Published in final edited form as:

Oncogene. 2014 March 20; 33(12): 1548–1557. doi:10.1038/onc.2013.101.

P63 regulates tubular formation via epithelial-to-mesenchymal transition

Y Zhang¹, W Yan¹, and X Chen

Center for Comparative Oncology, Schools of Medicine and Veterinary Medicine, University of California at Davis, Davis, CA, USA

Abstract

P63, a p53 family member, is expressed as TA and N isoforms. Interestingly, both TAp63 and Np63 are transcription factors, and regulate both common and distinct sets of target genes. p63 is required for survival of some epithelial cell lineages, and lack of p63 leads to loss of epidermis and other epithelia in humans and mice. Here, we explored the role of p63 isoforms in cell proliferation, migration and tubulogenesis by using Madin–Darby Canine Kidney (MDCK) tubular epithelial cells in two- or three-dimensional (2-D or 3-D) culture. We found that like downregulation of p53, downregulation of p63 and TAp63 decreases expression of growth-suppressing genes, including *p21*, *PUMA* and *MIC-1*, and consequently promotes cell proliferation and migration in 2-D culture. However, in 3-D culture, downregulation of p63, especially TAp63, but not p53, decapacitates MDCK cells to form a cyst structure through enhanced epithelial-to-mesenchymal transition (EMT). In contrast, downregulation of Np63 inhibits MDCK cell proliferation and migration in 2-D culture, and delays but does not block MDCK cell cyst formation and tubulogenesis in 3-D culture. Consistent with this, downregulation of Np63 markedly upregulates growth-suppressing genes, including *p21*, *PUMA* and *MIC-1*. Taken together, these data suggest that TAp63 is the major isoform required for tubulogenesis by maintaining an appropriate level of EMT, whereas Np63 fine-tunes the rate of cyst formation and tubulogenesis by maintaining an appropriate expression level of genes involved in cell cycle arrest and apoptosis.

Keywords

p63; the p53 family; tubular formation; epithelial-to-mesenchymal transition; cell proliferation; migration and invasion

Correspondence: Professor X Chen, Center for Comparative Oncology, Schools of Medicine and Veterinary Medicine, University of California at Davis, 2128 Tupper Hall, Davis, CA 95616, USA. xbchen@ucdavis.edu.

¹These authors contributed equally to this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Y Zhang and W Yan did the experiments and analyzed the data; X Chen supervised the project and analyzed the data; and Y Zhang, W Yan and X Chen wrote the paper. All authors read and commented on the draft versions of the manuscript and approved the final version.

Supplementary Information accompanies this paper on the *Oncogene* website (<http://www.nature.com/onc>)

INTRODUCTION

P63, a member of p53 family of transcription factors, bears a high similarity with p53 protein in sequence and structure.^{1,2} At the N-terminus, the *p63* gene is controlled by two promoters and is expressed as two major variants, TAp63 and Np63. Both TA and N variants are further subjected to alternative splicing, and produce at least five isoforms with an unique C-terminus, termed α , β , γ , δ and ϵ .³ TAp63, transcribed from the upstream promoter, contains a transactivation domain similar to the one in p53. In contrast, Np63, transcribed from the promoter in intron 3, lacks the activation domain but gains 14 unique residues in its N-terminus.² These 14 amino acids and the adjacent proline-rich region form an unique transactivation domain for Np63.⁴ Owing to the difference of transactivation domains, TAp63 and Np63 exhibit unique biological functions by regulating their own distinct target genes. For example, TAp63 can bind to p53-responsive elements to transactivate some p53 target genes, such as *p21* and *PUMA*, which mediate TAp63 to suppress cell proliferation and induce apoptosis.^{1,2} In contrast, Np63 can be dominant-negative over TAp63 and p53,² and consequently suppresses the expression of some p53 target genes, such as *p21* and *14-3-3 σ* .^{2,5} In addition, Np63 regulates a distinct set of target genes involved in growth suppression and promotion.^{2,6,7} Indeed, upregulation of Np63 was found in cancers of bladder, lung, head and neck, whereas TAp63 was concomitantly downregulated in these cancers.⁸⁻¹⁰

It is well-characterized that p63 is required for development of epidermis of the skin.¹¹ *p63*^{-/-} mice die shortly after birth due to lack of proper skin protection and exhibit other developmental deficiencies in multiple tissues, such as teeth, lachrymal, salivary, prostate, kidney, urothelia and mammary glands.¹¹⁻¹⁴ All these tissues are highly dependent on proper interactions between mesenchyme and epithelium. Similar phenotype has been observed in zebrafish, where disruption of p63 expression causes defects in epidermal morphogenesis and fin development.^{15,16} Significantly, germline p63 mutations in humans are associated with severe abnormalities of ectoderm-derived tissues, including ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome, Hay-Wells or ankyloblepharon-ectodermal dysplasia-clefting syndrome, and limb-mammary syndrome.^{2,17,18} Of note, type 3 ectodermal dysplasia and cleft lip/palate syndrome is found to contain a heterozygous mutation in the DNA-binding domain of p63 gene,^{17,18} and is associated with renal malformations.¹⁹⁻²¹

p63^{-/-} mice die at birth.^{11,12} Thus, the role of p63 in mature tissue cannot be examined in *p63*^{-/-} mice. However, several cell models, which recapitulate cell morphogenesis *in vivo*, have been developed and used to examine the function of specific genes during various differentiation programs.^{22,23} Among these models is the 3-D collagen gel culture of Madin–Darby Canine Kidney (MDCK) cells, a widely used system for epithelial cell biology.²⁴ Thus, 3-D culture of MDCK cells was utilized to examine the mechanism by which p63 modulates epithelial tissue organization and tumorigenesis. We found that downregulation of p53, p63 and TAp63 decreases the expression of growth-suppressing genes, including *p21*, *PUMA* and *MIC-1*. Interestingly, downregulation of p63, particularly TAp63, but not p53, disrupts the ability of MDCK cells to form a cyst structure in 3-D culture via heightened epithelial-to-mesenchymal transition (EMT). In contrast, knockdown

of Np63 in MDCK cells can still result in cyst structure and tubular formation, albeit substantially delayed. Consistently, downregulation of Np63 remarkably inhibits cell proliferation and migration via upregulation of growth-suppressing genes, such as *p21*, *PUMA*, *MIC-1*, *Bax*, and *14-3-3σ*. Thus, our study uncovers a novel role for TAp63 and Np63 in epithelial tubulogenesis.

