HHS Public Access

Author manuscript

Cell Stem Cell. Author manuscript; available in PMC 2015 March 25.

Published in final edited form as:

Cell Stem Cell. 2011 February 4; 8(2): 164–176. doi:10.1016/j.stem.2010.12.009.

ΔNp63α is an oncogene that induces Lsh expression and promotes stem-like proliferation

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SUMMARY

The p53 homolog p63 is essential for development, yet its role in cancer is not clear. We discovered that p63 deficiency evokes the tumor suppressive mechanism of cellular senescence, causing a striking absence of stratified epithelia such as the skin. Here we identify the predominant p63 isoform, ΔNp63α, as a protein that bypasses oncogene induced senescence to drive tumorigenesis *in vivo*. Interestingly, bypass of senescence promotes stem-like proliferation and maintains survival of the keratin 15-positive stem cell population. Furthermore, we identify the chromatin remodeling protein Lsh as a new target of ΔNp63α that is an essential mediator of senescence bypass. These findings indicate that ΔNp63α is an oncogene that cooperates with Ras to promote tumor-initiating stem-like proliferation, and suggest that Lsh-mediated chromatin remodeling events are critical to this process.

INTRODUCTION

Deregulation of intrinsic tumor protective mechanisms can convert normal epithelia into malignant carcinoma, and lies at the heart of the majority of human cancers. p53 is a pivotal regulator that protects from such events. Therefore, the discovery of the p53 family members p63 and p73 generated much interest that these proteins would also act to prevent cancer.

However, with regards to p63, a more complicated picture has emerged. p63 is expressed robustly in stem and progenitor cells within the proliferating basal layer of stratified epithelia such as the skin, breast and prostate. p63's critical importance in epithelia is highlighted by p63 deficient mouse models. In contrast to the viable and fertile, yet tumor-prone phenotype of p53 deficient mice (Donehower et al., 1992; Jacks et al., 1994), p63

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deficient mice die postnatally from severe developmental anomalies, including a lack of skin and associated derivatives, an absence of glands such as prostate and breast, and truncation of limbs (Mills et al., 1999; Yang et al., 1999). We previously circumvented this postnatal lethality by generating a p63 conditional mouse model (Mills et al., 2002), and reported that inducible ablation of p63 in primary keratinocytes in culture induces a program of cellular senescence, and that ablation of p63 in proliferating cells of the adult epidermis *in vivo* induces cellular senescence and accelerated aging, establishing that p63 deficiency-mediated cellular senescence is a cause of organismal aging (Keyes et al., 2005).

Despite these advances, the role of p63 in cancer has remained perplexing, in large part because multiple p63 isoforms exist. p63 proteins can be classified into two main groups, each of which is transcribed from distinct promoters: those possessing a full-length transactivation domain analogous to that of p53 (referred to as the TA isoform class), and those lacking this domain (referred to as the ΔN isoform class) (Yang et al., 1998). Alternative splicing at the carboxy terminus generates three splice variants (α, β, γ) within each group. The tumorigenic potential of p63 deficient mice has surprisingly not helped to clarify the role of p63 in cancer, as different groups have reported disparate results (Flores et al., 2005; Keyes et al., 2006). In addition, the p63 mouse models used for tumor studies thus far are deficient for all p63 isoforms, therefore the contribution of individual p63 proteins in cancer may have been masked in these studies. Indeed, it has been suggested that the TA isoforms are most similar to p53, whereas the ΔN isoforms have opposing functions. ΔNp63α is the predominant isoform expressed in stem cells as well as in proliferating basal cells of stratified epithelia (Yang et al., 1998). ΔNp63α is overexpressed in some types of human cancer, particularly squamous cell carcinoma (SCC) (Hibi et al., 2000; Tonon et al., 2005), and it has been suggested to have oncogenic functions through inhibition of p53 (Yang et al., 1998). However, in other tumor types, such as adenocarcinoma of the breast and prostate, ΔNp63α expression is lost during the tumorigenic process (Di Como et al., 2002).

Thus, the role of p63 in cancer, and in particular the role of individual p63 isoforms, merits further investigation. Given our previous findings that p63 deficiency induces cellular senescence, we hypothesized that p63 might also be a key modulator of the tumor suppressive mechanism of oncogene-induced senescence (OIS). We explored the role of p63 in OIS—specifically with activated Ras being the oncogene—using a system that circumvents the previously generated p63 deficient mouse models. Here we demonstrate that the predominant p63 isoform, ΔNp63α, is an oncogene that promotes stem-like proliferation and carcinoma development through induction of the chromatin remodeler Lsh.

RESULTS

Downregulation of ΔNp63α is required for Oncogene-Induced Senescence

To investigate a potential role for p63 in OIS, we induced senescence in primary mouse keratinocytes by retroviral infection with oncogenic H-Ras-V12 (hereafter referred to as Ras), and monitored p63 expression. As shown previously, Ras induced features of senescence, including proliferation arrest, a characteristic morphology and enhanced senescence-associated β-galactosidase (SA-β-gal) activity (Serrano et al., 1997) (Figure 1).

Interestingly, induction of senescence was concomitant with a significant decrease in expression of endogenous ΔNp63α expression at both the transcript and protein level, whereas expression of TAp63 isoforms was not affected (Figure 1A, Figure S1). This demonstrates that the predominant p63 isoform expressed in keratinocytes, ΔNp63α, is depleted during OIS.

To determine whether loss of ΔNp63α is a requirement for OIS, primary keratinocytes were co-infected with Ras and expression constructs encoding ΔNp63α, TAp63α, or mutant ΔNp63α (ΔNp63α^{R279H}). The R279H mutation within the DNA binding domain of p63 causes EEC syndrome in humans and corresponds to one of the most prevalent p53 point mutations found in human cancer (Celli et al., 1999). Whereas expression of ΔNp63α was sufficient to inhibit senescence and induce continued proliferation in Ras-expressing primary keratinocytes, neither TAp63α nor ΔNp63α^{R279H} had this ability (Figure 1B–D). These data demonstrate that downregulation of ΔNp63α is necessary for OIS, and that overexpression of this isoform bypasses senescence.

ΔNp63α cooperates with oncogenic Ras to promote stem-like proliferation

Keratinocytes expressing Ras and ΔNp63α (hereafter referred to as RΔN cells) had a specific cellular phenotype; whereas shortly after infection, they failed to senesce, and continued to proliferate slowly, approximately 2 weeks after infection, clonal populations of proliferating cells emerged from these cultures. Given that p63 has been proposed as a determinant of epithelial stem cells (Senoo et al., 2007), we asked whether the clonally proliferating RΔN cells that had bypassed senescence had characteristics of stem cells. We grew RΔN cells in three-dimensional (3D) culture conditions designed to assay for the self-renewal capability and proliferative potential of stem cells (Figure 2A)(Lawson et al., 2007). Whereas vector or Ras infected cells failed to proliferate in 3D culture, cells infected with ΔNp63α proliferated and formed clusters containing small numbers of cells. In contrast, the clonal RΔN cells that had bypassed senescence proliferated robustly in 3D culture, forming large multi-cellular spheres. To test whether this sphere-forming capability was a universal feature of cells that had escaped senescence, we inhibited OIS by shRNA-mediated knockdown of p53 in the presence of Ras (referred to as Rshp53 cells) and analyzed for growth in 3D culture. Rshp53 cells proliferated robustly and formed large irregular growths, but were devoid of the sphere-forming ability observed in RΔN cultures (Figure 2A). This suggested that the 3D sphere phenotype of RΔN keratinocytes resulted from a unique cooperation between ΔNp63α and Ras. Immunofluorescent analysis demonstrated that whereas RΔN spheres expressed markers of proliferating keratinocytes such as keratin 14(K14), indicating that they were relatively undifferentiated, Rshp53 cells expressed significantly lower levels of K14 (Figure 2B).

A hallmark feature of stem cells is their ability to self-renew. To assess the self-renewal potential of RΔN 3D cultures, primary spheres were either dissociated and re-plated directly into the 3D culture (data not shown), or were first expanded as a monolayer, and then replated into 3D culture (Figure S2A). In each case, RΔN cells retained their sphere forming capability, whereas Rshp53 cells did not have this property. This demonstrates that

ΔNp63α's ability to bypass senescence endows keratinocytes with intrinsic self-renewal capabilities that are characteristic of stem-like cells.

Genes whose expression is elevated in the keratinocyte stem cell populations of mouse skin have been identified (Blanpain et al., 2004; Mignone et al., 2007; Tumbar et al., 2004). Interestingly, real-time RT-PCR indicated that 3D cultures of RΔN cells expressed elevated levels of a number of stem cell markers relative to Rshp53 cells, including s100a6, Idb2, Notch and Tcf3 (Figure S3B). Using FACS analysis to identify the CD34/α-6 integrin double-positive stem cells, we found that the RΔN cultures expressed higher levels of the epithelial stem cell markers CD34 and α6-integrin than control cultures (not shown). This demonstrates that ΔNp63α acts in concert with oncogenic Ras to promote proliferation of cells with enhanced expression of stem cell-associated genes.

