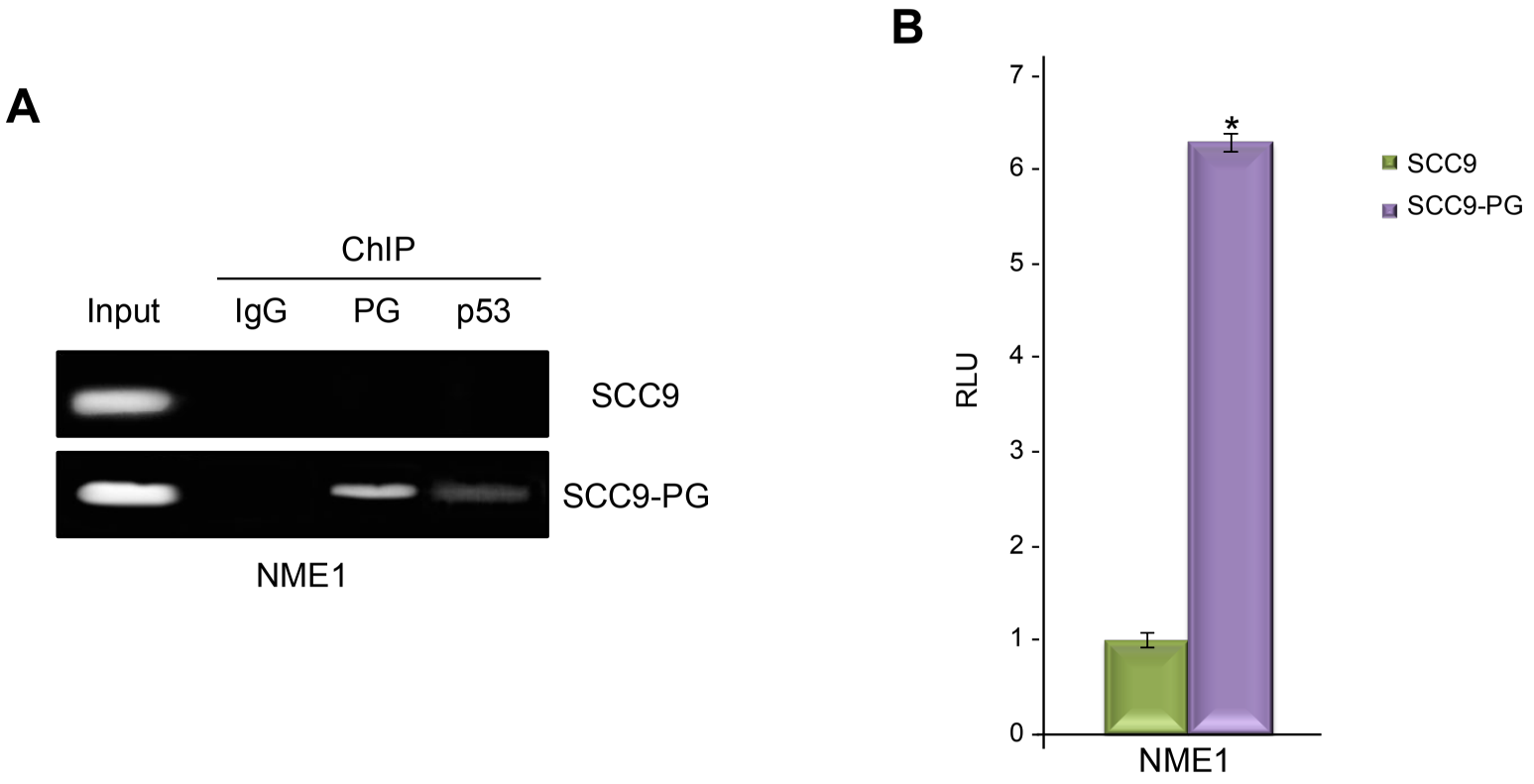

Figure 5-3. Plakoglobin associates with and activates the *NME1* promoter in SCC9-PG cells. (A) SCC9 and SCC9-PG cells were formaldehyde fixed and processed for chromatin immunoprecipitation. Following sonication, extracts were immunoprecipitated using control **IgG**, **plakoglobin** and **p53** antibodies. Following extensive washes, immunoprecipitated DNA was separated from the immune complexes and purified using standard DNA purification protocols. The purified DNA was then processed for PCR using **NME1** primers. As positive control, total cellular DNA (Input) was amplified using the same primers. **(B)** SCC9 and SCC9-PG cells were transfected with luciferase reporter constructs under the control of a 2 kb sequence of the *NME1* gene promoter. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector and NME1 reporter constructs were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. The *NME1* promoter activity was normalized to the corresponding vector activity for each cell line and then normalized to SCC9 (*$p < 0.01$). PG, plakoglobin; RLU, Relative Light Units.

plakoglobin expression resulted in increased *NME1* promoter activity. Taken together, these data suggest that while plakoglobin downregulates SATB1 levels, which may in turn result in increased *NME1* expression, plakoglobin also actively regulates *NME1* gene expression through its associations with the *NME1* promoter.

5.2.4. Plakoglobin regulates *NME1* in mammary epithelial cell lines.

We subsequently performed RT-PCR and western blot experiments to examine the levels of Nm23-H1 mRNA and protein in the mammary epithelial cell lines to confirm that plakoglobin-mediated regulation of *NME1* was not specific to squamous cell lines. Knockdown of plakoglobin in MCF-7 cells resulted in a notable decrease in Nm23-H1 mRNA, which was accompanied by a corresponding decrease in the levels of Nm23-H1 and -H2 protein (Figure 5-4A). In contrast, the levels of both Nm23-H1 mRNA and protein were increased considerably in MDA-231-PG cells compared to parental MDA-231 cells (Figure 5-4A). We also performed the RT-PCR experiments using primers specific to the Nm23-H2 (*NME2*) gene and observed that plakoglobin expression had no effect on *NME2* expression, since the levels of Nm23-H2 mRNA were not different between MCF-7 and MCF-7-shPG and MDA-231 and MDA-231-PG cells, respectively (Figure 5-4A). These results were consistent with the lack of *NME2* induction following plakoglobin expression in SCC9-PG cells (Aktary et al., 2010).