RESULTS

P53 is not required for normal cyst formation

MDCK epithelial cell line was derived from kidney tubules of a normal cocker spaniel²⁵ and possesses the ability to form cyst structures when cultured in 3-D collagen gel.²⁶ Upon stimulation with hepatocyte growth factor (HGF), these cyst structures develop into branching tubules through partial EMT, cell proliferation and redifferentiation, a process that resembles kidney tubulogenesis *in vivo*.^{27,28} Here, we showed that when cultured in 3-D collagen gel for 10 days, MDCK cells formed a polarized cyst structure with hollow lumen surrounded by a single layer of cells (Supplementary Figure S1A), consistent with previous published reports.^{26,27} Upon stimulation with HGF on day 10, the cyst ultimately formed tubular networks within 3 days by undergoing four distinct processes, including extension, and formation of chain, cord and tubules (Supplementary Figures S1B–S1H).

Previously, we have shown that wild-type p53 is required for formation of normal mammary acini with hollow lumen in 3-D culture of MCF10A cells.²³ To determine the role of p53 in tubular formation, we generated MDCK cell lines in which p53 was stably knocked down (Figure 1A, clones #2 and #8). We found that p53-KD MDCK cells still exhibited a cuboidal pebble-like morphology, as seen in parental MDCK cells in regular (2-D) culture (Figure 1B, compare b–c with a). In addition, p53-KD MDCK cells were able to form normal cysts, which then developed into tubules when induced with HGF in 3-D collagen gel culture (Figure 1C). Next, we performed colony formation and wound-healing assays, and found that downregulation of p53 promoted cell proliferation and migration (Figures 1D and E). Consistently, we found that downregulation of p53 led to decreased expression of growth-suppressing genes, such as *p21* and *PUMA* (Figure 1F, compare lanes 2–3 with 1). We would like to note that dog p21 is expressed as two isoforms, as previously described.²⁹

EMT is critical for tubulogenesis,^{27,28} which is characterized by decreased expression of E-cadherin, and increased expression of β -catenin and laminin V.³⁰ Thus, we measured these EMT markers and found that downregulation of p53 had little or mild effect on the expression of E-cadherin, β -catenin and laminin V (Figure 1G, compare lanes 2–3 with 1). Likewise, downregulation of p53 did not significantly alter the expression of EMT transcription factors, Snail-1, Slug and Twist (Figure 1H, compare lanes 2–3 with 1). Consistent with the above observations, immunofluorescence assay showed that downregulation of p53 had little if any effect on the expression patterns of epithelial makers E-cadherin and β -catenin, and a mild effect on the intensity of Claudin-1 staining (Supplementary Figures S2A–C). These data suggest that downregulation of p53 alone is not sufficient to alter tubular formation in 3-D culture, but promotes cell proliferation and migration through the loss of induction of growth-suppressing genes.

P63 is required for normal tubular formation

p63 is known to have an essential role in the development of some epithelial tissues and organs.¹² As several epithelial cell lineages, including keratinocytes and mammary epithelial cells, cannot survive without p63,³¹ whether p63 modulates cell polarity has not been examined. Here, 3-D culture of MDCK cells was used to explore the role of p63 in tubular epithelial cell morphogenesis. To test this, we generated multiple MDCK cell lines in which endogenous p63 is knocked down by short hairpin RNA. RT-PCR showed that the levels of transcripts for p63, TAp63 and Np63 were decreased in p63-KD MDCK cells (Figure 2a, clones #7 and #10). As the α isoform is the predominant form of TAp63 and Np63 in MDCK cells, TAp63 α and Np63 α proteins were measured, and used to represent the levels of TAp63 and Np63, respectively. Consistent with the transcript levels, the protein levels for both TAp63 α and Np63 α were decreased upon downregulation of p63 in MDCK cells (Figure 2b, clones #7 and #10). Interestingly, we found that p63-KD MDCK cells in 3-D culture proliferated at a random orientation and did not align with the axis perpendicular to the cellular plane (Figure 2c). We also found that p63-KD cells exhibited a proliferative, migratory and nonpolar morphology (Figure 2c). In addition, the percentage of 'normal' cysts decreased from 80–90% in MDCK cells to 10–15% in p63-KD cells, consistent with the report that the percentage of normal cysts dropped once cell division was randomized.³² These results indicate that downregulation of p63 promotes cell redistribution and invasion, which then leads to the formation of irregular cysts compared with the polarized ones formed by parental MDCK cells (Figure 2c). Furthermore, we found that downregulation of p63 significantly enhanced cell proliferation as measured by colony formation assay (Figure 2d), and cell motility as measured by wound-healing assay (Figure 2e).

Cell motility is a hallmark of a mesenchymal phenotype.³³ Thus, to determine whether EMT is involved in the alteration of cyst morphology, we measured the expression patterns of EMT markers. We found that the protein level of E-cadherin was decreased, whereas those of β -catenin and laminin V were increased in p63-KD cells (Figure 2f, compare lanes 2–3 with 1). In addition, we found that downregulation of p63 increased the protein levels of Snail-1, Slug and Twist (Figure 2g, compare lanes 2–3 with 1). Consistently, we found that downregulation of p63 decreased the mRNA level for E-cadherin, but increased those for Snail-1 and Twist (Figure 2h, compare lanes 2–3 with 1). However, downregulation of p63 had no significant effect on the mRNA level for β -catenin (Supplementary Figure S3A, compare lanes 2–3 with 1). To further examine whether downregulation of p63 is associated with EMT phenotype, we performed immunofluorescence assay and found that the intensity of E-cadherin and Claudin-1 staining was decreased (Supplementary Figures S3B–S3C), whereas the intensity of β -catenin staining was increased along with increased nuclear accumulation (Supplementary Figure S3D).