Another key feature of stem cells is their ability to differentiate. We asked whether RΔN cells have this capacity by taking advantage of the ability of primary keratinocytes to terminally differentiate and to withdraw from the cell cycle in the presence of elevated calcium (Dotto, 1999). Whereas wildtype keratinocytes lose their epithelial phenotype, acquire a differentiated morphology, and lose expression of proliferation markers such as PCNA upon calcium addition, cells transformed by Rshp53 fail to undergo such differentiation, proliferate robustly and readily form multiple foci (Figure 2C). Remarkably, RΔN keratinocytes were unique: they were similar to wildtype cells with regards to their ability to acquire phenotypic features of terminal differentiation in response to elevated calcium, yet in contrast to wildtype keratinocytes, they did not withdraw from the cell cycle and continued to proliferate. Consistent with these cellular phenotypes, real-time RT-PCR analyses for a panel of markers of epithelial proliferation and differentiation indicates that RΔN cells retain both proliferative and differentiation potential (Figure 2D, Figure S2C). These findings demonstrate that whereas both p53 loss and ΔNp63α overexpression facilitate bypass of the tumor suppressive mechanism of OIS, ΔNp63α-mediated bypass is unique in that it facilitates proliferation while retaining the capacity to differentiate.

The ability of ΔNp63α to enhance stem-like characteristics in the presence of oncogenic Ras could be due to expansion of the normal epithelial stem cell population, or alternatively could result from RΔN conferring stem-like properties onto the more differentiated transit-amplifying cell population. To determine if ΔNp63α was affecting the normal stem cell population, we used two different mouse models that mark two distinct CD34-positive epithelial stem cell populations of the skin *in vivo*. The first model is the Keratin 15 (K15)-GFP mouse, in which the K15 promoter drives GFP expression in stem cells of the hair follicle bulge that can give rise to all epidermal cell types (Morris et al., 2004). The second model is the Nestin-GFP mouse, in which the Nestin promoter drives GFP expression in multi-potent CD34-positive, K15-negative stem cells of the hair follicle bulge, which have the capacity to differentiate into multiple epidermal cell types, but also have neural, smooth muscle and adipogenic capacity (Mignone et al., 2007). In both models, GFP negative cells include committed, more differentiated transit amplifying cells. Although both K15 and Nestin-positive stem cell populations are prevalent in the hair follicles of the bulge region, these markers identify distinct stem cell populations in adult skin (Figure 3A). We evaluated the effect of RΔN on both models by establishing primary keratinocytes from newborn skin

from K15-GFP and Nestin-GFP mice, and infecting with control vectors or Ras plus ΔNp63α expression constructs. To evaluate whether the GFP or the non-GFP populations were being affected by ΔNp63α, we assessed the percentage of GFP-expressing cells, specifically looking both during senescence bypass, as well as during the clonal expansion stage (Figure 3B, C). As expected, the control vector did not confer a proliferative advantage to either the K15- or Nestin-marked stem cell pools, and these cells failed to proliferate 7 days following infection, as is typical for primary mouse keratinocyte cultures. In contrast, the K15-positive (but not the Nestin-positive) stem cell population was maintained in proliferating RΔN keratinocytes that had bypassed senescence, even 25 days following infection. These results indicate that ΔNp63α cooperates with oncogenic Ras to maintain the K15-positive stem cell population.

ΔNp63α is an oncogene that promotes tumor formation

Our results demonstrate that expression of ΔNp63α inhibits OIS and promotes proliferation of cells with properties of stem cells. Aberrantly proliferating stem cells, or cells that have acquired stem-like features have been suggested to be the cell-of-origin for a number of tumor types. Since mutations that impair senescence can facilitate malignancy, we asked whether primary cells infected with RΔN were transformed and tumorigenic *in vivo*. As positive controls, primary keratinocytes infected with Ras and shRNA-p53-GFP were injected subcutaneously into nude mice. Imaging and analysis of these mice revealed the growth of GFP-positive tumors with the expected high incidence for cells transformed by p53 deficiency in the context of Ras (overall tumor incidence, 17/18 sites, 94%) (Figure 4A and Table S1). Injection of cells infected with either vector, Ras alone or ΔNp63α alone did not yield any tumors, in agreement with previous results (Table S1)(Missero et al., 1996). Strikingly, RΔN cells also formed GFP-positive tumors, albeit with a lower overall incidence (12/24 sites, 50%) (Figure 4A and Table S1), indicating that ΔNp63α is an oncogene that induces tumorigenesis in cooperation with oncogenic Ras.

Histopathological analyses of tumors that developed *in vivo* revealed that RΔN expression caused tumors that were histologically distinct from those in which p53 was knocked down, even though the same preparations of cells that had been cultured under identical conditions in parallel were used. Tumors that developed from Rshp53 cells were large, rapidly growing hard masses and presented primarily as fibrosarcoma or poorly differentiated sarcoma, with features of having undergone epithelial-mesenchymal transformation (EMT) (Figure 4B, Table S1). In contrast, tumors that developed from RΔN cells were slower growing, often included smaller tumor masses surrounded by necrotic tissue, and presented predominantly as SCC, with some mice developing poorly differentiated sarcoma. Immunohistochemical analyses further demonstrated the distinction between the different tumor types, with the RΔN tumors expressing the epithelial markers K14 and β-catenin, while these epithelial markers were not detectable in Rshp53 tumors (Figure 4B, Figure S3A). Interestingly, N-cadherin, a marker of EMT, was detectable in only a few cells of RΔN tumors, but was expressed robustly throughout Rshp53 tumors (Figure S3A), suggesting that RΔN had not undergone EMT. Furthermore, whereas immunostaining for p53 and p19 showed that p53 deficient tumors had elevated p19 expression, indicating that p53-mediated feedback inhibition of p19 was impaired, RΔN tumors were also deficient for p53, but p19 was not

detectable in these tumors (Figure S3B). This further demonstrates the histological and molecular distinction between these tumors. These findings are in agreement with the high incidence and early expression of ΔNp63α in human SCC (Hibi et al., 2000), demonstrating that ΔNp63α is an oncogene capable of causing tumor development in a manner distinct from deficiency of p53.

Downregulation of p63 occurs in senescent cells of pre-malignant lesions *in vivo*

To extend our findings that p63 depletion is a prerequisite for OIS, we monitored expression of endogenous p63 *in vivo* in a model of OIS bypass-induced carcinogenesis. Topical treatment of mouse skin with DMBA induces Ras mutations, which facilitate development of pre-malignant papillomas with markers and features of OIS; inactivation of mediators of OIS facilitates senescence bypass and converts pre-neoplastic lesions to malignant SCC (Collado et al., 2005). We examined skin sections from DMBA-induced papillomas and SCC for expression of p63 and markers of senescence. p63 was detectable in premalignant lesions, as previously shown (Keyes et al., 2006), but notably was most robust in the proliferating basal layer, with expression decreasing in supra-basal layers (Figure 5), analogous to the graded pattern of p63 observed in normal skin. This contrasts markedly with p63 expression in SCC, which is abundant in transformed cells that escaped senescence and progressed to malignancy (Figure 5A). To examine whether the decrease in p63 expression in the pre-malignant lesions correlated with cells that had undergone OIS, tissues were co-stained for p63 as well as for markers of proliferation and senescence (Figure 5B). Whereas expression of p63 overlapped with the proliferation marker Ki67, it did not overlap with the senescence markers γH2AX and p19. This indicates that OIS *in vivo* occurs in p63-depleted cells, whereas cells that bypass senescence and develop into tumors express p63 robustly. The finding that the patterns of expression of p63 and markers of senescence are mutually exclusive *in vivo* confirms and extends our findings that downregulation of endogenous ΔNp63α is required for OIS.

Lsh is a new p63 target gene

To elucidate the mechanism by which ΔNp63α inhibits OIS, we examined primary keratinocytes that had been infected with RΔN or control vectors shortly following infection (see schematic, Figure 6A). This time point precedes the development of distinct cellular phenotypes and was chosen because we wanted to identify ΔNp63α-modulated pathways that initiated senescence bypass and led to stem-like proliferation and tumorigenesis. We examined expression of known senescence mediators at the transcript and protein level in RΔN cells; however, p53, p16, p19, p21 and PML were not reduced during the initial stages of senescence bypass (Figures 6B, Figure S4A). These analyses not only indicate that the initiating events by which ΔNp63α inhibits senescence does not likely occur via these pathways, it suggests that additional as yet uncharacterized senescence pathways are modulated by ΔNp63α.

