Plakoglobin interacts with the transcription factor p53 and regulates the expression of 14-3-3σ

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Summary

Plakoglobin (γ-catenin), a constituent of the adherens junction and desmosomes, has signaling capabilities typically associated with tumor/metastasis suppression through mechanisms that remain undefined. To determine the role of plakoglobin during tumorigenesis and metastasis, we expressed plakoglobin in human tongue squamous cell carcinoma (SCC9) cells and compared the mRNA profiles of parental SCC9 cells and their plakoglobin-expressing transfectants (SCC9-PG). We detected several p53-target genes whose levels were altered upon plakoglobin expression. In this study, we identified the p53 regulated tumor suppressor 14-3-3σ as a direct plakoglobin-p53 target gene. Coimmunoprecipitation experiments revealed that plakoglobin and p53 interact while chromatin immunoprecipitation and electrophoretic mobility shift assays revealed that plakoglobin and p53 associate with the 14-3-3σ promoter. Furthermore, luciferase reporter assays showed that p53 transcriptional activity is increased in the presence of plakoglobin. Finally, knockdown of plakoglobin in MCF-7 cells followed by luciferase assays confirmed that p53 transcriptional activity is enhanced in the presence of plakoglobin. Our data suggest that plakoglobin regulates gene expression in conjunction with p53 and that plakoglobin may regulate p53 transcriptional activity, which may account, in part, for the tumor/metastasis suppressor activity of plakoglobin.

Key Words: 14-3-3σ/gene expression/plakoglobin/p53/tumor suppressor/SCC9
Introduction

In epithelia, intercellular adhesive complexes limit proliferation and migration and maintain normal tissue architecture by connecting neighboring cells to each other. Disassembly of these adhesive complexes is a major contributing factor to the multi-step process of tumorigenesis and metastasis (Halbleib and Nelson, 2006; Jeanes et al., 2008; Makriilia et al., 2009; Yilmaz and Christofori, 2010). The main adhesive complexes in epithelial cells are the adherens junction and desmosomes, which are cadherin-based junctions. Cadherins are single-pass transmembrane glycoproteins that make homotypic interactions with cadherins on neighboring cells. Intracellularly, cadherins interact with catenin proteins, namely β-catenin or γ-catenin (plakoglobin), in a mutually exclusive manner. These proteins then interact with α-catenin, an actin binding protein, which tethers the cadherin-catenin complex to the actin cytoskeleton (Halbleib and Nelson, 2006; van Roy and Berx, 2008; Stemmler, 2008).

β-catenin and plakoglobin are structurally and functionally homologous: they both interact with classic cadherins, α-catenin, Axin and APC and both proteins are involved in Wnt signaling (Peifer et al., 1992; Zhurinsky et al., 2000b; Aktary and Pasdar, 2012). Plakoglobin also interacts with the desmosomal cadherins and is essential for desmosome formation, whereas β-catenin is not typically associated with the desmosomes (Parker et al., 1998; Chidgey and Dawson, 2007; Chidgey and Dawson, 2008). While both proteins interact with Tcf/Lef transcription factors, β-catenin-Tcf complexes are transcriptionally active whereas plakoglobin-Tcf complexes are inefficient in binding DNA (Simcha et al., 1998; Zhurinsky et al., 2000a). β-catenin’s oncogenic potential as part of the Wnt signaling pathway has been well described and mutations resulting in its stabilization and nuclear accumulation have been observed in a variety of tumors (Huang and He, 2008; MacDonald et al., 2009; Valenta et al., 2009).
Aktary et al. 2012. In contrast, plakoglobin has often been associated with tumor/metastasis suppressor activity, although the mechanisms underlying this activity remain unclear. A number of studies have suggested that plakoglobin decreases cell proliferation, migration and invasion and induces apoptosis (Simcha et al., 1996; Parker et al., 1998; Pantel et al., 1998; Charpentier et al., 2000; Winn et al., 2002; Reiger-Christ et al., 2005; Yin et al., 2005; Dusek et al., 2007; Kanazawa et al., 2008; Narkio-Makela et al., 2009; Todorovic et al., 2010; Aktary et al., 2010; Aktary and Pasdar, 2012; Bailey et al., 2012; Holen et al., 2012; Franzen et al., 2012; Lam et al., 2012).