Next, we measured the expression pattern of Cadherin-6, MIC-1, p21 and Puma. Cadherin-6 is a kidney differentiation maker and functions as an inhibitor of tubulogenesis.^{34,35} Macrophage inhibitory cytokine 1 (*MIC-1*) is a p53 target gene and acts as a growth inhibitory molecule.³⁶ Here, we found that downregulation of p63 led to an increase in the expression of Cadherin-6 (Figure 2h). In contrast, downregulation of p63 markedly decreased the expression of p21, PUMA and MIC-1 (Figure 2i).

TAp63 is necessary for tubular formation

p63 is expressed as TA and N isoforms. Thus, to determine the role of each p63 isoform in tubulogenesis of MDCK cells, we generated multiple MDCK cell lines in which TA, but not N, isoform was specifically knocked down (Figures 3a and b, clones #6 and #28). We found that in 3-D culture, TAp63-KD MDCK cells formed abnormal structures with an EMT phenotype, which is similar to that formed by p63-KD cells (Figure 3c). By day 6 in 3-D culture, TAp63-KD cells rapidly proliferated and randomly migrated to form irregular structures (Figure 3c, upper panel), which ultimately developed into cell aggregates without polarity by day 12–14 (Figure 3c, middle and lower panels). Consistently, cell proliferation (Figure 3d) and cell motility (Figure 3e) were markedly enhanced by downregulation of TAp63. The EMT phenotype was confirmed by decreased protein level of E-cadherin and by increased protein levels of β -catenin, laminin V, Snail-1, Slug and Twist due to downregulation of TAp63 (Figures 3f and g, compare lanes 2–3 to 1). Likewise, the level of mRNA for E-cadherin was decreased, whereas the ones for Snail-1 and Twist were increased by downregulation of TAp63 (Figure 3h). However, downregulation of TAp63 had no obvious effect on β -catenin transcription (Supplementary Figure S4A, compare lanes 2–3 with 1). Moreover, the mRNA level of Cadherin-6 was increased (Figure 3h, compare lanes 2–3 with 1), whereas the protein levels of p21, PUMA and MIC-1 were decreased (Figure 3i, compare lanes 2–3 to 1) by downregulation of TAp63 in MDCK cells. Consistent with these observations, we found that TAp63-KD MDCK cells displayed a weak staining for E-cadherin and Claudin-1 (Supplementary Figure S4B and C), but a strong staining for β -catenin at the cell–cell junction, along with increased nuclear accumulation (Supplementary Figure S4D). These results suggest that TAp63 is primarily responsible for the ability of p63 to mediate tubular formation via maintaining an appropriate level of EMT, cell proliferation and cell mobility.

Np63-KD delays tubular formation via growth suppression

To determine the effect of Np63 on tubular formation, we generated multiple MDCK cell lines, in which N, but not TA, isoform was specifically knocked down. The decreased levels of Np63 mRNA and protein in these cell lines were verified by RT-PCR and western blotting, respectively (Figures 4A and B, Clones #44 and #66). Interestingly, we found that unlike the downregulation of TAp63, downregulation of Np63 did not obviously alter cell morphology in 2-D culture (Figures 4C,b and c). However, downregulation of Np63 delayed cyst formation and markedly decreased the size of the cysts compared with that formed by parental MDCK cells (Figure 4D). It is known that the delay and suppression of cyst formation can be caused by decreased cell proliferation and/or increased cell death. Indeed, we found that downregulation of Np63 repressed cell proliferation and migration (Figures 4E and F). As Np63 α acts as a transcriptional repressor *in vivo* and *in vitro*,^{5,15,16} we examined alteration of gene expression by downregulation of Np63. We found that downregulation of Np63 increased the expression of several growth-suppressing genes, including *p21*, *PUMA*, *MIC-1*, *Bax* and *14-3-3 σ* (Figures 4G and H, compare lanes 2–3 to 1). Interestingly, the level of Cadherin-6 mRNA was decreased, whereas those of E-cadherin protein and mRNA were slightly increased by downregulation of Np63 (Figures 4I and K, compare lanes 2–3 to 1). However, downregulation of Np63 had a mild or no effect on the levels of protein and mRNA for laminin V, β -catenin, Snail-1, Slug and Twist (Figures 4I–K,

Supplementary Figure S5A, compare lanes 2–3 with 1). Moreover, downregulation of Np63 had no or a mild effect on the staining patterns for E-cadherin, β -catenin and Claudin-1 (Supplementary Figure S5B–D).

We showed above that in response to HGF stimulation, MDCK cysts undergo partial EMT, proliferation and redifferentiation to start extensions and form chains, cords and tubules (Supplementary Figure S1). To test whether Np63 is required for HGF-induced tubulogenesis, cysts were exposed to HGF in 3-D culture. Using parental MDCK cells as a control, we found that 24 h after exposure to HGF, cord formation was initiated from most of the MDCK cysts (Figure 5a, left panel). Upon exposure to HGF for 36–48 h, the cells in the cords further migrated and proliferated, leading to expanded luminal surface (Figure 5a, left panels). Seventy-two hours following HGF treatment, all of the cysts were developed into mature tubule networks (Figure 5a, left panel). However, upon downregulation of Np63, cord and tubule formations were markedly delayed and incomplete (Figure 5a, middle and right panels, arrow headed). These observations suggest that decreased cell proliferation by downregulation of Np63 delays tubular formation, but is not sufficient to block tubulogenesis.