To identify genes whose expression was altered by ΔNp63α expression and hence might reveal novel ΔNp63α-mediated pathways facilitating senescence bypass, we performed cDNA microarray analyses on primary keratinocytes at an early stage following infection with RΔN and controls. This led to the identification of 26 genes whose expression was

either increased (23 genes) or decreased (3 genes) in RΔN cells relative to controls (Figure S4B). Real-time RT-PCR confirmed that expression of a number of these genes was altered in RΔN cells, indicating that these genes are induced during the initial phase of senescence bypass.

To determine whether any of the candidate genes were direct targets of ΔNp63α, we analyzed the regions upstream of the transcriptional start sites for p53/p63 consensus binding sites. We identified a p63-consensus binding site in the mouse Lsh (lymphoid-specific helicase) (Figure S5A), which encodes a member of the SNF2 family of chromatin remodeling proteins. Like p63, Lsh has been implicated in both embryonic development and cellular senescence (Geiman et al., 2001). Analysis of the human LSH promoter identified putative p63 consensus binding sites similar to those described previously (Figure S5B) (Yang et al., 2006). To determine whether p63 binds the Lsh promoter, we performed chromatin immunoprecipitation (ChIP) in RΔN cells using an antibody specific for p63. Primers for p21, a previously identified ΔNp63α target gene were used as a positive control (Figure 6C). Using multiple primer pairs flanking the p63 consensus binding site identified in Lsh, we found that p63 bound this region, whereas under similar conditions, we did not detect binding to the promoter region of 2 other genes (Figure 6C, Figure S5C). The p63 antibody used for ChIP was raised against a peptide common to each of the six p63 isoforms (Yang et al., 1998). To determine which p63 isoform was likely directly regulating Lsh, we performed western analyses of lysates that had been immunoprecipitated with the p63 antibody. This analysis revealed that ΔNp63α is the predominant isoform detectable in these cells (Figure S5D), supporting the idea that Lsh is a direct target of this isoform.

To determine whether ΔNp63α and Lsh are expressed in the same cell type in an *in vivo* setting, we assessed the pattern of Lsh expression in embryonic day 17.5 skin, a time point when ΔNp63α levels are especially robust (Figure 6D). Co-immunostaining revealed a clear overlap between p63 and Lsh expression in proliferating basal keratinocytes of the epidermis, further supporting that Lsh is a target of ΔNp63α. To assess whether ΔNp63α is required for Lsh expression, we ablated p63 in primary keratinocytes obtained from the p63 conditional mouse model that we described previously (Keyes et al., 2005; Mills et al., 2002). Indeed, Cre-mediated disruption caused loss of p63, while simultaneously reducing Lsh expression at the transcript level (Figure 6E). These findings demonstrate that the pattern of Lsh expression overlaps with that of ΔNp63α *in vivo*, and that Lsh expression is dependent upon p63 expression, providing additional evidence for Lsh being a direct target of ΔNp63α in primary keratinocytes.

Lsh is required for ΔNp63α-mediated senescence bypass

Having identified Lsh levels as being upregulated in RΔN cells at the transcript level, we examined Lsh expression at the protein level. We prepared protein lysates from ΔNp63α, RΔN and control cells six days following infection, at a time point prior to senescence bypass when all cultures were phenotypically similar (see Figure 6A). Western analyses indicated that Lsh protein levels were higher in cells overexpressing ΔNp63α (Figure 7A). In addition, we found that in contrast to control cultures, RΔN keratinocytes could be maintained for an extended time in culture, and these cells expressed robust levels of Lsh

(Figure S6A). These findings further support our observations that ΔNp63α mediates Lsh expression, and that Lsh is induced prior to, and is maintained following, senescence bypass.

Our findings identify *Lsh* as a novel p63 target whose expression is induced during senescence bypass and tumor initiation. To determine whether Lsh is required for ΔNp63α-mediated inhibition of OIS, we generated multiple shRNAs for Lsh. We identified one hairpin (977) that was capable of reducing Lsh levels by about 95% (Figure S6B, Figure 7B). To test the specificity of this hairpin, we assayed for Lsh activity by assessing reactivation of repetitive elements throughout the genome—integrated retrotransposons silenced by Lsh (Figure S6C) (Sun et al., 2004). A number of repeat elements (including ERVL, MaLR, Major and minor satellites, LINE1) were significantly upregulated in Lsh depleted cells, providing functional evidence that Lsh activity is compromised by RNAi-mediated knock down.

To functionally test whether Lsh was required for ΔNp63α-mediated bypass of senescence, keratinocytes were infected with combinations of vector controls, Ras, ΔNp63α and shRNA-Lsh and the extent of RΔN-mediated bypass of senescence was evaluated (Figures 7B, C). Interestingly, whereas knockdown of Lsh in vector control cells impaired proliferation (Figure 7C), in agreement with the finding that deficiency of Lsh induces senescence *in vivo* (Sun et al., 2004), knockdown of Lsh in the context of ΔNp63α produced a stronger senescent-like morphology, irrespective of Ras expression, a finding consistent with *Lsh* being a target of ΔNp63α. In agreement, a specific hairpin that did not affect Lsh levels did not have this effect (not shown). BrdU incorporation shortly after infection indicated that proliferation was significantly compromised when Lsh was knocked down in the presence of ΔNp63α, with remaining cells failing to proliferate when maintained for longer periods. These data indicate that the ΔNp63α target Lsh is an essential mediator of senescence bypass.

To assess the relationship between endogenous p63 and Lsh in senescence inhibition and tumorigenesis in an *in vivo* setting, we analyzed the pattern of Lsh expression in DMBA-induced pre-malignant papillomas. Whereas Lsh was not detectable in normal skin of these adult animals (Figure S7A), it was robustly expressed in the p63-positive proliferative zone of pre-malignant lesions; in contrast, Lsh was not detectable in the p63-negative senescent zone (Figure 7D). This co-expression of p63 and Lsh in chemically induced carcinomas of the skin was in agreement with our analyses of RΔN and Rshp53 tumors obtained in nude mice, as RΔN tumors had significantly higher expression of Lsh (Figure 7E). To examine whether ΔNp63α and LSH expression are also co-regulated in human carcinomas, we analyzed HNSCC cell lines previously shown to rely on enhanced ΔNp63α expression (Rocco et al., 2006). Interestingly, in these cell lines that overexpress ΔNp63α, we found that LSH is also upregulated relative to normal human keratinocytes, supporting our observations in made in mouse cells (Figure 7F, Figure S7B). These data demonstrate that ΔNp63α induces expression of Lsh and support our findings that ΔNp63α-mediated Lsh expression bypasses Ras-induced senescence and promotes stem-like proliferation, setting the stage for carcinoma development *in vivo*.

DISCUSSION

Our study identifies ΔNp63α as an oncogene in the skin, capable of inhibiting oncogene induced senescence and promoting development of squamous cell carcinoma. There has been much confusion about the role of p63 in tumorigenesis. Unlike p53, point mutations in p63 are rarely associated with cancer, but cause a spectrum of developmental birth defects (Brunner et al., 2002). In further contrast to p53, the predominant p63 isoform detectable in proliferating epithelia, ΔNp63α, is frequently overexpressed in a variety of human tumors, particularly SCC of the skin, head and neck, and lung (Hibi et al., 2000; Tonon et al., 2005), although an oncogenic role for ΔNp63α had not been demonstrated previously. In the context of cultured cells, ΔNp63α was suggested to promote oncogenesis by inhibiting p53 activity (Yang et al., 1998), though this has not been demonstrated *in vivo*. However, we found that primary epithelial cells transformed with these different genetic lesions give rise to cells and tumors with different properties, suggesting that ΔNp63α promotes tumor development through p53-independent mechanisms. Our data now conclusively shows that gain of ΔNp63α activity is a causative lesion that cooperates with oncogenic Ras to promote tumorigenesis. Furthermore, the ability of ΔNp63α to inhibit OIS provides mechanistic insight that explains why overexpression of this isoform in human tumors is an early and highly penetrant event.