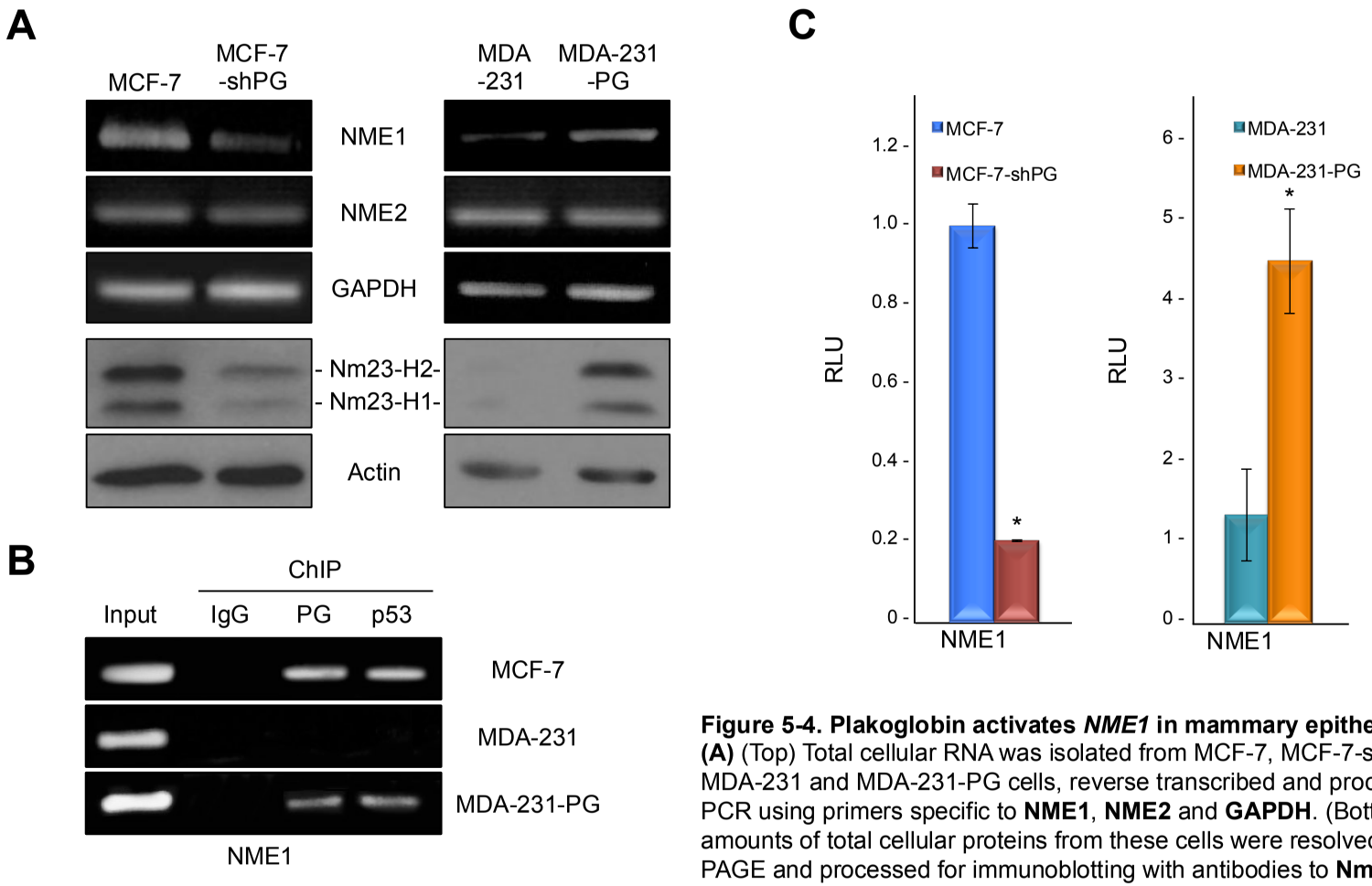

Figure 5-4. Plakoglobin activates *NME1* in mammary epithelial cell lines. **(A)** (Top) Total cellular RNA was isolated from MCF-7, MCF-7-shPG, MDA-231 and MDA-231-PG cells, reverse transcribed and processed for PCR using primers specific to **NME1**, **NME2** and **GAPDH**. (Bottom) Equal amounts of total cellular proteins from these cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to **Nm23-H1**, **-H2** and **Actin**. **(B)** MCF-7, MDA-231 and MDA-231-PG cells were formaldehyde Fixed and processed for chromatin immunoprecipitation. Following sonication, extracts were immunoprecipitated using control **IgG**, **plakoglobin** and **p53** antibodies. Following extensive washes, immunoprecipitated DNA was separated from the immune complexes and purified using standard DNA purification protocols. The purified DNA was then processed for PCR using **NME1** primers. As positive control, total cellular DNA (Input) was amplified using the same primers. **(C)** MCF-7, MCF-7-shPG, MDA-231 and MDA-231-PG cells were transfected with luciferase reporter constructs under the control of a 2 kb sequence of the *NME1* gene promoter. Luciferase activities were measured 48 hours post-transfection. The levels of luciferase activities from the vector and NME1 reporter constructs were determined from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector. The *NME1* promoter activity was normalized to the corresponding vector activity for each cell line and then normalized to MCF-7 or MDA-231, respectively (*$p < 0.01$). PG, plakoglobin; RLU, Relative Light Units.

Next, ChIP experiments were conducted with chromatin from MCF-7, MDA-231 and MDA-231-PG cells using plakoglobin and p53 antibodies. The results from these experiments showed that plakoglobin and p53 associated with the *NME1* promoter in both MCF-7 and MDA-231-PG cells, but not MDA-231 cells (Figure 5-4B). In addition, luciferase reporter assays using these cell lines were performed to determine the role of plakoglobin in the regulation of the *NME1* promoter. While minimal luciferase activity was observed in MDA-231 cells, promoter activity was induced over 3-fold in MDA-231-PG cells (compared to parental MDA-231 cells; Figure 5-4C). In contrast, luciferase activity was decreased by ~5-fold in MCF-7-shPG cells compared to MCF-7 cells (Figure 5-4C). Taken together, these results suggest that plakoglobin and p53 positively regulate the expression of the *NME1* gene and that plakoglobin expression has no effect on the *NME2* gene.

5.2.5. Changes in SATB1 target gene expression in response to plakoglobin levels.

Since SATB1 is a major global regulator of gene expression, we argued that the alteration in SATB1 levels based on plakoglobin expression would result in alterations in the expression of various SATB1 target genes in addition to Nm23. More specifically, we focused on a select number of SATB1 target genes that are known to participate in tumorigenesis and metastasis (e.g. tumor/metastasis suppressors BRMS1, Kiss1, Claudin-1; tumor/metastasis promoters c-Abl, MMP3, ErbB2 and Snail). We

performed qRT-PCR experiments and observed that the levels of c-Abl, Snail, ErbB2 and MMP3 mRNA were all increased in MCF-7-shPG cells, compared to MCF-7 cells. Consistent with the increased mRNA levels, western blot experiments showed that protein levels of these tumor/metastasis promoters were also increased in MCF-7-shPG cells (Figure 5-5A-B, top). Furthermore, the mRNA and protein levels of tumor/metastasis suppressors BRMS1, Kiss1 and Claudin-1 were decreased in MCF-7-shPG cells relative to MCF-7 cells (Figure 5-5A-B, bottom).

5.2.6. Plakoglobin suppresses cancer cell growth, migration and invasion.

The results so far suggested that plakoglobin plays a role in promoting the expression of various genes involved in suppression of tumorigenesis/ metastasis, while suppressing the expression of those genes that promote these processes. In order to determine whether plakoglobin's regulation of gene expression resulted in a biologically discernable effect on the *in vitro* growth and the migratory and invasive properties of cells, MCF-7 and MCF-7-shPG cells we processed for growth, migration and invasion assays (as described in Chapter Two). The results of the growth assay showed a significant increase (~2.5-fold) in the growth of MCF-7-shPG relative to MCF-7 cells (Figure 5-6A). As additional controls, we also assessed the growth rate of SCC9 and MDA -231 cells and their plakoglobin expressing transfectants SCC9-PG and MDA-231-PG