DISCUSSION

p63 is known to have a role in tumor suppression and promotion, as well as development and differentiation of specific tissues and organs. However, how p63 regulates cell behaviors is still not fully understood. Here, by taking the advantage of 3-D culture model of MDCK cells, we reveal a distinct role for TAp63 and Np63 in the process of branching morphogenesis of tubular epithelium, a common feature of vertebrate organogenesis.^{27,28} First, we found that downregulation of p53 has little, if any, effect on tubular formation of MDCK cells. Second, we found that downregulation of p63, particularly TAp63, disrupts cyst formation coupled with a proliferative, migratory and nonpolar phenotype, suggesting that TAp63 is the major isoform required for cyst formation, an initiating and potentially rate-limiting stage of tubulogenesis. Third, we found that the effect of p63 on tubulogenesis is at least in part via EMT, as downregulation of p63 alters the expression pattern of EMT markers along with increased cell proliferation and migration. Fourth, we found that downregulation of Np63 markedly increases the expression of many growth-suppressing genes, which in turn delays tubular formation through repressing cell proliferation and migration. Taken together, our data indicate that TAp63 is the major isoform required for tubulogenesis by maintaining an appropriate level of EMT, whereas Np63 fine-tunes the rate of cyst formation and branching morphogenesis by maintaining an appropriate expression level of genes involved in cell cycle arrest and apoptosis (Figure 5b).

Epithelial tissues, such as kidney and mammary gland, are commonly developed from spherical cysts and cylindrical tubules.³⁷ The formation of cysts and tubules is known as branching morphogenesis or tubulogenesis. This process is regulated by a wide range of growth factors and their receptors, transcription factors and cell-extracellular matrix adhesion molecules, all of which coordinately regulate cellular behaviors, such as proliferation, differentiation, apoptosis, migration and polarization.³⁸ The phenotype of p63-deficient animals indicates that p63 has a role in developmental processes that heavily rely

on proper epithelial–mesenchymal interactions.^{11,12} In line with this, in adult p63^{+/-} mice, several defects were observed in the epithelia of the tongue, rectal prolapse, vaginal cervicitis and cornea of the eye.¹⁴ In this study, we found that TAp63 and Np63 differently regulate tubular formation and gene expression in MDCK cells. Particularly, downregulation of p63 and TAp63, but not Np63, renders MDCK cells to proliferate randomly into a single layer, instead of initiating polarized cyst formation in 3-D collagen gel culture. Cadherin-6, also called K-cadherin, is considered as a differentiation maker for kidney.³⁴ Cadherin-6 is expressed at a relatively high level in MDCK cells and functions as an inhibitor of tubulogenesis.³⁵ Here, we found that downregulation of p63 and TAp63 increased, whereas that of Np63 decreased, Cadherin-6 in MDCK cells. These data are consistent with a recent report that loss of E-cadherin inhibits lumen formation, whereas ablation of Cadherin-6 leads to tubular formation in MDCK cells.³⁵ Thus, we speculate that TAp63 is the major isoform to control the cell polarity in 3-D culture, loss of which disrupts cell–cell adhesion and promotes cells to invade into the adjacent structure to form irregular aggregates, leading to developmental defects and tumor formation. Indeed, TAp63-deficient and p63-heterozygous mice have defects in epithelial tissues, including kidney,^{13,14} and are prone to tumor formation.^{39,40} In contrast, Np63 is the main isoform required for cell growth and survival. Consistent with this, Np63-deficient humans and mice are prone to developmental defects.^{11,18}

EMT is a process in which immotile epithelial cells transit into motile fibroblastic-like cells. EMT is involved in normal embryogenesis and tissue morphogenesis, and in adults, it is required for the maintenance of the epithelium for wound healing and tissue repair. However, aberrant activation of EMT can cause cellular invasion and metastasis in cancer. It has been reported that adult epithelial cells of kidney, eye, lung, peritoneum and colon can undergo EMT in chronic inflammatory diseases associated with fibrosis, as well as carcinogenesis in a broad range of epithelia.^{41,42} Especially, EMT is an important contributor to the progression of renal disease in an animal model of kidney fibrosis.⁴³ The pathological EMT is characterized by downregulation of epithelial markers and acquisition of mesenchymal properties, including loss of E-cadherin, a prerequisite for epithelial cells to acquire migratory properties.⁴⁴ Previous study also proved that ablation of E-cadherin does not induce tubulogenesis.³⁵ In this study, we found that downregulation of p63 and TAp63 disrupts cyst formation, accompanied by loss of E-cadherin, increased expression of β -catenin, laminin V, Snail, Slug and Twist. However, downregulation of Np63 and p53 causes little, if any, alteration on the expression of these EMT markers. These data indicate that MDCK cells deficient in p63 and TAp63, but not Np63 and p53, have undergone EMT, which leads to acquisition of enhanced migratory activity and formation of abnormal structures. Consistent with this, previous reports showed that p63 regulates cell–cell and cell–matrix interactions, and p63 deficiency leads to decreased cell adhesion and cell cycle arrest, but increased invasion and metastasis potentials.^{31,45,46} Of note, p63 is found to activate several micro RNAs, including miRNA-200 family and miRNA-205, to suppress transcription factors ZEB1, ZEB2 and vimentin, and consequently inhibits EMT.^{47–49} On the other hand, EMT markers, including Snail and Slug, are found to up- and downregulate TAp63 and Np63 expression, respectively.⁵⁰ Thus, it is likely that a regulatory feedback loop exists between EMT markers and p63. Taken together, these studies suggest that p63 is

necessary for the induction and maintenance of epithelial cell fate. It is worth to note that in 3-D culture of MCF10A cells, p53 is required for the formation of normal acini with hollow lumen,²³ suggesting that p53 has a cell/tissue-specific effect on cell polarity.