We had previously discovered that the apparent tumor protection in p63-compromised mice comes at the expense of reduced lifespan (Keyes et al., 2005). Furthermore, conditional ablation of p63 in stratified epithelia does not induce tumorigenesis (Mills et al., 2002) (Keyes and Mills, personal observation), but in contrast, activates the tumor suppressive mechanism of cellular senescence, interestingly, with features of accelerated aging (Keyes et al., 2005). This provided functional evidence that cellular senescence and aging are linked *in vivo*, and exemplified p63's role in this process. Since all p63 proteins were ablated in these studies, the contribution of individual p63 isoforms was not established. We hypothesized that loss of ΔNp63α was responsible for p63 deficiency-induced cellular senescence, an idea supported by the finding that ΔNp63α regulates epithelial stem/progenitor cells within the epidermis (Senoo et al., 2007). A recent report suggests that the TAp63 isoforms also function to regulate senescence within a different stem cell niche, inhibiting cellular senescence in skin-derived progenitor cells (SKPs) (Su et al., 2009)—a progenitor population that gives rise to neuronal and mesenchymal cell types, but not to epithelial cell types such as keratinocytes (Fernandes et al., 2008; Toma et al., 2005). In yet another setting —fibroblasts—TAp63 isoforms induce cellular senescence in a p53-independent manner (Guo et al., 2009). These data highlight the roles of p63 in specific stem/progenitor cell populations of distinct tissues, as we now show occurs in tumor-initiating stem-like cells. The findings that different p63 isoforms regulate cellular senescence differently within diverse settings suggests that although senescence is a program that has common mediators and pathways, there are underlying tissue-specific mechanisms at play.

A major finding of this study is that it identifies ΔNp63α as a new inhibitor of OIS. OIS is a tumor suppressive mechanism that curtails aberrant proliferation of cells responding to DNA damage or oncogenic signaling, a program mediated predominantly by tumor suppressors such as p53, p16 and Rb (Narita and Lowe, 2005). We had previously linked p63 deficiency

to cellular senescence (Keyes et al., 2005) and more recently found that TAp63 isoforms are essential inducer of OIS that halts both the initiation and progression of Ras-driven p53 deficient tumors *in vivo* (Guo et al., 2009). This ability of TAp63 to induce OIS in mesenchymal cells contrasts vividly with the potent senescence-inhibitory activity of ΔNp63α in epithelial cells presented here, underscoring the unique role of different p63 isoforms in distinct cellular contexts.

We used an unbiased genetic screen to uncover mechanisms by which ΔNp63α bypasses senescence and induces stem-like proliferation. This approach revealed a number of interesting candidates, including stem cell associated genes (Brca1, Geminin, Lsh, TGFβ), DNA damage and repair genes (Brca1, Chk1, Exo1, Rad51, Cdc6) and others, suggesting that ΔNp63α affects cellular proliferation on several levels. Interestingly, mouse models deficient for three of these candidates have phenotypes of cellular senescence or organismal aging: Lsh (Sun et al., 2004), Exo1 (Schaetzlein et al., 2007) and Brca1 (Cao et al., 2003). Here we identify Lsh, a chromatin remodeler of the SNF2 family, as a novel p63 target required for ΔNp63α-mediated senescence bypass. Lsh plays a key role in senescence and aging, with Lsh-deficient mice exhibiting either postnatal lethality (Geiman et al., 2001) or accelerated aging and enhanced cellular senescence (Sun et al., 2004). Lsh regulates DNA methylation and transcriptional silencing (Muegge, 2005), as its deficiency causes DNA hypomethylation, altered histone modification, alterations in heterochromatin (Dennis et al., 2001; Muegge, 2005). In particular, Lsh is required for methylation-induced silencing of repeat elements and transposons in the pericentromeric regions (Huang et al., 2004; Sun et al., 2004), and also causes transcriptional silencing of individual loci including *Cdkn1c/p57* (Sun et al., 2004) and *Hox* genes (Xi et al., 2007). Lsh functions by recruiting DNA methyltransferases (Myant and Stancheva, 2008), or through direct interaction with members of the Polycomb repressive complex 1 (Xi et al., 2007). Although the mechanism by which ΔNp63α-driven Lsh expression facilitates senescence bypass and tumor development remain to be elucidated, a recent report that Lsh is overexpressed in human HNSCC (Gemenetzidis et al., 2009) supports our finding that ΔNp63α promotes tumorigenesis via induction of Lsh-mediated bypass of OIS.

Another major finding of this work is that ΔNp63α's oncogenic role is associated with its ability to maintain the K15-positive stem cell population. There is accumulating evidence that p63, and in particular the ΔNp63α isoform, maintains the proliferative capacity of epithelial stem cells of the skin, thymus, prostate, breast and eye (Pellegrini et al., 2001; Senoo et al., 2007; Xin et al., 2007). However, the mechanisms by which p63 mediates stem-like proliferation has not been established. Our identification of Lsh, a chromatin remodeling protein robustly expressed in human embryonic stem cells (Assou et al., 2007), as a ΔNp63α target suggests that p63 plays a role in epigenetic regulation of stemness. Our findings also raise interesting questions regarding the role of stem or stem-like cells in tumorigenesis. In some tumors, the cell of origin is suggested to be a normal somatic stem cell that has acquired mutations that allow it to transform (Barker et al., 2009). In other tumors, however, the cell of origin is suggested to be a more mature progenitor or committed daughter cell that acquired mutations that make it function more like a stem cell, thereby promoting tumorigenesis (Jamieson et al., 2004). Our data supports that ΔNp63α

promotes aberrant proliferation of the K15 population of stem cells in the skin, providing evidence that these are the tumor initiating population in SCC. In contrast with tumors such as pancreatic intra-epithelial neoplasia and malignant astrocytomas, in which Nestin-positive stem/progenitor cells are the source of malignancy following oncogene activation or tumor suppressor loss (Alcantara Llaguno et al., 2009; Carriere et al., 2007; Dai et al., 2001), our data suggests that ΔNp63α does not target the Nestin population in this context. Our finding that ΔNp63α affects a multi-potent stem cell population and identification of Lsh as a critical component of senescence bypass concomitant with the acquisition/maintenance of stem-like features and tumorigenesis, suggests that epigenetic events play a critical role in this process.

MATERIALS AND METHODS

Keratinocyte culture, retroviral infection and hairpin design

Primary keratinocyte cultures were prepared as previously described (Keyes et al., 2005). Differentiation was induced in media + 1.5mM $CaCl_2$ for 6–96 hours. For GFP-stem cell assays, primary keratinocytes from K15-GFP (Morris et al., 2004) and Nestin-GFP transgenic mice (Mignone et al., 2004; Mignone et al., 2007) were prepared and infected as described. Nestin-GFP and K15-GFP quantitation was performed using Guava Easycyte (Guava Technologies) and LSRII (BD Biosciences) FACS analyzer and FloJo software, respectively. Human HNSCC cells were previously described (Rocco et al., 2006). Western blotting and QRT-PCR were performed as described (Keyes et al., 2005) using primers listed (S Materials and Methods). SA-β-gal activity was performed as described (Keyes et al., 2005). Mouse Genome 430A 2.0 microarrays (Affymetrix) were used. siRNA's targeting *Lsh* (CCGTCGGAAATTAGTAAAGAA) were designed using the Biopredsi (http://www.biopredsi.org/design.html) website, cloned into LMP vectors (Dickins et al., 2005), and evaluated for efficiency in mouse embryonic fibroblasts (mefs) and assessed by western blotting 2 days alter drug selection. Keratinocyte infections were performed essentially as described previously (Keyes et al., 2005). Briefly, retrovirus was produced by transiently transfecting the ecotropic Phoenix packaging cell line (G. Nolan, Stanford University, Stanford, CA, USA). Two days alter transfection, viral supernatant was collected, filtered and added to the keratinocyte cultures. Two infections of two hours each were performed. After two days, keratinocytes were drug selected according to the different selection markers.

Three-dimensional tissue culture

Three-dimensional tissue culture was performed as previously described (Aranda et al., 2006), using 2.5% collagen:matrigel (BD biosciences) 1:1 mix as matrix. 5×10^3 cells were seeded onto 8-well chamber slides (BD Falcon) and fresh media containing collagen:matrigel was added every two days. Morphology was assessed by phase microscopy. For immunofluorescence, cultures were fixed and stained as described (Aranda et al., 2006), using anti-K14 polyclonal antibody AF64 (Covance Research Products Inc.) and Alexa Fluor Seconday antibodies (Molecular Probes).

Tumor study

Cells were injected subcutaneously into athymic nude mice as outlined in Supplementary Table 1. Tumor development was monitored by whole body GFP-imaging. Histopathology was performed by H.V. Immunohistochemistry was performed as described (Keyes et al., 2005).