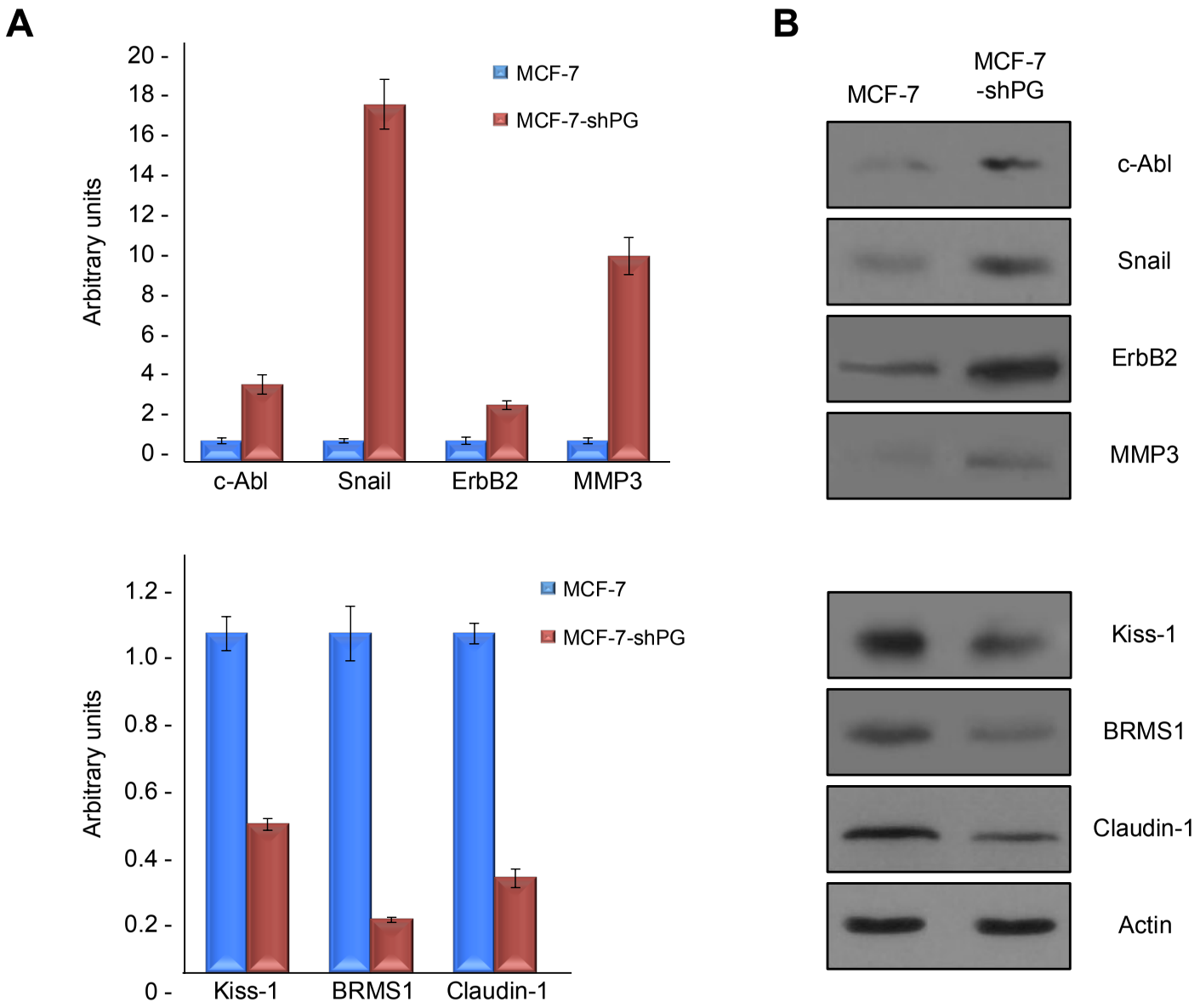

Figure 5-5. Plakoglobin knockdown changes the levels of SATB1 target genes. (A) Total cellular RNA was isolated from MCF-7 and MCF-7-shPG cells, reverse transcribed and processed for PCR using primers specific to SATB1 target genes c-Abl, MMP3, ErbB2, Snail, BRMS1, Kiss1 and Claudin-1. **(B)** Equal amounts of total cellular proteins from these cells were resolved by SDS-PAGE and processed for immunoblotting with antibodies to c-Abl, MMP3, ErbB2, Snail, BRMS1, Kiss1 and Claudin-1. PG, plakoglobin.

respectively. In contrast to MCF-7-shPG, the growth rate of SCC9-PG cells was reduced ~2.5-fold relative to parental SCC9 cells, whereas MDA-231-PG cells showed a 2-fold reduction in growth relative to parental MDA-231 cells, which was consistent with what we had observed previously (Figure 5-6A; Parker et al., 1998; Lam et al., 2012).

We then used BrdU labeling to verify if the differences observed at the end of the 7-day growth assay among different cell lines with various levels of plakoglobin expression were due to differences in cell proliferation. Cells from various cell lines were plated and allowed to grow for 6 days at which time they were labeled with BrdU for 24 hours and processed for confocal microscopy as described in Materials and Methods. The results showed that SCC9 and MDA-231 cells were highly proliferative as almost all cells displayed BrdU incorporation. In contrast, we detected very little or no BrdU incorporation in the plakoglobin expressing MCF-7, SCC9-PG and MDA-231-PG cells (Figure 5-6B), whereas there was significant BrdU incorporation in the plakoglobin knockdown MCF-7-shPG cells (Figure 5-6B).

The migratory properties of the various cell lines were assessed using transwell chambers. Cells were allowed to migrate through transwell filters for 48 hours, after which the migrated cells were fixed and counted.