Previous studies showed that in 3-D culture of MDCK cells, tubulogenesis undergoes a partial epithelial–mesenchymal transition (p-EMT), which then modulates cell proliferation and cell death required for cyst maturation, lumen formation, and subsequent redifferentiation.^{51,52} Interestingly, Np63 is found to be involved in maintaining undifferentiated phenotype in renal carcinoma cells.⁵³ Here, we found that downregulation of Np63 suppresses cell proliferation and migration, and dramatically delays the process of tubular formation, coupled with increased expression of growth-suppressing genes, such as *p21*, *PUMA*, *MIC-1*, *Bax* and *14-3-3σ*. However, downregulation of p63 or TAp63 repressed the expression of *p21*, *PUMA* and *MIC-1*. Like p21, *MIC-1* acts as a growth inhibitory molecule, which can be induced by p53.³⁶ *MIC-1* is an early mediator of the injury response in kidney and lung, and may regulate inflammation, cell survival, proliferation and apoptosis.⁵⁴ Thus, in addition to p21 and PUMA, decrease expression of *MIC-1* may contribute to the increased proliferation due to downregulation of p63 or TAp63. Considering that *MIC-1*, *p21* and *PUMA* are p53 target genes and can mediate p63-induced cell cycle arrest and apoptosis,^{2,35} we hypothesize that Np63 maintains a proper level of expression for these growth-suppressing genes, and fine-tunes the rate of cyst formation and tubulogenesis. Nevertheless, additional work is still needed to address how these genes mediate the function of p63 in tubular formation in 3-D culture of MDCK cells.

MATERIALS AND METHODS

Reagents

Bovine collagen solution (3.2 mg/ml) was purchased from Advanced Biomatrix (Poway, CA, USA). MEM medium and non-essential amino acid were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human HGF was purchased from Sigma (St Louis, MO, USA).

Cell culture

The MDCK cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in MEM medium supplemented with 10% fetal bovine serum and 1% non-essential amino acid. The overlay 3-D culture was carried out as described previously with some modifications.⁵⁵ Briefly, 12-well culture plates were precoated evenly with 1.0 mg/ml premixed collagen gel and then incubated at 37 °C for 30 min to allow the collagen gel to solidify. MDCK cells (5000 cells) suspended in 1.0 ml collagen gel mixture were seeded on the top of pre-gelled layer, and then incubated for 30 min at 37 °C. Complete growth medium was gently added to the top of each gel and incubated at 37 °C in a humidified 5% CO₂. Culture medium was renewed every third day. For induction of tubulogenesis, culture medium plus 10 ng/ml of HGF was added to the culture plate.

Plasmid construction and cell line generation

To generate expression vectors for short hairpin RNAs against p63, Np63 or TAp63 under the control of the U6 promoter, two 62-base oligos were annealed and then cloned into pBabe-U6 short hairpin RNA expression vector.⁵⁶ The resulting plasmids were designed as pBabe-U6-shp63, pBabe-U6-sh Np63 and pBabe-U6-shTAp63. The short hairpin RNA oligos along with the siRNA-targeting region shown in uppercase are listed in the Supplementary Table 1. pBabe-U6-shp53 was used as previously described.²³ To generate stable cell lines in which target genes can be downregulated, pBabe-U6-shp53, pBabe-U6-shp63, pBabe-U6-sh Np63 or pBabe-U6-shTAp63 was transfected into MDCK cells. The resulting cell lines were selected with puromycin and confirmed by RT-PCR and western blot analysis.

RT-PCR analysis

Total RNA was extracted from cells using TRIzol (Invitrogen Life Technologies, Grand Island, NY, USA), according to the manufacturer's instructions. cDNA was synthesized using M-MLV Reverse Transcriptase Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The mRNA levels of p63 and its isoforms were measured by PCR with *p63* and *p63* isoform-specific primers. The primers to detect *p63* are sense 5'-TTTCGACGTGTCCTTCCAGCA-3' and antisense 5'-CACTTCA GTAACATGTTACAG-3'. The primers to detect *Np63* are sense 5'-GACAGCG GCATTGATCAATC-3' and antisense 5'-TGCGCGTGGTCTGTGTTGTA-3'. The primers to detect *TAp63* are sense 5'-TGTTTCAGTTCAGCCTATTGAC-3' and antisense 5'-TGCGCGTGGTCTGTGTTGTA-3'. The *Actin* gene was chosen as a loading control and detected with primers 5'-CTGAAGTACCCCATCGA GCACGGCA-3'⁵⁷ and 5'-GGATAGCACAGCCTGGATAGCAACG-3' (antisense). The primers to detect *Snail-1*, *Twist*, *E-cadherin*, *cadherin-6* and β -catenin were showed in Supplementary Table 2.

Western blot analysis

Western blot was performed as described.⁵⁸ Antibodies were purchased from Millipore (anti-laminin γ 2, Temecula, CA, USA; anti-MIC-1, Billerica, MA, USA), Sigma (anti-actin), Santa Cruz Biotechnology (anti-p63 (4A4), anti- β -catenin (E-5), anti-Snail-1, anti-Twist and anti-p21 (H-164), Santa Cruz, CA, USA), Cell Signaling (anti-Slug, Danvers, MA, USA), BD Transduction Laboratories (anti-E-cadherin, anti-Bax, anti-14-3-3 σ , San Jose, CA, USA), ProSci Inc. (anti-PUMA, Poway, CA, USA), and Bio-Rad (secondary antibodies against rabbit or mouse IgG conjugated with HRP, Life Science Research, Hercules, CA, USA).

Colony formation assay

MDCK cells were cultured in a six-well plate for ~12 days and then fixed with methanol/glacial acetic acid (7:1), followed by staining with 0.1% crystal violet. Experiments were conducted in triplicate.

Wound-healing assay

Cells were grown in a six-well plate for 24 h. The monolayers were wounded by scraping with a P200 micropipette tip and washed two times with phosphate-buffered saline (PBS). At specified time points after the scraping, cell migration was captured using phase contrast microscopy and cell monolayers were photographed using a Canon EOS 40D digital camera (Canon, Lake Success, NY, USA). Migration rate of cells was measured by averaging the time required to close the borders of cells. Six regions were analyzed in each well, and the result was expressed as the mean $\pm$ s.d.