Chromatin immunoprecipation

RΔN keratinocytes were fixed in 1% formaldehyde, 10′, room temperature, crosslinking stopped in 0.125 M glycine/PBS, washed in cold PBS, centrifuged 2,000 rpm, 2′, and resuspended to ~3×10^6 cells/ml in cell lysis buffer (5 mM HEPES pH 7.9, 85 mM KCl, 0.5% NP40) + protease inhibitors and incubated on ice, 15′. Homogenates were centrifuged at 5,000 rpm, 5′, 4°C, pellets resuspended in nuclear lysis buffer (50 mM Tris pH 8, 10 mM EDTA pH 8, 1% SDS) + protease inhibitors and incubated on ice, 20′. Nuclear lysates were sonicated using a Diagenode Sonicator and centrifuged at 12,000 rpm, 10′, 4°C. Supernatants were diluted in IP buffer (150 mM NaCl, 20 mM Tris pH 8, 2 mM EDTA) with Protein-A/G beads (previously pre-blocked with sheared salmon sperm DNA and BSA and pre-cleared for >2 hours at 4°C). Pre-blocked protein A/G beads were incubated with 4A4 antibody (Santa Cruz, sc-8431) or α-integrin 1α antibody (Santa Cruz, SC-8978) in IP buffer (2 hours, 4°C), centrifuged, washed with IP buffer and incubated with pre-cleared chromatin overnight at 4°C. Beads were pelleted, washed sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP40, 1% Na^{+2} deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8), TE buffer. Chromatin was eluted in elution buffer (100 mM $NaHCO_3$, 1% SDS) overnight at 65°C, collected by centrifugation, and incubated with proteinase K (30′, 37°C). DNA was extracted and PCR amplified using specific primers (see Figure S5, Supplemental Methods).

p63 consensus-binding sites *WDD**CNDG**HHD {1–25} WDD**CNDG**HHD* were identified using modifications of previously published motifs (Ortt and Sinha, 2006; Yang et al., 2006). The Lsh promoter was identified using the TRED database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and p63 consensus sites were identified ~1300 bp upstream of the TSS (38284774 of NM_008234) and ~2500 bp upstream of the TSS (47110038 of NT_030059) of mouse and human LSH, respectively (see Figure S5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Johannes Zuber, Michael Hemann and Scott Lowe for assistance and advice, Yuri Lazebnik and Dominik Duelli for DMBA-treated tissues, Chris Johns and Sohail Khan for microarray analysis, Leif Ellisen and James W. Rocco for HNSCC cell lines.

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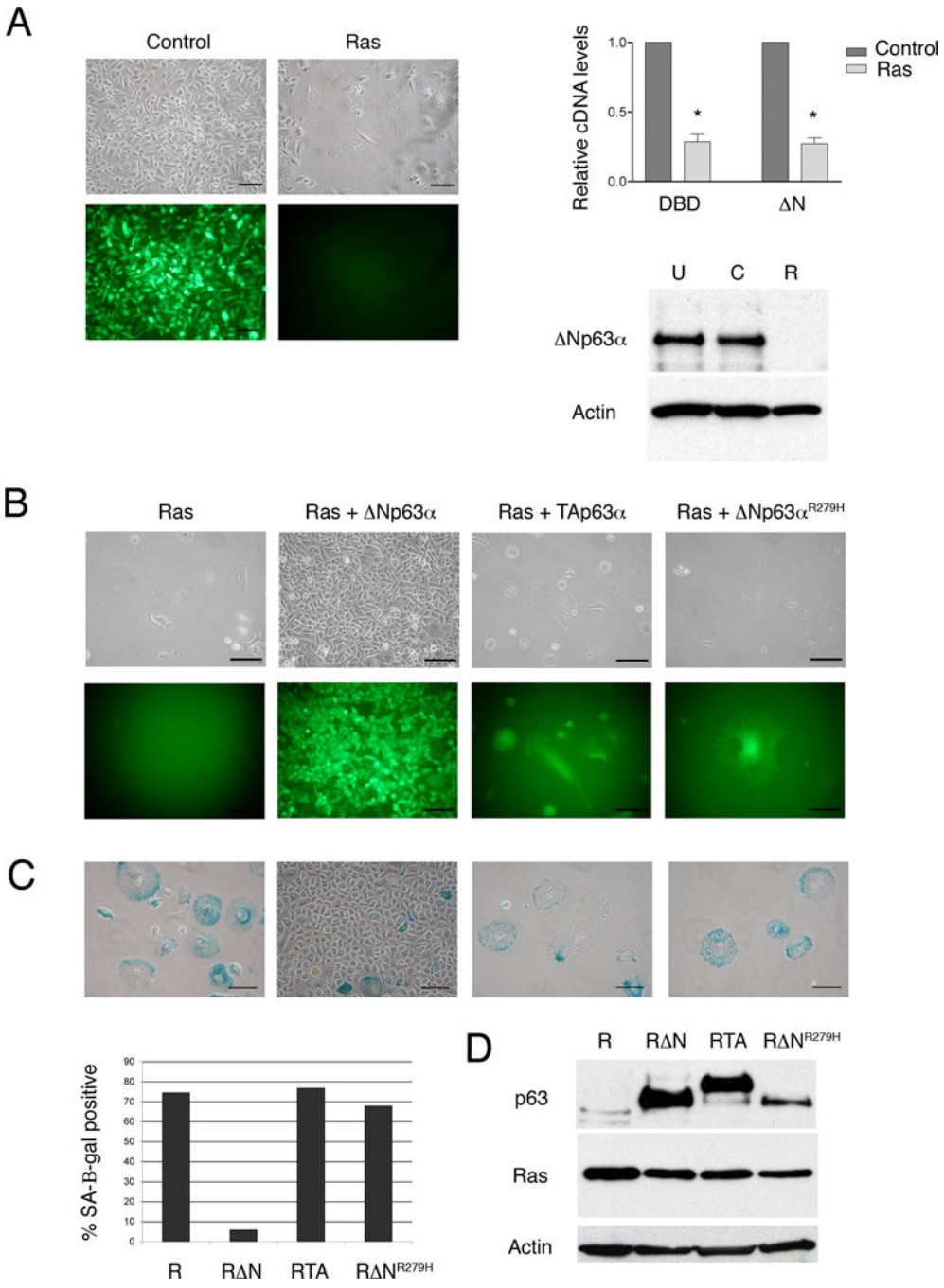

Figure 1. Downregulation of ΔNp63α is required for oncogene-induced senescence

(A) Primary mouse keratinocytes were infected with retrovirus expressing GFP-vector (control) or oncogenic HRasV12 (Ras), with Ras-infected cells undergoing oncogene-induced senescence (left). QPCR (upper right) and western-blot (lower right) analysis 7 days post-infection indicates that ΔNp63 is downregulated at both the transcript and the protein level in senescent cells. DNA binding domain, DBD; ΔN, ΔNp63 isoforms; Uninfected, U; Control, C; Ras, R; cDNA. Quantitation ± S.D. Scale bar, 50μM.

(B) Keratinocytes were infected with retroviral Ras (R), or were co-infected with Ras and GFP-expressing ΔNp63α (RΔN), TAp63α (RTA) or ΔNp63α^{R279H} (RΔN^{R279H}) constructs. Cells expressing Ras and ΔNp63α continue to proliferate. Scale bar, 50μM.
(C) Senescence-associated-β Galactosidase (SA-β-Gal) assay and quantitation shows decreased senescence in cells expressing Ras+ΔNp63α. Scale bar, 50μM.
(D) Western blotting showing expression of p63, Ras, and actin in infected cells.