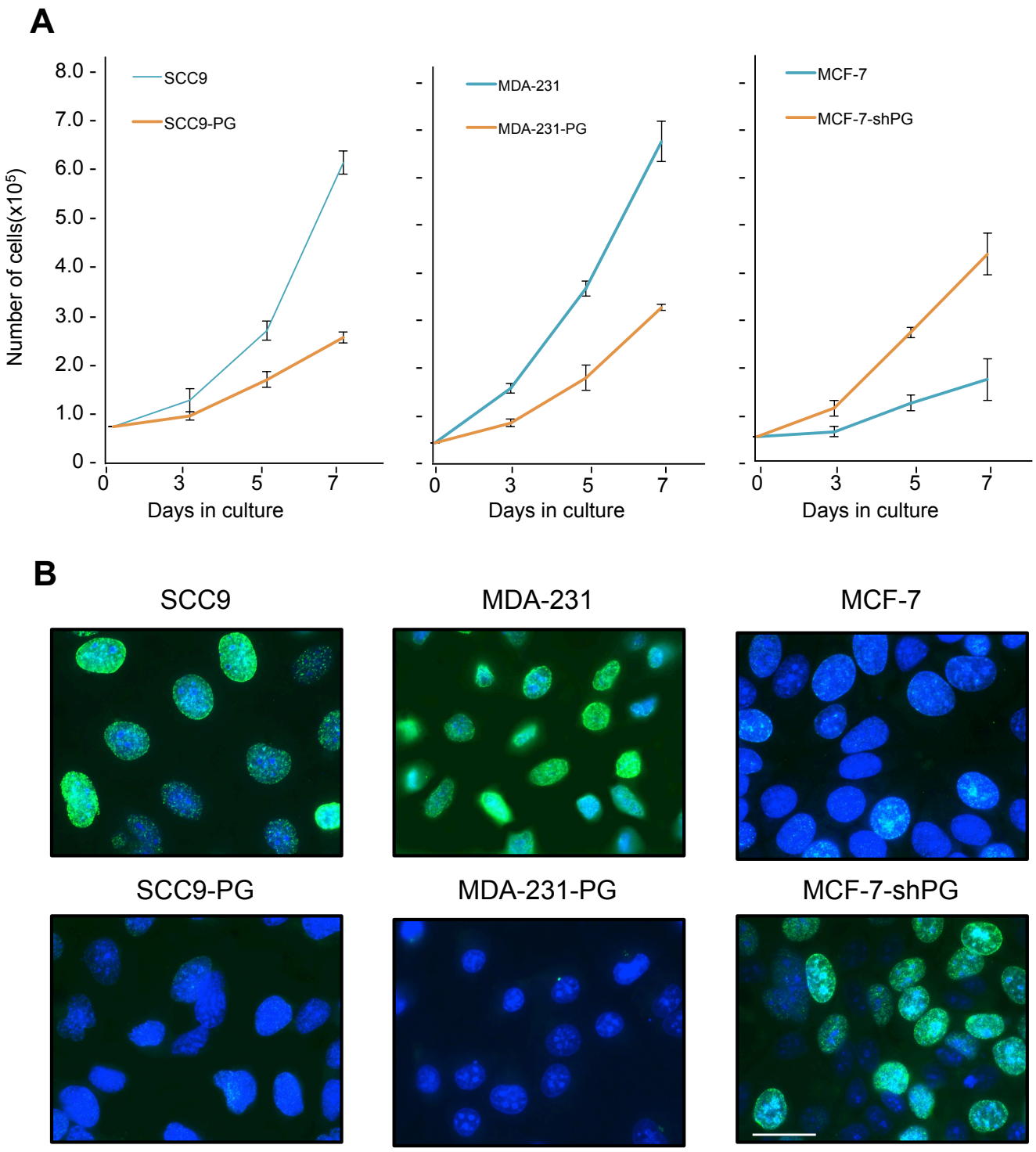

Figure 5-6. Plakoglobin decreases *in vitro* cell growth and proliferation. (A) Replicate cultures of SCC9, SCC9-PG, MDA-231, -231-PG, MCF-7 and MCF-7-shPG cells were established at single cell density and cells were counted at 3, 5 and 7 days. Each time point represents the average of three independent experiments. **(B)** The absence of error bars at some time points is due to the small differences among the experiments. SCC9, SCC9-PG, MDA-231, -231-PG, MCF-7 and MCF-7-shPG cells were plated on glass coverslips and allowed to grow for 6 days at which time BrdU was added to the cell cultures for 24 hours. BrdU incorporation was then assessed by immunofluorescence staining using BrdU antibodies. Nuclei were counterstained with DRAQ5 and cells viewed using a 63X objective of an LSM510 META (Zeiss) laser scanning confocal microscope. Bar, 20 μm.

Consistent with our previous observations, MDA-231-PG cells displayed ~ 40% less migration than MDA-231 cells (Figure 5-7A; Lam et al., 2012). Similarly, SCC9 cells were approximately 10-fold more migratory than SCC9-PG cells, whereas MCF-7-shPG cells showed a 4-fold increase in migration compared to MCF-7 cells (Figure 5-7A). To rule out the possibility that the increased migration in SCC9, MDA-231 and MCF-7-shPG could be due to their higher cell proliferation rate, we repeated the migration assays for 12 hours, since our growth data showed that none of the cell lines had a doubling time less than 24 hours (Figure 5-6A). The results of these experiments were consistent with those of the 48 hours assays and showed that SCC9, MDA-231 and MCF-7-shPG cells were considerably more migratory than their plakoglobin-expressing counterparts (SCC9-PG, MDA-231-PG, MCF-7; Figure 5-7A).

The invasive properties of the various cell lines were assessed using matrigel-coated transwell chambers. Similar to the migration experiments, cells were allowed to migrate through the matrigel matrix, after which the invaded cells were fixed and counted. These experiments showed that in addition to being more migratory, SCC9, MCF-7-shPG and MDA-231 cells were more invasive than SCC9-PG, MCF-7 and MDA-231-PG cells (approximately 6-, 7- and 2-fold, respectively; Figure 5-7B; Lam et al., 2012). Taken together, these results suggest that plakoglobin, regulates the expression of genes involved in cell growth, migration and invasion concurrent with a suppression of *in vitro* migration and invasion.

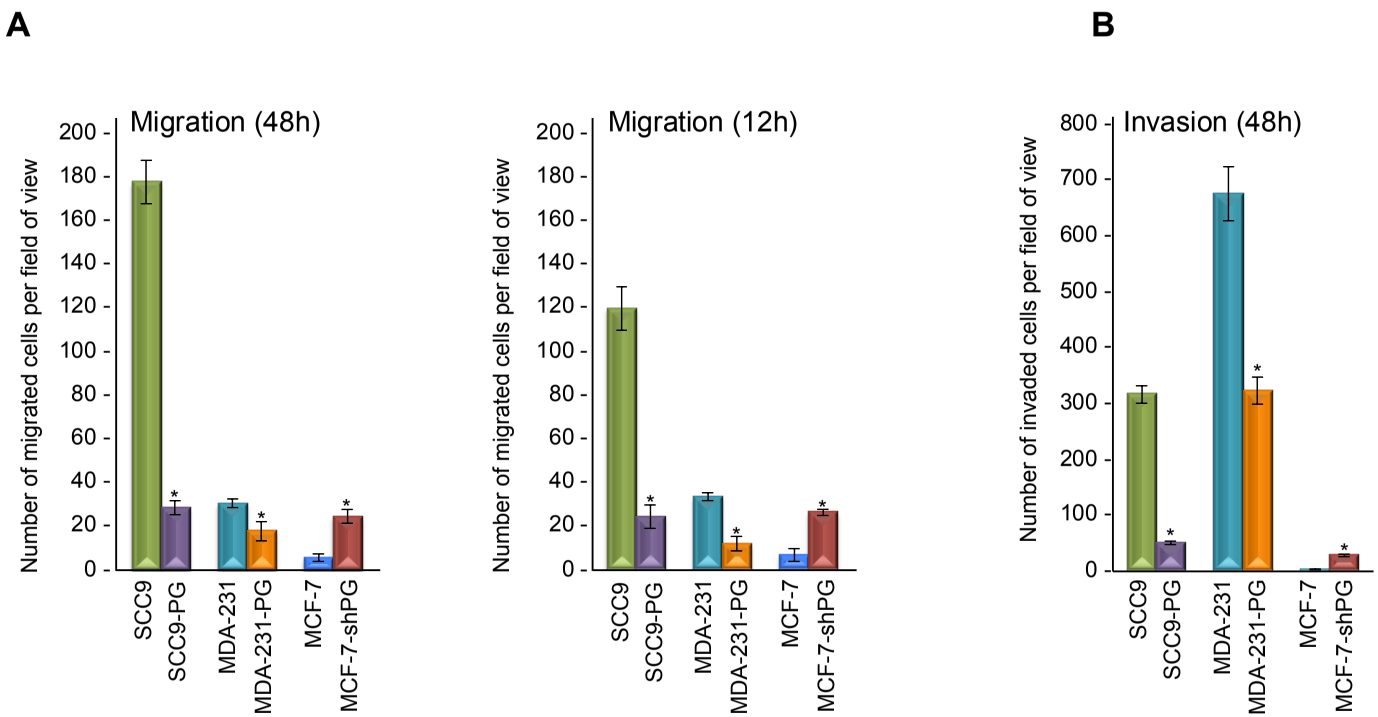

Figure 5-7. Plakoglobin decreases *in vitro* migration and invasion. (A) Forty-eight- and twelve-hour Transwell migration assays were performed in triplicates for SCC9, SCC9-PG, MDA-231, MDA-231-PG, MCF-7 and MCF-7-shPG cell lines. The membranes were fixed, stained, cut and mounted on slides and viewed under an inverted microscope. **(B)** Forty-eight-hour matrigel invasion assays were performed as described in (A) using matrigel coated transwell membranes. The number of migrated/invaded cells in five random fields for each membrane were calculated using the ImageJ Cell Counter program and averaged. Histograms represent the average $\pm$ SD of the number of migrated/invaded cells for each cell line. *$p < 0.01$). PG, plakoglobin.