Immunofluorescence staining

MDKC cells were grown to near confluence in four-well chamber slides. Cells were rinsed with PBS and then fixed with 3.7% formaldehyde in PBS buffer for 15 min at room temperature. After rinsing three times with PBS, cells were incubated in 10% bovine serum albumin in PBS for 1 h, followed by incubation with primary antibodies at 4°C. After washing with PBS, FITC-conjugated anti-rabbit IgG or anti-mouse IgG was applied and incubated at 4°C for 1 h. After washing with PBS, nuclear counterstaining was performed by adding 4', 6-diamidini-2-phenylindole (DAPI, 0.25 mg/ml) and slides were mounted with coverslips.

Statistical analysis

Data are presented as mean $\pm$ s.d. Statistical significance was determined by Student's *t*-test. Values of *P*<0.05 were considered significant.

Acknowledgments

This work was supported in part by the National Institutes of Health grants CA102188, CA108122 and CA076069. We thank Jin Zhang for helpful comments on this manuscript.

References

1. Osada M, Ohba M, Kawahara C, Ishioka C, Kanamaru R, Katoh I, et al. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nat Med.* 1998; 4:839–843. [PubMed: 9662378]
2. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell.* 1998; 2:305–316. [PubMed: 9774969]
3. Vanbokhoven H, Melino G, Candi E, Declercq W. p63, a story of mice and men. *J Invest Dermatol.* 2011; 131:1196–1207. [PubMed: 21471985]
4. Dohn M, Zhang S, Chen X. p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene.* 2001; 20:3193–3205. [PubMed: 11423969]
5. Westfall MD, Mays DJ, Sniezek JC, Pietenpol JA. The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters *in vivo* and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. *Mol Cell Biol.* 2003; 23:2264–2276. [PubMed: 12640112]
6. Sasaki Y, Ishida S, Morimoto I, Yamashita T, Kojima T, Kihara C, et al. The p53 family member genes are involved in the Notch signal pathway. *J Biol Chem.* 2002; 277:719–724. [PubMed: 11641404]
7. Lo Iacono M, Di Costanzo A, Calogero RA, Mansueto G, Saviozzi S, Crispi S, et al. The Hay Wells syndrome-derived TAp63alphaQ540L mutant has impaired transcriptional and cell growth regulatory activity. *Cell Cycle.* 2006; 5:78–87. [PubMed: 16319531]

8. Park BJ, Lee SJ, Kim JI, Lee CH, Chang SG, Park JH, et al. Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res.* 2000; 60:3370–3374. [PubMed: 10910040]
9. Sniezek JC, Matheny KE, Westfall MD, Pietenpol JA. Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. *Laryngoscope.* 2004; 114:2063–2072. [PubMed: 15564824]
10. Massion PP, Taflan PM, Jamshedur Rahman SM, Yildiz P, Shyr Y, Edgerton ME, et al. Significance of p63 amplification and overexpression in lung cancer development and prognosis. *Cancer Res.* 2003; 63:7113–7121. [PubMed: 14612504]
11. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature.* 1999; 398:708–713. [PubMed: 10227293]
12. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature.* 1999; 398:714–718. [PubMed: 10227294]
13. Su X, Paris M, Gi YJ, Tsai KY, Cho MS, Lin YL, et al. TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell.* 2009; 5:64–75. [PubMed: 19570515]
14. Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, Mills AA. p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev.* 2005; 19:1986–1999. [PubMed: 16107615]
15. Bakkers J, Hild M, Kramer C, Furutani-Seiki M, Hammerschmidt M. Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. *Dev Cell.* 2002; 2:617–627. [PubMed: 12015969]
16. Lee H, Kimelman D. A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev Cell.* 2002; 2:607–616. [PubMed: 12015968]
17. Brunner HG, Hamel BC, Bokhoven HvH. P63 gene mutations and human developmental syndromes. *Am J Med Genet.* 2002; 112:284–290. [PubMed: 12357472]
18. Celli J, Duijf P, Hamel BC, Bamshad M, Kramer B, Smits AP, et al. Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell.* 1999; 99:143–153. [PubMed: 10535733]
19. Allen LM, Maestri MJ. Three-dimensional sonographic findings associated with ectrodactyly ectodermal dysplasia clefting syndrome. *J Ultrasound Med.* 2008; 27:149–154. [PubMed: 18096742]
20. Rinne T, Hamel B, van Bokhoven H, Brunner HG. Pattern of p63 mutations and their phenotypes--update. *Am J Med Genet.* 2006; 140:1396–1406. [PubMed: 16691622]
21. van Bokhoven H, Hamel BC, Bamshad M, Sangiorgi E, Gurrieri F, Duijf PH, et al. p63 Gene mutations in EEC syndrome, limb-mammary syndrome, and isolated split hand-split foot malformation suggest a genotype-phenotype correlation. *Am J Hum Genet.* 2001; 69:481–492. [PubMed: 11462173]
22. Lindsay J, McDade SS, Pickard A, McCloskey KD, McCance DJ. Role of DeltaNp63-gamma in epithelial to mesenchymal transition. *J Biol Chem.* 2011; 286:3915–3924. [PubMed: 21127042]
23. Zhang Y, Yan W, Chen X. Mutant p53 disrupts MCF-10A cell polarity in three-dimensional culture via epithelial-to-mesenchymal transitions. *J Biol Chem.* 2011; 286:16218–16228. [PubMed: 21454711]
24. Simons K, Fuller SD. Cell surface polarity in epithelia. *Annu Rev Cell Biol.* 1985; 1:243–288. [PubMed: 3939606]
25. Leighton J, Estes LW, Mansukhani S, Brada Z. A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. *Cancer.* 1970; 26:1022–1028. [PubMed: 4248968]
26. McAteer JA, Evan AP, Gardner KD. Morphogenetic clonal growth of kidney epithelial cell line MDCK. *Anat Rec.* 1987; 217:229–239. [PubMed: 3578840]
27. Montesano R, Schaller G, Orci L. Induction of epithelial tubular morphogenesis *in vitro* by fibroblast-derived soluble factors. *Cell.* 1991; 66:697–711. [PubMed: 1878968]
28. Saxen L, Sariola H. Early organogenesis of the kidney. *Pediatr Nephrol.* 1987; 1:385–392. [PubMed: 3153305]