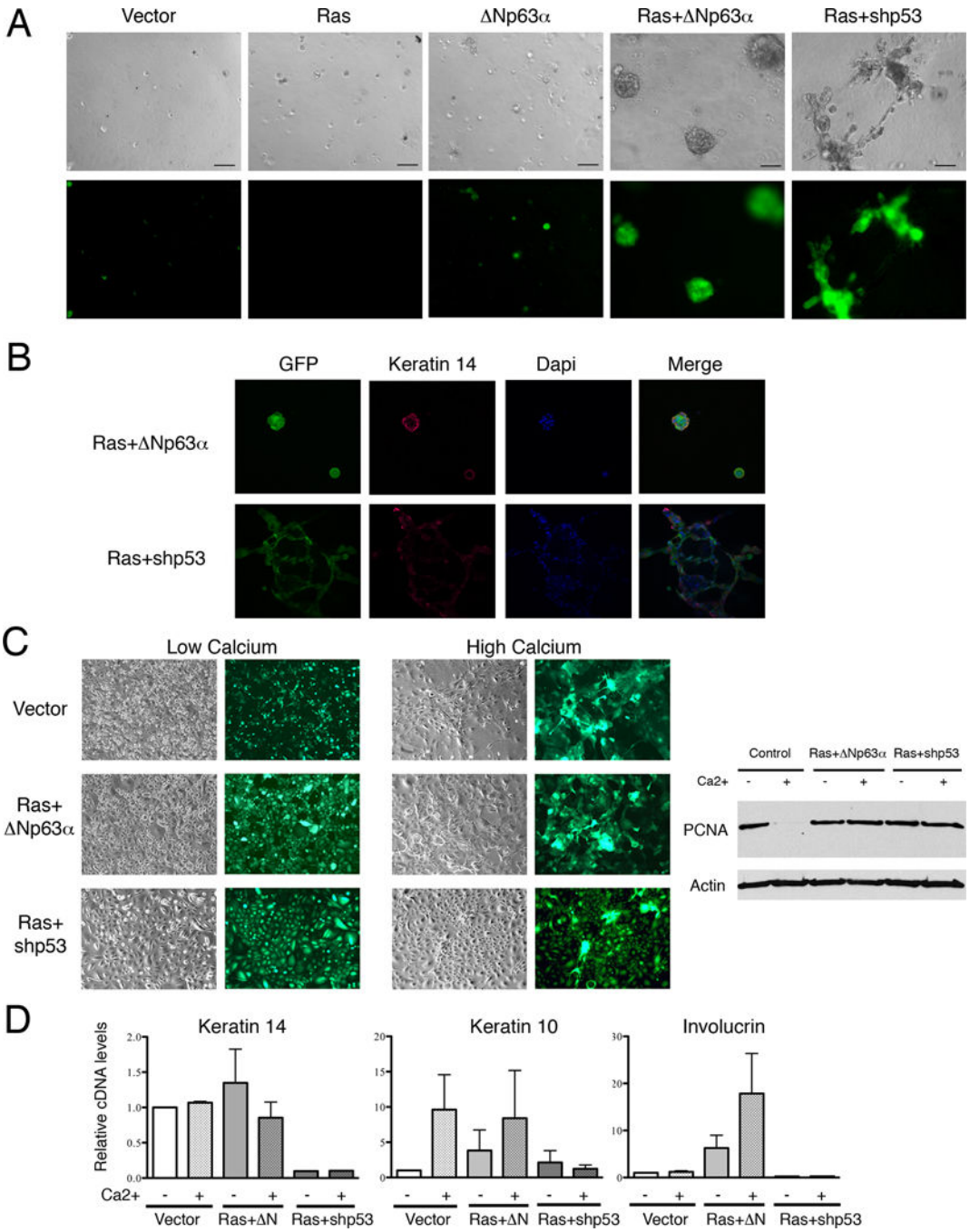

Figure 2. ΔNp63α's ability to bypass OIS leads to proliferation of cells with stem-like properties

(A) Primary keratinocytes infected with Ras+ΔNp63α-GFP form spheres in 3D-tissue culture, while cells infected with vector, Ras, ΔNp63α, or Ras and a short-hairpin specific for p53 (shp53) do not have this ability.

(B) Immunofluorescence on 3D cultures shows elevated K14 expression in Ras+ΔNp63α spheres compared to Ras+shp53 cultures.

(C) Primary keratinocytes expressing a GFP construct (vector) proliferate in low calcium media but terminally differentiate and stop proliferating in high calcium conditions, as

shown with western blot for PCNA (right). Cells infected with Ras+ΔNp63α acquire the same morphological changes with calcium treatment, but do not stop proliferating, whereas cells expressing Ras+shp53 do not acquire the same differentiation features or stop proliferating.

(D) QPCR for markers of basal epithelia (keratin 14), early differentiation (keratin 10) and late differentiation (involucrin) show cells expressing Ras+ΔNp63α differentiate in response to calcium, similar to early differentiation in vector expressing cells. Ras+shp53 cells have decreased keratin markers and do not differentiate.

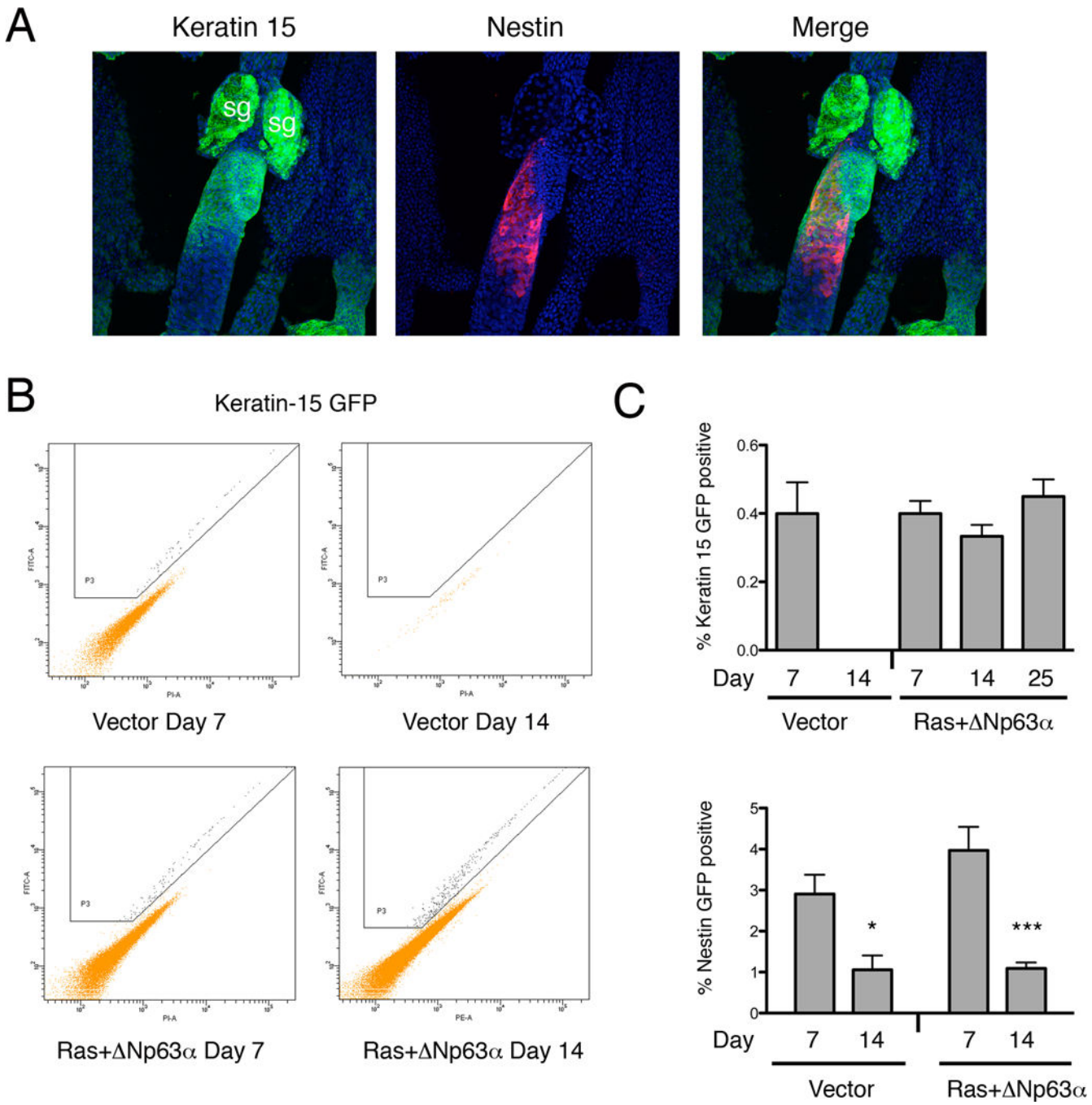

Figure 3. Ras and ΔNp63α expression maintains survival of K15-expressing stem cells

(A) Whole mount immunofluorescence of the bulge region of adult mouse skin identifies keratin15 (green)- and Nestin (red)-positive stem cell populations in the bulge region of the hair follicle. The K15-positive population is visualized with an anti-K15 antibody; the Nestin-positive population is co-visualized by immunofluorescence using a GFP-specific antibody in Nestin-GFP transgenic mice. Sebaceous glands (sg) label non-specifically.

(B) FACS analyses of primary keratinocyte cultures derived from K15-GFP transgenic mice after infection with vector or Ras+ΔNp63α. After 14 days, cells infected with vector are not

maintained and GFP-positive stem cells are depleted. However, cells infected with Ras +ΔNp63α maintain the GFP-positive stem cells 14 days after infection.
(C) Quantification of FACS experiments (K15, n=5; Nestin n=9) showing a loss of K15-positive stem cells infected with vector control after 14 days. In contrast, the GFP-positive stem cell population is maintained by Ras+ΔNp63α (top). Similar experiments with Nestin-GFP positive stem cells (bottom) are not maintained by either vector or Ras+ΔNp63α.