5.3. Discussion

In the present study, we have further investigated the underlying mechanisms for plakoglobin's role in tumorigenesis and metastasis (also see Lam et al., 2012; Aktary et al., 2013). Our data showed that plakoglobin associated with the promoter of the oncogenic DNA binding protein SATB1 and downregulated its expression. The decreased expression of *SATB1* following plakoglobin expression was associated with its decreased protein levels and in turn, altered expression of SATB1 target genes with an overall effect of decreased cell growth and *in vitro* migration and invasion. Conversely, knockdown of plakoglobin in MCF-7 cells resulted in the upregulation of SATB1 and increased cell proliferation, migration and invasion.

SATB1's ability to regulate gene expression was initially identified in the thymus, where several studies showed that it was essential for T-cell development and differentiation (de Belle et al., 1998; Alvarez et al., 2000; Kumar et al., 2007). These studies demonstrated that SATB1 regulates gene expression by organizing target gene loci into distinct domains/chromatin loop structures and by recruiting different chromatin remodeling enzymes to promote gene expression and T-cell differentiation. Since then, SATB1 has been established as a contributing factor to the development and progression of many different types of cancer, including breast, lung, prostate, colon and ovarian (Han et al., 2008; Li et al., 2010; Zhao et al., 2010; Chen et al., 2011; Xiang et al.,

2012; Tu et al., 2012; Nodin et al., 2012; Chu et al., 2012; Huang et al., 2013). SATB1 has also been shown to participate in the epidermis differentiation as SATB1$^{-/-}$ mice showed defects in epidermal differentiation (Fessing et al., 2011). These defects were associated with the improper activation of genes found within the epidermal differentiation complex locus, to which SATB1 was shown to bind. Other studies have demonstrated that SATB1 regulates the expression of at least 10% of genes in both T-cells and non T-cells, including genes involved in apoptosis, cell-extracellular matrix attachment, cellular metabolism, calcium signaling and the Wnt, Notch, and TGF-β pathways, suggesting that it plays a role in the global regulation of gene expression (Kumar et al., 2005; Notani et al., 2011).

SATB1 has been suggested to regulate gene expression in conjunction with β-catenin as part of the Wnt signaling pathway (Purbey et al., 2009; Notani et al., 2010; Burute et al., 2012), since during T-cell differentiation, SATB1 associates with and recruits p300/CBP histone acetyltransferase and β-catenin to the promoters of Wnt target genes, resulting in the increased expression of genes such as *IL-2* and *MYC* (Notani et al., 2010). SATB1 also associated with the major breakpoint region (mbr) in the 3'-UTR of the *BCL2* gene and promoted the expression of this anti-apoptotic protein, whose expression is also regulated by β-catenin, through the induction of c-Myc and E2F1 (Ramakrishnan et al., 2000; Zhang et al., 2006; Ma et al., 2007; Li et al., 2007b). We previously

showed that plakoglobin is also able to regulate the levels of Bcl-2 through the modulation of the signaling activity of β-catenin (Li et al., 2007a). The data presented here clearly demonstrates that plakoglobin associates with the *SATB1* promoter and downregulates its expression. Taken together, these observations suggest that plakoglobin may regulate Wnt β-catenin and SATB1 signaling in multiple ways. First, plakoglobin downregulates the expression of *SATB1*, which would result in the decreased expression of SATB1 target genes. The decreased levels of SATB1 may also alter/reduce β-catenin recruitment to its target promoters and therefore reduce the expression of those genes. Second, nuclear plakoglobin decreases the interaction between β-catenin and TCF and results in inhibition of TCF/β-catenin signaling (Miravet et al., 2002; Li et al., 2007). Third, expression of physiological levels of plakoglobin results in decreased levels of β-catenin (Salomon et al., 1997; Parker et al., 1998). Finally, plakoglobin associates with and inhibits the expression of the *MYC* promoter (Williamson et al., 2006), a β-catenin and SATB1 target gene (He et al., 1998; MacDonald et al., 2009; Notani et al., 2010).

More recent studies have suggested that SATB1 plays a role in breast tumorigenesis and metastasis. Indeed, SATB1 expression in SATB1 deficient SKBR3 breast cancer cells resulted in increased tumor growth and a more migratory and invasive phenotype that was concurrent with increased expression of tumor/metastasis promoter genes such as c-Abl, Snail, MMP3, TGF-β1, ErbB2 and decreased expression of

tumor/metastasis suppressors including Nm23, Claudin-1, Kiss1, BRMS1, KAI1. Conversely, knockdown of SATB1 in the highly invasive MDA-231 cells had the opposite effect: tumor/metastasis promoting genes were downregulated whereas inhibitors of these processes were upregulated (Han et al., 2008).

Plakoglobin also appears to have a role in regulating tumorigenesis and metastasis through the modulation of gene expression. We recently showed that plakoglobin interacts with the transcription factor p53 and regulates the expression of the tumor suppressor *SFN* (14-3-3σ; Aktary et al., 2013). Furthermore, we showed that p53-transcriptional activity is enhanced in the presence of plakoglobin and that mutant p53 proteins may, in association with plakoglobin, be functional in regulating their wild-type target genes. In the current study, we have identified *SATB1* as another target gene of plakoglobin and p53, as ChIP experiments clearly demonstrated an association of both proteins with the *SATB1* promoter (Figures 5-1, 5-2). However, as opposed to *SFN, SATB1* is negatively regulated by p53 and plakoglobin. While we have shown that plakoglobin and p53 interact with one another (Aktary et al., 2013), whether these interactions are direct or involve other cofactors is not clear and warrants further investigation. Furthermore, although plakoglobin is known to associate with TCF/LEF and regulate gene expression (Zhurinsky et al., 2000a, b; Miravet et al., 2002; Williamson et al., 2006), neither the human *SATB1* nor the *NME1* genes contain potential TCF/LEF binding sites,

therefore it is likely that plakoglobin-mediated regulation of these genes is independent of TCF/LEF. It was previously shown that p63 is a transcriptional activator of *SATB1* during epidermal differentiation (Fessing et al., 2011), however, to the best of our knowledge, the present work is the first to show that p53 also regulates *SATB1* expression, albeit opposite to p63. What other co-factors are involved in the regulation of p53 and plakoglobin target genes and to what extent these co-factors differ based on whether the complex is activating or repressing gene expression remains unknown and warrants further investigation.

Along with repressing *SATB1* expression, plakoglobin increased the expression of *NME1*, a potential SATB1 target gene. We previously showed that Nm23-H1 mRNA and protein as well as Nm23-H2 protein were upregulated in SCC9-PG cells and that plakoglobin and Nm23 interacted in both the soluble and cytoskeleton-associated pools of cellular proteins (Chapter Three; Aktary et al. 2010). In this chapter, we further characterized the role of plakoglobin in the regulation of the *NME1* gene and showed that plakoglobin and p53 associated with the *NME1* promoter and activated its expression (Figures 5-3, 5-4). While the association of plakoglobin with the *NME1* promoter is novel, it is supported by a previous report that showed decreased Nm23-H1 mRNA levels following plakoglobin knock down in breast cancer cells (Holen et al., 2012), In addition, while it has been previously suggested that *NME1* is a transcriptional target of p53 (Chen et al., 2003; Rahman-Roblick et al.,

2007), our ChIP data unequivocally shows that p53 associated with the *NME1* promoter and regulated its expression. Taken together, these data suggest that plakoglobin can alter the levels of its potential target genes through different mechanisms, including direct regulation of gene expression (e.g. *SFN, NME1*) and through protein-protein interactions that result in increased protein levels (e.g. Nm23-H2; Chapter Three; Aktary et al., 2010).