We previously expressed physiological levels of plakoglobin in SCC9 cells, a human squamous cell carcinoma cell line derived from the tongue, which expresses β-catenin but lacks endogenous expression of plakoglobin as well as E-cadherin and observed a mesenchymal-to-epidermoid phenotypic transition concurrent with stabilization of N-cadherin, downregulation of β-catenin and formation of desmosomes (Parker et al., 1998). In order to elucidate the mechanism underlying this phenotypic transition, we performed microarray experiments and identified various genes differentially expressed between SCC9 cells and their plakoglobin-expressing transfectants (SCC9-PG; Supplementary Table I). Among these differentially expressed genes was the metastasis suppressor Nonmetastatic protein 23 (Nm23), a recently identified p53 target gene, whose protein and RNA levels were increased in SCC9-PG cells (Rahman-Roblick et al., 2007; Aktary et al., 2010). In addition to Nm23, we identified several other p53 target genes, including 14-3-3σ, that were differentially expressed in SCC9-PG cells, suggesting that plakoglobin may be involved in p53-mediated regulation of gene expression.

The p53 tumor suppressor is regarded as one of the most important tumor suppressors. 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.
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). As a tumor suppressor, p53’s most documented role is that of a transcription factor, regulating the expression of genes involved in tumorigenesis, metastasis, cell-cycle control and apoptosis (Harris and Levine, 2005; Juntila and Evan, 2009; Menendez et al., 2009; Meek, 2009; Cicalese et al., 2009; Goh et al., 2011). In addition, p53 has recently been shown to regulate apoptosis at the mitochondrial level through interactions with various Bcl-2 family proteins thereby inducing mitochondrial outer membrane permeabilization (Mihara et al., 2003; Vaseva and Moll, 2009; Lindenboim et al., 2011). p53 protein stability is regulated by several post-translational modifications, including ubiquitination, phosphorylation, acetylation, and sumoylation (Boehme and Blattner, 2009; Collavin et al., 2010), as well as by its interactions with different cytoplasmic and nuclear proteins, which also alter its activity and function (Juntila and Evan, 2009; Menendez et al., 2009; Boehme and Blattner, 2009; Collavin et al., 2010; Goh et al., 2011).

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 (Obsilova et al., 2008; Morrison, 2008; Van Heusden, 2009). 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. 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 (Lodygin and Hermeking, 2006; Lee and Lozano, 2006).

In the present study, 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 results in the induction of 14-3-3σ mRNA and protein. Furthermore, we show that plakoglobin interacts with p53 in squamous (SCC9-PG, A431) and mammary (MCF-10-2A, MCF-7) epithelial cell lines, that plakoglobin and p53 both associate with the 14-3-3σ gene promoter, and that plakoglobin promotes 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).

**Results**

**14-3-3σ levels are induced in SCC9-PG cells.** SCC9 cells do not express PG (Fig. 1A,B; Parker et al., 1998). We initially observed that plakoglobin expression in SCC9 cells (SCC9-PG)
resulted in a mesenchymal-to-epidermoid phenotypic transition (Parker et al., 1998), suggesting that plakoglobin acts as a tumor suppressor. In order to gain insight as to the possible mechanisms underlying this phenotypic transition, we performed transcription microarray experiments and identified several differentially expressed genes in SCC9-PG relative to SCC9 cells. Among these differentially expressed genes, we noticed that the levels of several p53 target genes were altered. 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 RNA 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 (Fig. 1B,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 (Fig. 1A). 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).

**Plakoglobin interacts with p53 in SCC9-PG cells.** The results from Fig. 1A-C showed that plakoglobin expression results 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 interact 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 (Fig. 1D, IP: PG/IB: PG and p53). Reciprocal coimmunoprecipitation experiments using p53 antibodies coprecipitated plakoglobin from SCC9-PG cells but not SCC9 cells (Fig. 1D, IP: p53/IB: PG and p53). Since plakoglobin and β-catenin have common interacting partners (Peifer et al., 1992; Zhurinsky et al., 2000b; Stemmler, 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 does not interact with p53 (Fig. 1E) and that the plakoglobin-p53 interaction is specific to these two proteins.

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; Fig. 2). We first confirmed that these cell lines express 14-3-3σ by Western blot analysis (Fig. 2A, TCE). Next, reciprocal coimmunoprecipitation experiments using plakoglobin (Fig. 2B), p53 (Fig. 2C) and preimmune (Fig. 2D) antibodies followed by immunoblotting demonstrated that plakoglobin and p53 are coprecipitated in non-epidermoid as well as epidermoid cell lines by plakoglobin and p53 but not preimmune antibodies. We have performed these experiments in human and mouse fibroblast cell lines and observed plakoglobin-p53 interactions, supporting that these interactions are not cell type specific (data not shown).

Finally, reciprocal coimmunoprecipitation experiments using β-catenin and p53 antibodies in
these cell lines showed that these two proteins do not interact, further demonstrating the
specificity of the plakoglobin-p53 interaction (Fig. 2E).