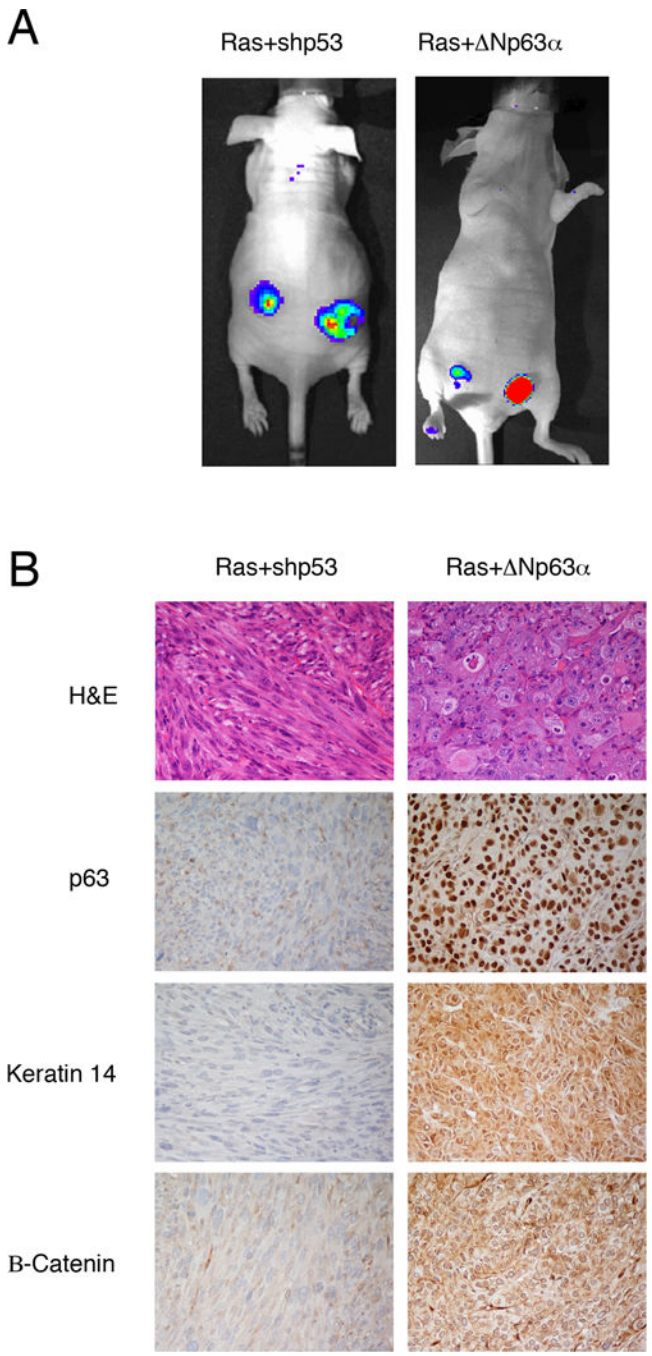

Figure 4. ΔNp63α is an oncogene that cooperates with oncogenic Ras *in vivo*

(A) Whole animal imaging of nude mice injected sub-cutaneously with primary keratinocytes expressing Ras+shp53-GFP or Ras+ΔNp63α-GFP shows the development of GFP-expressing tumors.

(B) Pathological and immunohistological analyses demonstrate distinct tumor types. Ras +shp53 tumors present mainly as fibrosarcoma lacking expression of epithelial markers, whereas Ras+ΔNp63α tumors are squamous cell carcinomas expressing p63, K14 and β-catenin.

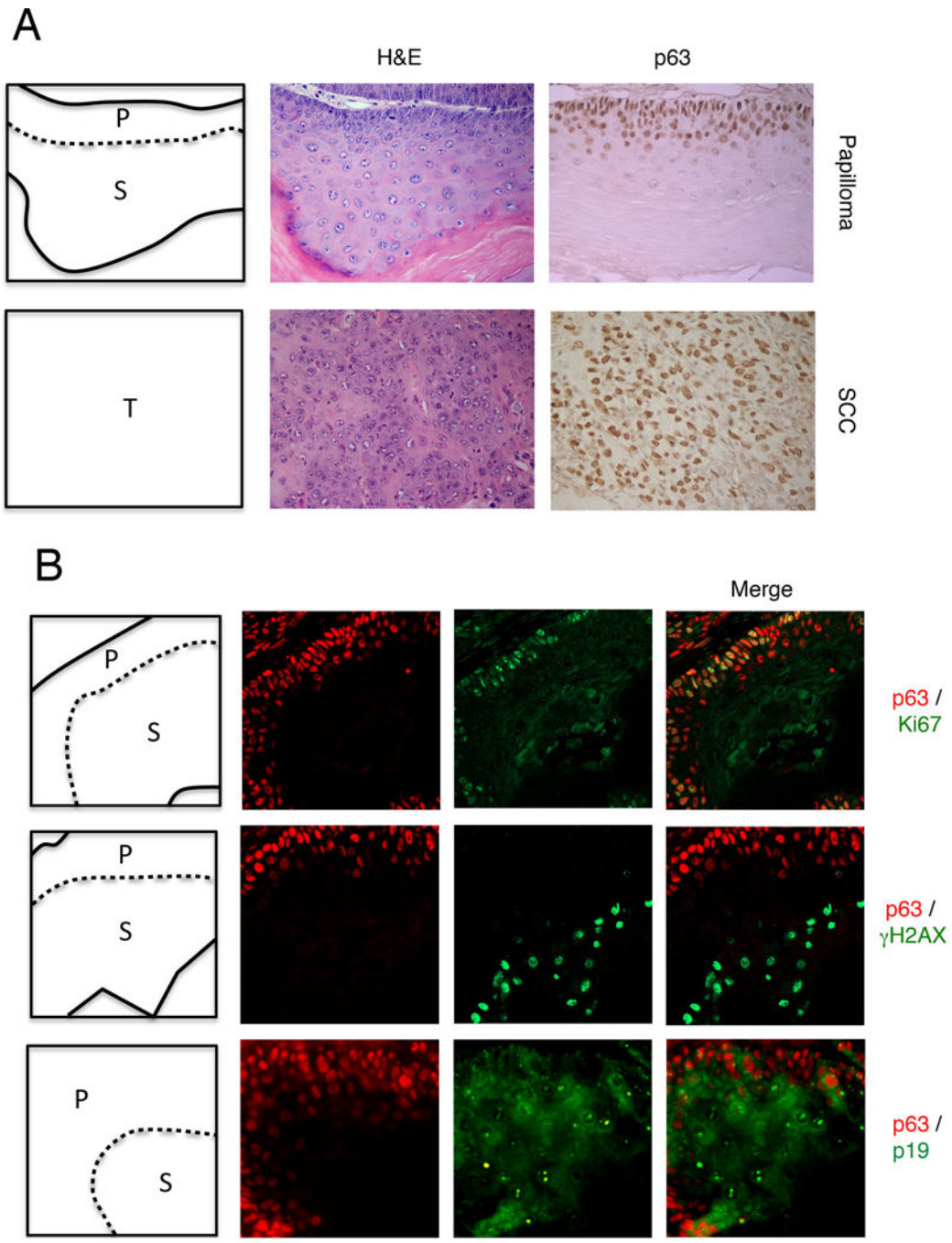

Figure 5. Downregulation of endogenous p63 occurs in senescent cells of premalignant lesions *in vivo*

(A) Papilloma and SCC were induced by treating mouse skin with DMBA. In papillomas, p63 expression is restricted to the basal layer, but decreases in the lesion, whereas p63 is expressed in all cells that progressed to squamous cell carcinoma (SCC). The schematic highlights areas of proliferation (P) and senescence (S).

(B) Immunohistochemical analyses of premalignant papillomas indicates that Ki67 and p63 are co-expressed in the basal layer, but are absent in the non-proliferative region where the

senescence markers γH2AX and p19 are expressed. The schematic highlights areas of proliferation (P) and senescence (S).