In addition to *NME1*, we also observed alterations in the mRNA and protein levels of other SATB1 target genes. More specifically, knockdown of plakoglobin in MCF-7 cells resulted in the increased mRNA and protein levels of the tumor/metastasis promoters c-Abl, Snail, ErbB2 and MMP3 and the decreased levels of tumor/metastasis suppressors BRMS1, Kiss1 and Claudin-1 (Figure 5-5). Whether plakoglobin may alter the expression of these SATB1 target genes by altering the expression of SATB1 itself and/or by associating with the promoters of these target genes and promoting/repressing their expression requires further investigation.

To confirm that the regulation of tumorigenesis and metastasis associated genes by plakoglobin had a biological consequence, we performed cell growth, migration and invasion assays and showed that plakoglobin suppressed cell growth as well as *in vitro* migration and invasion (Figures 5-6, 5-7). These results were in agreement with other studies that have previously shown that plakoglobin suppresses these processes and promotes a more “epithelial” phenotype, consistent with its

role as a tumor and metastasis suppressor (Holen et al., 2012; Bailey et al., 2012; Lam et al., 2012).

Increasing evidence suggests that plakoglobin regulates tumorigenesis independent of its cell-cell adhesion function. Plakoglobin regulates the expression of genes such as *MYC, DSC2* and *SFN* (Williamson et al., 2006; Tokonzaba et al., 2013; Aktary et al., 2013) and also suppresses Ras-mediated oncogenesis through increased HDAC4 mRNA levels (Yim et al., 2013). In addition to regulation of gene expression, plakoglobin has been shown to act as a tumor/metastasis suppressor by modulating Rho, Fibronectin and Vitronectin-dependent Src signaling (Todorovic et al., 2010; Franzen et al. 2012).

Our findings are significant in that they clearly point to a role of plakoglobin in regulating a variety of genes that are involved in tumor development and progression. Our data also suggests that plakoglobin may regulate a number of genes (both positively and negatively) under normal cellular conditions (i.e. in the absence of cell stress or activation of different growth pathways), implying that plakoglobin may be a “basal” and more global type of regulator of gene expression. As such, our results have larger implications in that plakoglobin may have a potential as a new therapeutic target for the treatment of various cancers.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE STUDIES

6.1. Summary of Research

6.1.1. Overview

Despite its initial discovery nearly thirty years ago and many studies that have suggested that plakoglobin acts as a suppressor of tumorigenesis and metastasis, the exact molecular mechanisms by which plakoglobin regulates these processes has, until recently, remained unclear. The focus of this thesis has been characterizing, at the molecular level, potential mechanisms by which plakoglobin may suppress tumor formation and metastatic progression.

We expressed plakoglobin in the plakoglobin-null SCC9 cell line and examined the protein and RNA profiles of SCC9-PG transfectants in order to identify proteins and transcripts that were differentially expressed following plakoglobin expression. From these studies, we identified several growth and metastasis regulating proteins/genes as potential plakoglobin targets, some of which were characterized in this thesis.

6.1.2. Nm23-H1 and Nm23-H2

We observed that the levels of Nm23-H1 and -H2 protein were both significantly increased in SCC9-PG cells compared to SCC9 cells, with Nm23-H2 levels being more notably increased. Interestingly, while the levels of both Nm23-H1 and -H2 protein were increased, only the levels of Nm23-H1 mRNA were upregulated in SCC9-PG cells (Figure 3-1). We verified these observations using both MDA-231 cells and their plakoglobin expressing transfectants (MDA-231-PG) and MCF-7 cells

along with MCF-7 cells in which plakoglobin was knocked down (MCF-7-shPG). These experiments showed that while the levels of both Nm23-H1 and -H2 proteins were altered upon plakoglobin expression, only the *NME1* gene was affected (Figure 5-3). Furthermore, plakoglobin interacted with both Nm23-H1 and -H2 in various epithelial cell lines and this interaction was dependent on α-catenin (Figures 3-4, 3-5). Finally, we showed that plakoglobin (in conjunction with p53) interacted with and increased the expression of the *NME1* promoter (Figures 5-3, 5-4).

6.1.3. p53 and 14-3-3σ

We identified several p53-target genes (including the tumor suppressor 14-3-3σ) that were differentially expressed in SCC9-PG cells, which led us to examine whether plakoglobin and p53 interact. We showed that plakoglobin and p53 interacted in both the cytoplasm and the nucleus (Figures 4-2, 4-3, 4-4) and that both proteins were associated with the 14-3-3σ gene (*SFN*) promoter (Figure 4-5). We subsequently showed that both wild-type and mutant p53 transcriptional activity was increased in the presence of plakoglobin (Figures 4-8 and 4-9). Furthermore, we observed that mutant p53, only in the presence of plakoglobin, was able to associate with the *SFN* promoter and promote its expression (Figure 4-5, 4-8).

6.1.4. SATB1

Our microarray experiments showed that SATB1, the chromatin remodeling factor and oncogene, was one of the genes whose levels were downregulated in SCC9-PG cells. We showed that plakoglobin expression

in both SCC9 and MDA-231 cells resulted in decreased *SATB1* expression and protein levels, whereas knockdown of plakoglobin in MCF-7 cells resulted in increased SATB1 mRNA and protein. We also showed that plakoglobin and p53 associated with the *SATB1* promoter and repressed its activity (Figures 5-1, 5-2). Finally, we showed that SCC9, MDA-231 and MCF-7-shPG cells displayed increased growth, migratory and invasive properties compared to their plakoglobin-expressing counterparts (Figure 5-5, 5-6, 5-7).

6.1.5. Model for regulation of tumorigenesis and metastasis by plakoglobin

The results of this thesis suggest that plakoglobin can regulate its potential targets in a variety of ways (Figure 6-1). First, plakoglobin can interact with various intracellular partners and alters their levels, localization or function. In support of this scenario, we recently showed that plakoglobin interacted with nucelophosmin (NPM), the nucleolar phosphoprotein whose role in tumorigenesis is largely dependent on its subcellular distribution (Grisendi et al., 2006; Brady et al., 2009; Falini et al., 2008; Shandilya et al., 2009). We showed that plakoglobin expression in MDA-231 cells resulted in increased NPM protein levels and its redistribution from the cytoplasm and nucleoplasm, where it is thought to function as an oncogene, (Brady et al., 2009; Falini et al., 2008; Shandilya et al., 2009) into the nucleolus, where it is typically localized in untransformed cells (Grisendi et al., 2006). Therefore, plakoglobin,