29. Zhang J, Chen X, Kent MS, Rodriguez CO. Establishment of a dog model for the p53 family pathway and identification of a novel isoform of p21 cyclin-dependent kinase inhibitor. *Mol Cancer Res.* 2009; 7:67–78. [PubMed: 19147538]
30. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest.* 2009; 119:1429–1437. [PubMed: 19487819]
31. Carroll DK, Carroll JS, Leong CO, Cheng F, Brown M, Mills AA, et al. p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol.* 2006; 8:551–561. [PubMed: 16715076]
32. Zheng Z, Zhu H, Wan Q, Liu J, Xiao Z, Siderovski DP, et al. LGN regulates mitotic spindle orientation during epithelial morphogenesis. *J Cell Biol.* 2010; 189:275–288. [PubMed: 20385777]
33. Prindull G, Zipori D. Environmental guidance of normal and tumor cell plasticity: epithelial mesenchymal transitions as a paradigm. *Blood.* 2004; 103:2892–2899. [PubMed: 15070660]
34. Kim D, Dressler GR. Nephrogenic factors promote differentiation of mouse embryonic stem cells into renal epithelia. *J Am Soc Nephrol.* 2005; 16:3527–3534. [PubMed: 16267156]
35. Jia L, Liu F, Hansen SH, Ter Beest MB, Zegers MM. Distinct roles of cadherin-6 and E-cadherin in tubulogenesis and lumen formation. *Mol Biol Cell.* 2011; 22:2031–2041. [PubMed: 21508319]
36. Tan M, Wang Y, Guan K, Sun Y. PTGF-beta, a type beta transforming growth factor (TGF-beta) superfamily member, is a p53 target gene that inhibits tumor cell growth via TGF-beta signaling pathway. *Proc Natl Acad Sci USA.* 2000; 97:109–114. [PubMed: 10618379]
37. Lipschutz JH, Guo W, O'Brien LE, Nguyen YH, Novick P, Mostov KE. Exocyst is involved in cystogenesis and tubulogenesis and acts by modulating synthesis and delivery of basolateral plasma membrane and secretory proteins. *Mol Biol Cell.* 2000; 11:4259–4275. [PubMed: 11102522]
38. Dressler G. Tubulogenesis in the developing mammalian kidney. *Trends Cell Biol.* 2002; 12:390–395. [PubMed: 12191916]
39. Guo X, Keyes WM, Papazoglu C, Zuber J, Li W, Lowe SW, et al. TAp63 induces senescence and suppresses tumorigenesis *in vivo*. *Nat Cell Biol.* 2009; 11:1451–1457. [PubMed: 19898465]
40. Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL, et al. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. *Nature.* 2010; 467:986–990. [PubMed: 20962848]
41. Zeisberg M, Shah AA, Kalluri R. Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. *J Biol Chem.* 2005; 280:8094–8100. [PubMed: 15591043]
42. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell.* 2009; 139:871–890. [PubMed: 19945376]
43. Cook HT. The origin of renal fibroblasts and progression of kidney disease. *Am J Pathol.* 2010; 176:22–24. [PubMed: 20008128]
44. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer.* 2007; 7:415–428.
45. Ihrle RA, Marques MR, Nguyen BT, Horner JS, Papazoglu C, Bronson RT, et al. Perp is a p63-regulated gene essential for epithelial integrity. *Cell.* 2005; 120:843–856. [PubMed: 15797384]
46. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, et al. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell.* 2009; 137:87–98. [PubMed: 19345189]
47. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* 2008; 22:894–907. [PubMed: 18381893]
48. Tucci P, Agostini M, Grespi F, Markert EK, Terrinoni A, Vousden KH, et al. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. *Proc Natl Acad Sci USA.* 2012; 109:15312–15317. [PubMed: 22949650]
49. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol.* 2008; 10:593–601. [PubMed: 18376396]

50. Herfs M, Hubert P, Suarez-Carmona M, Reschner A, Saussez S, Berx G, et al. Regulation of p63 isoforms by snail and slug transcription factors in human squamous cell carcinoma. *Am J Pathol*. 2010; 176:1941–1949. [PubMed: 20150431]
51. O'Brien LE, Tang K, Kats ES, Schutz-Geschwender A, Lipschutz JH, Mostov KE. ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. *Dev Cell*. 2004; 7:21–32. [PubMed: 15239951]
52. Pollack AL, Runyan RB, Mostov KE. Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis. *Dev Biol*. 1998; 204:64–79. [PubMed: 9851843]
53. Cao Y, Wang L, Nandy D, Zhang Y, Basu A, Radisky D, et al. Neuropilin-1 upholds dedifferentiation and propagation phenotypes of renal cell carcinoma cells by activating Akt and sonic hedgehog axes. *Cancer Res*. 2008; 68:8667–8672. [PubMed: 18974107]
54. Zimmers TA, Jin X, Hsiao EC, McGrath SA, Esquela AF, Koniaris LG. Growth differentiation factor-15/macrophage inhibitory cytokine-1 induction after kidney and lung injury. *Shock*. 2005; 23:543–548. [PubMed: 15897808]
55. O'Brien LE, Yu W, Tang K, Jou TS, Zegers MM, Mostov KE. Morphological and biochemical analysis of Rac1 in three-dimensional epithelial cell cultures. *Methods Enzymol*. 2006; 406:676–691. [PubMed: 16472697]
56. Liu G, Chen X. DNA polymerase eta, the product of the xeroderma pigmentosum variant gene and a target of p53, modulates the DNA damage checkpoint and p53 activation. *Mol Cell Biol*. 2006; 26:1398–1413. [PubMed: 16449651]
57. Sensen CW, Charlebois RL, Chow C, Clausen IG, Curtis B, Doolittle WF, et al. Completing the sequence of the *Sulfolobus solfataricus* P2 genome. *Extremophiles*. 1998; 2:305–312. [PubMed: 9783178]
58. Zhang Y, Shu L, Chen X. Syntaxin 6, a regulator of the protein trafficking machinery and a target of the p53 family, is required for cell adhesion and survival. *J Biol Chem*. 2008; 283:30689–30698. [PubMed: 18779328]