Plakoglobin and p53 interact in both the cytoplasm and nucleus. Figs 1 and 2 demonstrated
that plakoglobin interacts 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 interact 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 (Fig. 3A,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 (Fig. 3A,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 (Fig. 3A,B, IP: p53/IB: PG).

Plakoglobin and p53 associate with the 14-3-3σ gene promoter. Taken together, the results so
far showed that plakoglobin expression results in induction of 14-3-3σ mRNA and protein
expression and that plakoglobin and p53 interact in the nucleus as well as in the cytoplasm.
These results suggested that plakoglobin and p53 may coordinate ly 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 promoter (Supplementary
Table II) showed that both plakoglobin and p53 associated with the 14-3-3σ promoter in SCC9-
PG cells only (Fig. 4A, 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 (Fig. 4A, 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., 2007). In agreement with the coimmunoprecipitation data, we did not observe an association between the 14-3-3σ gene promoter and β-catenin in any cell line (Fig. 4B). 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. 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 NFI gene (negative control), and no amplification was observed, whereas the same p53 ChIP sample clearly amplified the 14-3-3σ promoter in both SCC9-PG and MCF-10-2A cells (Fig. 4C).

Plakoglobin binds the p53-consensus sequence in the 14-3-3σ promoter. Since plakoglobin interacts with p53 and regulates 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 corresponds 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 (Fig. 5A, Lane 2) that was inhibited by the addition of a specific oligonucleotide competitor (unlabeled probe in 50-fold excess; Fig. 5A, Lane 6) but not by a non-specific oligonucleotide (corresponding to the NFY gene; Fig. 5A, 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 (Fig. 5A, Lane 3). When plakoglobin antibodies were added to the reaction mixture, a supershift was observed (Fig. 5A, Lane 4), whereas the addition of IgG to the reaction mixtures had no effect on the band shift (Fig. 5A, Lane 5). In contrast to plakoglobin antibodies, when β-catenin antibodies were added to the reaction mixtures, no effect was observed (Fig. 5B, Lane 3).

Similarly, in Fig. 6, 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 (Fig. 6, Lane 2) that was inhibited by the addition of a specific competitor but not by a non-specific oligonucleotide (Fig. 6, 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 (Fig. 6, 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 (Fig. 6, 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 (Fig. 6, Lanes 5 and 6).

**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; Supplementary Table III). SCC9 cells transfected with the control vector, wild-type p53 or mutant p53 containing plasmids showed minimal luciferase activity regardless of the plasmid (Fig. 7A, 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 (Fig. 7A, 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 plasmids (Fig. S1A-B), suggesting that mutant p53 could bind to both the wild type and mutant response element and is 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 (Fig. S1C) in these cells.

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 (Fig. 7B) 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 (Fig. 7C). In MCF-7 cells, knockdown of plakoglobin resulted in a significant (~21-fold) decrease in luciferase activity from the wild-type construct (Fig. 7C, PG shRNA), demonstrating that p53 transcriptional activity is enhanced in the presence of plakoglobin.

To verify that plakoglobin’s regulation of p53 transcriptional activity is 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 (Fig. 7D), while luciferase activity was significantly induced only from the wild-type plasmid in MCF-7 cells (Fig. 7E, MCF-7). Finally, knockdown 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 (Fig. 7E, MCF-7 shPG).
Discussion

Our microarray studies identified several p53-target genes whose levels were altered upon plakoglobin expression in SCC9 cells. Among these 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., 2007), 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 interact 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 $\beta$-catenin and p53, a relationship between them exists whereby p53 regulates the stability of $\beta$-catenin, via the upregulation of the ubiquitin ligase Siah-1, which in turn degrades $\beta$-catenin. Furthermore, $\beta$-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 interact in both the cytoplasm and nucleus suggested that perhaps these proteins regulate gene expression concurrently. ChIP and EMSA experiments showed that plakoglobin and p53 are both associated with the 14-3-3$\sigma$ gene promoter (Figs 4-6). 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., 2007; 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., 2007; 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 $\beta$-catenin than with its own independent function. More recently, it has been shown that in addition to regulating the signaling activity of $\beta$-catenin itself, plakoglobin is also capable of regulating $\beta$-catenin oncogenic signaling by interacting with and promoting the nuclear export of the transcription factor SOX4, which interacts with $\beta$-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, 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 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., 2007). 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 interact in the cytoplasm as well as the nucleus (Fig. 3), 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 associates 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 (Fig. 3). 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 (Supplementary Table V). 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 cells (Lin and Chang, 1996; Wu et al., 2011). Following its expression, the
mutant p53 protein accumulated in 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 (Fig. S2). Collectively, these results suggest that this mutant p53 is capable of regulating 14-3-3σ 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 (Fig. 4). 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 (Fig. 7A,D). 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 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 knockdown 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 (Fig. S1B), suggesting that the mutant p53 protein in these cells is 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 (Fig. S1C). 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 suggest that in addition to wild-type p53-binding sequences, the mutant p53 protein in SCC9-PG cells can 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 (Nielsen 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; Juntila 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 (Fig. 7C,E), demonstrating that wild-type p53 can only activate gene expression from wild-type p53-binding sequences. Interestingly, knockdown 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 (Fig. 1D). Furthermore, MCF-7 shPG transfectants had lower levels of p53 compared to parental MCF-7 cells (Fig. 7B). 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 (Fig. 4A).
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 Fig. 3, 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 (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 (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 report is the first demonstration of 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 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.