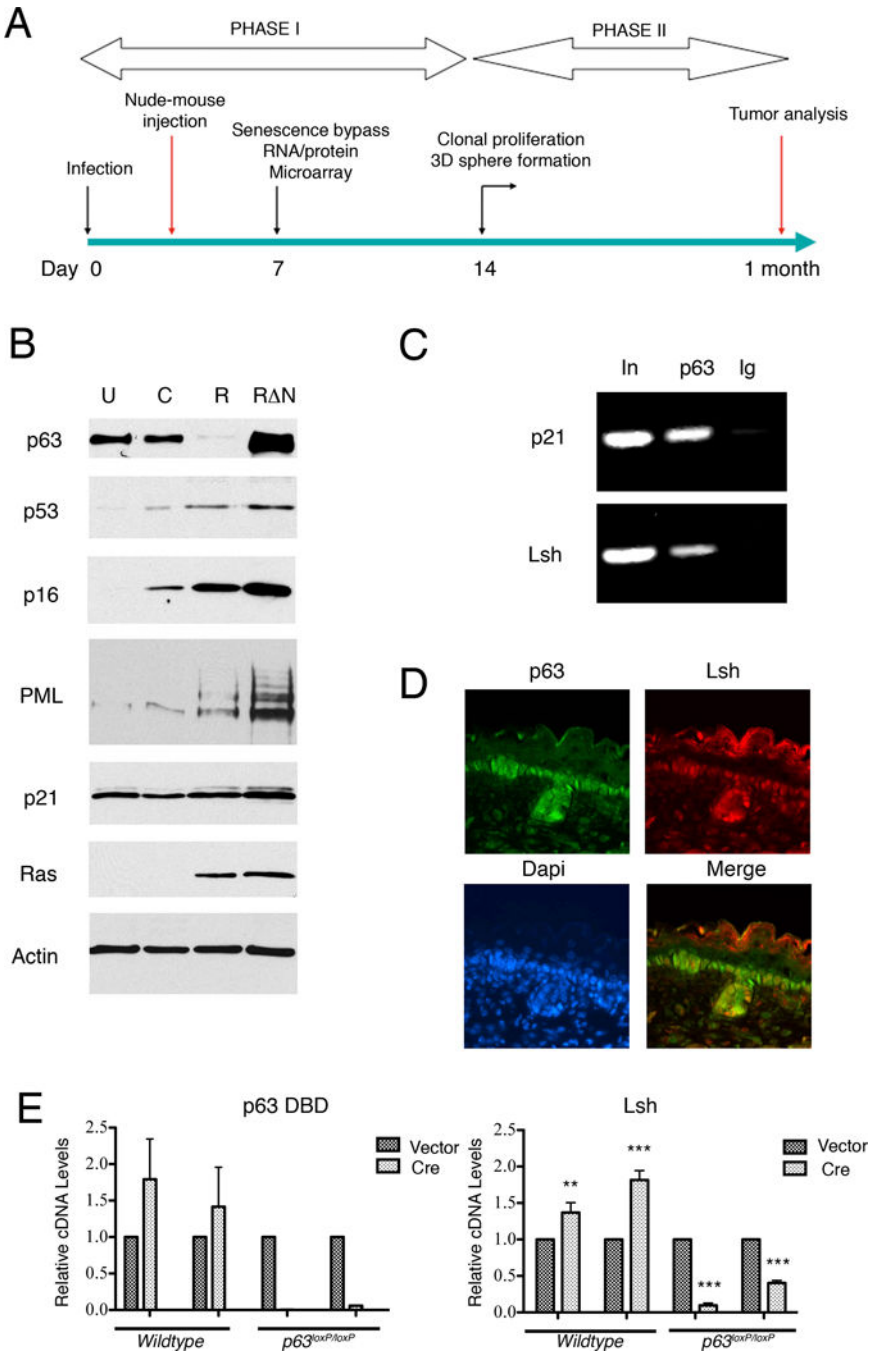

Figure 6. Lsh is a p63 target gene

(A) Timeline schematic of the experimental procedure. Phase I corresponds with initiating events leading to senescence bypass, whereas Phase II involves clonal proliferation of stem-like cells and tumor development.

(B) Western blotting for p63 and senescence mediators in cells expressing Ras+ΔNp63α during phase 1 of senescence bypass indicates that expression of p53, p16, PML, or p21 are not reduced.

(C) Chromatin Immunoprecipitation (ChIP) analysis shows p63 is bound to the Lsh promoter. p21 binding serves as a positive control. (Input, In; Immunoglobulin negative control, Ig).

(D) Immunofluorescence for p63 and Lsh in E17.5 mouse skin shows overlap in the basal epithelia.

(E) Primary mouse keratinocytes from 2 individual wildtype and $p63^{loxP/loxP}$ mice were infected with retroviral vector or Cre. Expression of p63 DNA-binding domain (DBD) is lost in $p63^{loxP/loxP}$ cells with Cre, and Lsh expression is decreased coordinately.

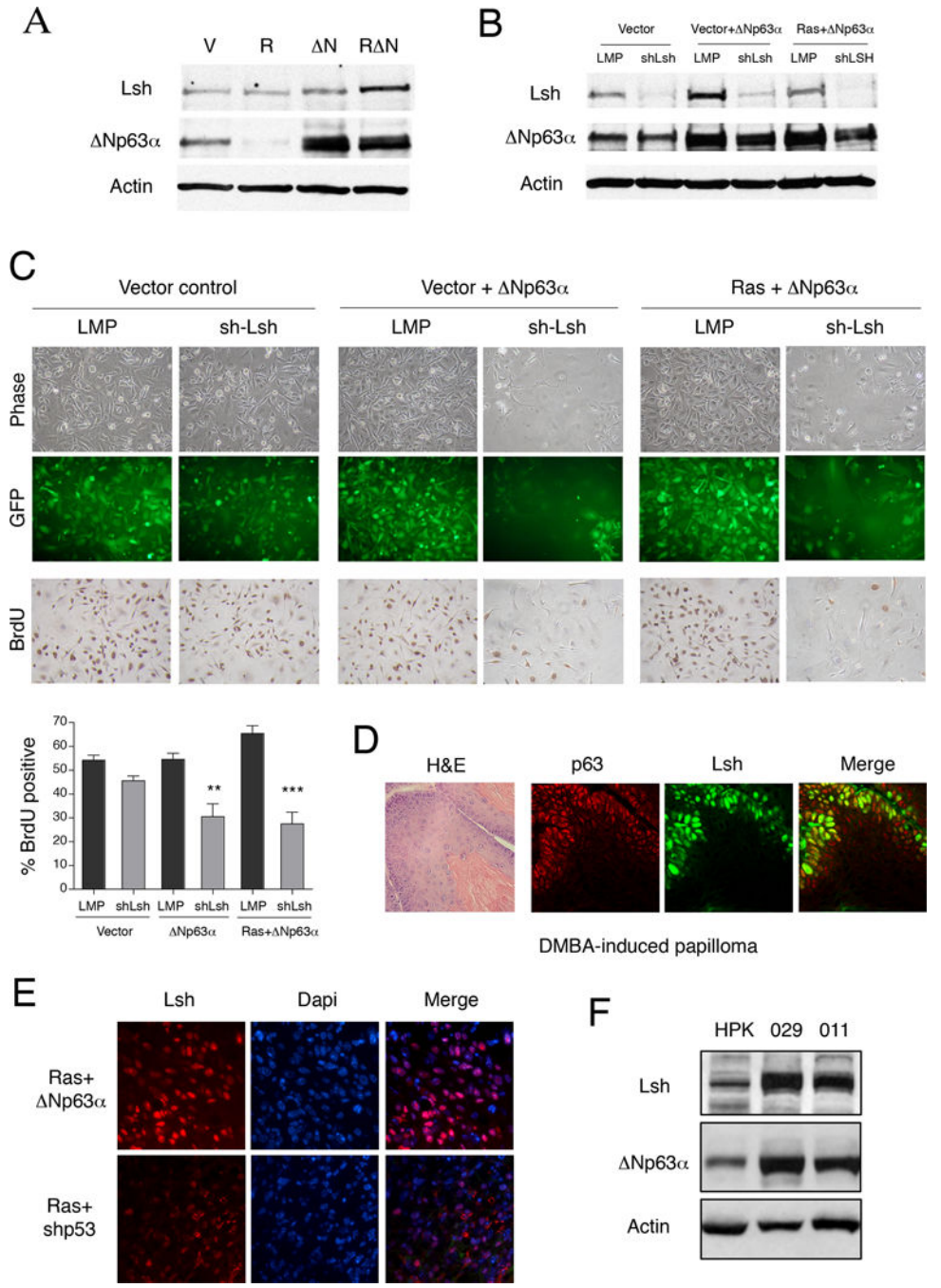

Figure 7. Lsh is required for ΔNp63α's OIS-inhibiting activity

(A) Western blotting for Lsh and p63 in primary mouse keratinocytes expressing vector (V), Ras (R) ΔNp63α (ΔN) and Ras+ΔNp63α (RΔN) during phase I senescence bypass indicates that Lsh expression is increased in ΔNp63α-expressing cells.

(B) Western blotting for Lsh and p63 in primary mouse keratinocytes expressing vector (V), ΔNp63α (ΔN) and Ras+ΔNp63α (RΔN) co-infected with retroviral vector (LMP) or shRNA-Lsh.

(C) Cell morphology by phase contrast and GFP-fluorescence shows fewer cells and senescent-like morphology in Lsh-knockdown cells. Immunostaining for BrdU incorporation and quantitation shows that Lsh-knockdown cells expressing ΔNp63α proliferate less. (BrdU quantitation, ± S.E.M: one way ANOVA/Tukey's t-test).
(D) Immunostaining indicates that p63 and Lsh are coexpressed in DMBA-induced premalignant papillomas *in situ*.
(E) Immunostaining indicates that Lsh is expressed robustly in tumors arising from sub-cutaneous injection of RΔN keratinocytes, but not those arising from sub-cutaneous injection of Rshp53 keratinocytes.
(F) Western blotting shows increased Lsh expression in 2 squamous cell carcinoma cell lines (029, 011) that have increased p63 expression, compared to human primary keratinocytes (HPK).