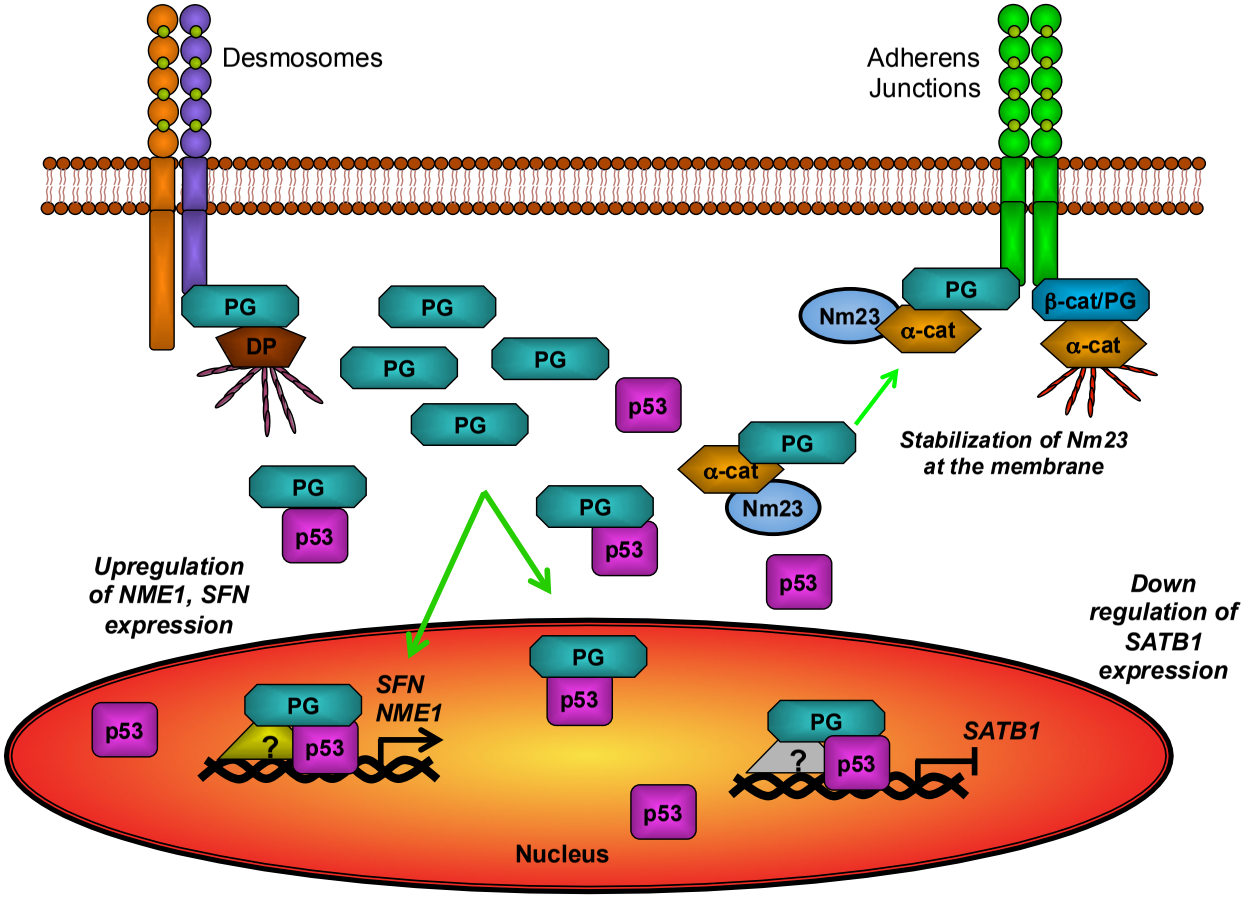

Figure 6-1. Potential model for tumor/metastasis suppressor activity of plakoglobin. Cytoplasmic, non-junctional plakoglobin may regulate tumorigenesis and metastasis by multiple mechanisms. Plakoglobin interacts with the metastasis suppressor Nm23 and increases its protein levels and localization at the membrane. Plakoglobin also interacts with the transcription factor p53 and promotes the expression of tumor and metastasis suppressor genes (e.g. *NME1, SFN)* and suppresses the expression of oncogenes (e.g. *SATB1)*. α-cat, α-catenin; β-cat, β-catenin; DP, desmoplakin; Nm23, nonmetastatic protein 23; PG, plakoglobin; SFN, stratifin

through its interactions with NPM, altered NPM protein levels and localization, which was concurrent with the decreased growth, invasive and migratory properties of MDA-231-PG cells (Lam et al., 2012). Furthermore, since plakoglobin expression resulted in increased Nm23-H2 protein levels, but not increased mRNA, it is likely that plakoglobin's interactions with Nm23-H2, which also resulted in the subsequent redistribution of Nm23-H2 to the membrane, contributed to its increased protein levels.

Second, plakoglobin can interact with transcription factors, such as p53, and regulate the expression of various target genes. Plakoglobin and p53 interact in the cytoplasm and then translocate into the nucleus, where they most likely associate with various other co-factors to regulate gene expression. In this case, plakoglobin-mediated transcriptional regulation can either be activating (in the case of the *NME1* and *SFN* genes) or inhibitory (e.g. *SATB1*). Plakoglobin also appears to help recruit p53 to its target gene promoters and promotes p53 transcriptional activity.

The work presented in this thesis suggests that plakoglobin regulates tumorigenesis and metastasis through at least two mechanisms: regulation of gene expression and interactions with different cellular partners. However, plakoglobin's tumor and metastasis suppressor activity is likely not limited to these described mechanisms and further studies are needed to clearly define other functions of plakoglobin in regulation of these processes.

6.2. Future Studies

6.2.1. *In vivo* corroboration of research findings

While the data presented in this thesis clearly demonstrates that plakoglobin suppresses tumorigenesis and metastasis *in vitro*, these studies need to be expanded to examine the role of plakoglobin in regulating these processes *in vivo.* As such, different studies should be undertaken to compliment our *in vitro* findings.

First, immunohistochemistry (IHC) experiments using tumor specimens from patients with tumors of different origins and stages should be examined for the expression and subcellular localization of plakoglobin, p53, Nm23, 14-3-3σ and SATB1. While the expression of these proteins has been examined in various independent studies, analysis of their levels and localization has not been performed in the same tumor samples. The results of the IHC experiments can then be analyzed together with patient clinicopathological parameters (e.g. recurrence, lymph node status, etc.) using different statistical and computing science techniques in order to identify potential markers that can be useful in the diagnosis and prognosis of different cancers. Recently using an autonomous machine learning technique and data from 66 primary invasive ductal breast carcinomas, we were able to generate a simple and efficient decision-tree prognostic classifier, based on the levels and subcellular distribution of 6 junctional proteins, 8 standard clinical features and 4 diagnostic markers that could predict whether a novel breast cancer patient would relapse.

We showed that a decision-tree classifier, which incorporated a combination of only 4 features (nuclear α- and β-cat levels, the total level of the tumor suppressor PTEN, and the number of involved axillary lymph nodes), is able to correctly predict patient outcomes 80% of the time (Asgarian et al., 2010).

Second, *in vivo* tumorigenesis and metastasis assays should be performed using transgenic animal models. While we were able to show that plakoglobin expressing cells were considerably less migratory and invasive and had decreased growth rates compared to their non-plakoglobin expressing counterparts, these types experiments need to be performed using animal models in order to assess the role of plakoglobin in the regulation of tumorigenesis and metastasis in a more biological setting. As such, subcutaneous injection of cell lines expressing different levels of plakoglobin (e.g. MDA-231 and MDA-231-PG) in immunodeficient mice will be useful in assessing the growth suppressive effects of plakoglobin. In contrast, tail vein or intracardial injection of the same cell lines and examination of metastasis formation in organs such as the lungs, liver or brain will help to determine whether plakoglobin can suppress tumor cell migration and invasion (i.e. metastasis).

6.2.2. Modulation of the metastasis suppressor activity of Nm23 by plakoglobin

We have shown that plakoglobin interacted with the metastasis suppressors Nm23-H1 and -H2 and increased their expression (H1) and

protein levels (H1 and H2). These increases in Nm23-H1 and -H2 protein levels in plakoglobin expressing cell lines resulted in an overall decrease in *in vitro* migration and invasion, which most likely results (at least in part) from the increased levels of Nm23 proteins in these cells. However, the exact role of plakoglobin in regulating the metastasis suppressor activity of Nm23 remains unknown.