Materials and Methods

Cell culture and conditions

All tissue culture reagents were purchased from Invitrogen (Burlington, Canada) unless stated otherwise. The human tongue squamous cell carcinoma cell line SCC9, plakoglobin-expressing SCC9, MCF-7, MCF-10-2A and SW620 cells have been described previously (El-Bahrawy et al., 2004, Aktary et al., 2010; Lam et al., 2010). The vulvar carcinoma cell line A431 was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and was maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 1% glucose and 1% antibiotics. The p53-null H1299 lung carcinoma cell line (Lin and Chang, 1996; Wu et al., 2011) was maintained in Minimum Essential Medium supplemented with 10% FBS and 1% antibiotics.

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.

Antibodies

A list of antibodies and their respective dilutions in specific assays is presented in Supplementary Table IV.
RNA isolation and RT-PCR

RNA was isolated from 150 mm confluent 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 DNase I 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 are outlined in Supplementary Table II. 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.

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.

Immunoprecipitation

Confluent cultures (150 mm) were washed twice with cold PBS containing 1mM NaF, Na$_3$VO$_4$ 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$_3$VO$_4$ 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, to which antibodies and 40 μl protein A Sepharose CL-4B beads (Pierce, Nepean,
Canada) were added 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 50 μl 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,
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 MgCl2, 1 mM DTT, 0.2% Nonidet P-40, 1 mM NaF, 1
mM Na3VO4 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 MgCl2, 1 mM
DTT, 1 mM NaF, 1 mM Na3VO4 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.

Chromatin Immunoprecipitation
Chromatin immunoprecipitation experiments were performed as previously described in detail (Peng and Jahroudi, 2003). Briefly, cells were fixed, lysed, sonicated and samples were immunoprecipitated overnight at 4°C. Following immunoprecipitation, the supernatants were removed and the beads washed extensively. After washing, the protein-DNA complexes were eluted and the DNA purified and processed for PCR as described above.

**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, Na₃VO₄ and CaCl₂, 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.

**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 ³²P-ATP (adenosine 5'-triphosphate; Perkin Elmer). Nuclear extracts (5 μg) 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 MgCl₂, 5 mM EDTA, 5% glycerol and 1 μg 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 polyacrylimide gels and exposed to film overnight.

**Luciferase Reporter Assays**

Confluent 35 mm cultures were transfected with 4 μg of luciferase reporter plasmids downstream of either the wild-type p53-binding sequence within the SFN gene or a consensus p53 sequence, mutants of these sequences, or control vectors (Supplementary Table III; 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. Forty-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.

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References


Aktary et al. 30


Figure Legends

Fig. 1. 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. SCC9 and SCC9-PG cell extracts were processed for immunoprecipitation using plakoglobin or p53 or preimmune antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to plakoglobin and p53.

E. SCC9 and SCC9-PG cell extracts were processed for immunoprecipitation using β-catenin or p53 or preimmune antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with antibodies to β-catenin and p53.

**Fig. 2. 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. TCE, total cell extract.

**Fig. 3. 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.

**Fig. 4. 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 NFI and 14-3-3σ (C) primers. As positive control, total cellular DNA (Input) was amplified using the same primers. PG, plakoglobin.

**Fig. 5. 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.

**Fig. 6. 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.

**Fig. 7. Plakoglobin regulates 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 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. 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.

C. MCF-7, MCF-7 shControl and shPG cells were transfected with luciferase reporter constructs as in (A).

D. SCC9 and SCC9-PG 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 from a minimum of three independent transfections and normalized for transfection efficiency by co-transfection with a β-galactosidase expression vector.

E. MCF-7 and shPG cells were transfected with luciferase reporter constructs as in (D) (*p < 0.01, as compared to SCC9 or 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.
Aktary et al., Figure 1
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Aktary et al. Figure 4
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