Nm23-H1 interacts with h-prune, a nucleotide phosphodiesterase that inhibits Nm23's metastasis suppressor activity and results in increased cell migration and invasion (D'Angelo et al., 2004; Galasso and Zollo, 2009). In our microarray experiments, we identified the h-prune gene as being downregulated in SCC9-PG cells. We have preliminary evidence showing that the levels of h-prune protein are decreased in SCC9-PG cells compared to SCC9 cells and that plakoglobin expression results in changes in h-prune subcellular distribution from cytoplasmic to what appears to be Golgi-localized. These findings suggest that perhaps plakoglobin not only interacts with Nm23 proteins, but that it also alters their intracellular interactions. Therefore, coimmunoprecipitation experiments examining the interactions between plakoglobin, Nm23 and h-prune will be an interesting next step to determine whether plakoglobin does in fact promote Nm23 metastasis suppressor activity by altering its interactions with h-prune.

6.2.3. Characterization of plakoglobin-p53 interactions

We showed that plakoglobin and p53 interacted with one another, however we have yet to characterize which domains of each protein are necessary for these interactions. In order to do so, coimmunoprecipitation experiments will need to be (and currently are being) performed using cell lines expressing different mutants of either plakoglobin or p53. By expressing either mutant plakoglobin or mutant p53 proteins missing one specific domain in cell lines that express the other protein endogenously, we can accurately assess which domain of each protein is necessary for these interactions.

To complement these coimmunoprecipitation experiments, we plan to perform 3-dimensional (3-D) modeling experiments based on the amino acid sequences of both plakoglobin and p53 and use these generated models to map the sequences between plakoglobin and p53 that mediate their interactions. Since we found that plakoglobin interacted with both wild-type and mutant p53 proteins, we can also use these 3-D modeling experiments to identify the amino acid sequences that mediate the interactions of plakoglobin with both wild-type and mutant p53.

6.2.4. Activation of p53 transcriptional activity in cancer cell lines

The observation that mutant p53, in the presence of plakoglobin, was recruited to the promoters of its target genes, suggests that plakoglobin can interact with a mutant p53 that does not function properly and allow it to regain some of its wild-type functions. This phenomenon has numerous

therapeutic possibilities. By identifying the minimum amino acid sequence in plakoglobin that potentiates its interaction with p53 (through the 3-D modeling experiments), we can use this sequence to activate mutant p53 in various cancer cell lines.

In order to accomplish this, we will need to design cell permeable peptides containing the identified plakoglobin sequence, as was recently accomplished for Nm23 (Lim et al., 2011). These cell permeable plakoglobin peptides can then be administered to various cancer cell lines (with low or no plakoglobin expression) and their interactions with p53 can be assessed. In addition, following administration of the plakoglobin peptides, p53 transcriptional activity and associations with its target gene promoters both in the absence and presence of cellular stressors (e.g. UV irradiation, staurosporine treatment, etc.) can be examined. Furthermore, the migratory, invasive and apoptosis-inducing properties of these cells should be determined. If these cell permeable plakoglobin peptides result in the increased transcriptional activity of p53 and a decreased transformed phenotype, their effectiveness in activating p53 and suppressing tumorigenesis and metastasis *in vivo* can be assessed.

6.2.5. Role of plakoglobin in p53-mediated apoptosis

Our studies describing the interactions between plakoglobin and p53 were all done in the absence of cellular stressors and as such represent a more “basal” or normal cell function for these two proteins in the regulation of gene expression. However, multiple studies have suggested that

plakoglobin stimulates apoptosis in response to cell stressors (Charpentier et al., 2000; Hakimelahi et al., 2000; Dusek et al., 2007). While the mechanisms behind the apoptosis-promoting activity of plakoglobin remain unclear, Dusek et al. demonstrated that plakoglobin-null keratinocytes were deficient in apoptosis, which corresponded with decreased cytochrome c release from the mitochondria. Our results suggest that plakoglobin may promote apoptosis through its interactions with p53. Importantly, we observed plakoglobin-p53 interactions in both the nucleus and cytoplasm. Since p53 interacts with pro- and anti-apoptotic proteins at the mitochondria and regulates mitochondrial membrane permeabilization (Mihara et al., 2003; Green and Kroemer, 2009; Vaseva and Moll, 2009), it is possible that plakoglobin is involved in these interactions. To test this possibility, apoptosis can be induced in cells lacking and expressing plakoglobin (e.g. by UV irradiating the cells to induce DNA damage) and apoptosis induction can be measured. Furthermore, p53's interactions with its apoptosis inducing target genes promoters can be assessed, as can its (and plakoglobin's) interactions with pro- and anti-apoptotic proteins at the mitochondria.

6.2.6. Plakoglobin-mediated regulation of gene expression

Our data clearly demonstrates that plakoglobin regulates gene expression in collaboration with p53. While we have identified three plakoglobin-p53 target genes, further studies are necessary to identify a

larger subset of plakoglobin target genes. As such, ChIP-chip experiments will be valuable in identifying plakoglobin targets on a large scale.

While our data shows that plakoglobin and p53 coordinately regulate gene expression, it is entirely possible that plakoglobin can regulate gene expression with other transcription factors either in conjunction with, or independent of, p53. For example, plakoglobin may regulate gene expression through its interactions with Nm23 proteins. A recent study showed that knockdown of Nm23-H1 in the NL9980 human large cell lung cancer cell line resulted in alterations in the expression of approximately 1000 genes, many of which are involved in tumorigenesis and metastasis (Ma et al., 2008). Interestingly, comparison of the mRNA profiles of Nm23-H1 knockdown cells with SCC9-PG cells revealed that several genes may potentially be regulated by both plakoglobin and Nm23. For example, the *BCL2A1, CXCL2, JUN,* and *MMP1* genes were all decreased in SCC9-PG cells and were increased in NL9980-Nm23-H1 knockdown cells, whereas the *BEX5, HDAC5* and *NME1* genes were increased in SCC9-PG cells and decreased in NL9980-Nm23-H1 knockdown cells. Yet another study showed that overexpression of Nm23-H1 in the highly invasive and Nm23-H1-deficient MDA-MB-435 cell line resulted in the downregulation of various genes involved in tumorigenesis and metastasis, including *EDG2* and *PTN* (Horak et al., 2007), both of which were downregulated in SCC9-PG cells.

Considering that previous studies have shown that both Nm23-H1 and -H2 can regulate gene expression by binding to DNA (Postel et al., 1993, 2000; Ma et al., 2002; Postel, 2003; Cervoni et al., 2006; Thakur et al., 2009; Choudhuri et al., 2010) and our observations of interactions between plakoglobin and Nm23-H1 and -H2 in SCC9-PG-NLS cells, it is possible that plakoglobin and Nm23 proteins regulate gene expression concurrently. Future studies aimed at determining whether plakoglobin and Nm23 associate with the same target gene promoters and identifying these potential target genes would be of great interest.

6.3. Conclusions

Overall, the studies described in this thesis are the first detailed analysis of mechanisms underlying plakoglobin's growth/metastasis inhibitory function. Our results clearly demonstrate that plakoglobin plays an active role in suppressing tumorigenesis and metastasis through both the regulation of gene expression and by interacting with and altering the levels, localization and function of various intracellular proteins involved in these processes. The larger implication of this work is that plakoglobin, as an important player in tumorigenesis and metastasis, may be a useful marker for diagnosis and prognosis as well as a therapeutic target for the treatment of various cancers.

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