Figure 1.

p53 is not required for tubular formation in 3-D culture. (A) Generation of MDCK cell lines in which wild-type p53 (clones #2 and #8) was knocked down. The level of p53 was determined by western blotting. (B) Representative images of MDCK or p53-KD MDCK cells in 2-D culture (200×). (C) Representative images of MDCK or p53-KD MDCK cells in 3-D culture. Scale bar: 1 μM. (D) Top panel: colony formation assay was performed with MDCK and p53-KD MDCK cells. Cells were cultured for 12 days, then fixed and stained with crystal violet. Bottom panel: the number of colonies was counted and presented as mean±s.d. from three separate experiments. (E) Top panel: wound-healing assay was performed with MDCK and p53-KD MDCK cells. Bottom panel: The time required for wound closure was measured and presented as mean±s.d. from three separate experiments.

(F–H) Western blots were prepared using extracts from MDCK cells (lane 1) and p53-KD MDCK cells (lanes 2 and 3). The blots were probed with antibodies against p21 (F), PUMA (F), laminin V (G), β -catenin (G), E-cadherin (G), Snail (H), Slug (H), Twist (H) and actin (F–H), respectively.

Figure 2.

p63 is required for tubular formation in 3-D culture. **(a, b)** Generation of MDCK cell lines, in which endogenous p63 was knocked down (clones #7 and #10). The levels of p63, Np63 and TAp63 transcripts were determined by RT-PCR **(a)**. The levels of Np63α and TAp63α proteins were measured by western blotting **(b)**. **(c)** Representative images of MDCK or p63-KD MDCK cells in 3-D culture for 6 or 12 days. Scale bar: 1 μM. **(d)** Top panel: colony formation assay was performed with MDCK or p63-KD MDCK cells. Bottom panel: the number of colonies was counted and presented as mean±s.d. from three separate experiments. **(e)** Top panel: wound-healing assay was performed with MDCK or p63-KD

MDCK cells. Bottom panel: The time required for wound closure was measured and presented as mean $\pm$ s.d. from three separate experiments. (**f**, **g**, **i**) Western blots were prepared using extracts from MDCK (lane 1) and p63-KD MDCK cells (lanes 2–3). The blots were probed with antibodies against laminin V (**f**), E-cadherin (**f**), β -catenin (**f**), Snail (**g**), Slug (**g**), Twist (**g**), p21 (**i**), PUMA (**i**), MIC-1 (**i**) and actin (**f**, **g**, **i**), respectively. (**h**), The mRNA levels of E-cadherin, Cadherin-6, Snail, Twist and actin were determined by RT–PCR by using total RNAs from MDCK (lane 1) and p63-KD MDCK cells (lanes 2–3).

Figure 3.

TAp63 is the main isoform of p63 required for tubular formation in 3-D culture. **(a, b)** Generation of MDCK cell lines in which TAp63 isoform (clones #6 and #28) was stably knocked down. The levels of p63, Np63 and TAp63 transcripts were determined by RT-PCR **(a)**. The levels of Np63α and TAp63α proteins were measured by western blotting **(b)**. **(c)** Representative images of MDCK or TAp63-KD MDCK cells in 3-D culture for 6, 12 and 14 days. Scale bar: 1 μm. **(d)** Top panel: colony formation assay was performed with MDCK or TAp63-KD MDCK cells. Bottom panel: the number of colonies was counted and presented as mean±s.d. from three separate experiments. **(e)** Top panel: wound-healing assay

was performed with MDCK or TAp63-KD MDCK cells. Bottom panel: the time required for wound closure was measured and presented as mean $\pm$ s.d. from three separate experiments. **(f, g, i)** Western blots were prepared using extracts from MDCK (lane 1) and TAp63-KD MDCK cells (lanes 2–3). The blots were probed with antibodies against laminin V (**f**), E-cadherin (**f**), β -catenin (**f**), Snail (**g**), Slug (**g**), Twist (**g**), p21 (**i**), PUMA (**i**), MIC-1 (**i**) and actin (**f, g, i**), respectively. **(h)** The mRNA levels of E-cadherin, Cadherin-6, Snail, Twist and actin were determined by RT–PCR by using total RNAs from MDCK (lane 1) and TAp63-KD MDCK cells (lanes 2–3).

Figure 4.

Downregulation of Np63 delays cyst formation through inhibition of cell proliferation and migration. (A–B) Generation of MDCK cell lines in which Np63 isoform (clones #44 and #66) was stably knocked down. The levels of p63, Np63 and TAp63 transcripts were determined by RT-PCR (A). The levels of Np63 α and TAp63 α proteins were measured by western blotting (B). (C) Representative images of MDCK and Np63-KD MDCK cells in 2-D culture (200 $\times$). (D) Representative images of MDCK and Np63-KD MDCK cells in 3-D culture for 12 days. Scale bar: 1 μ M. (E) Top panel: colony formation assay was performed with MDCK and Np63-KD MDCK cells. Bottom panel: the number of colonies was counted and presented as mean $\pm$ s.d. from three separate experiments. (F) Top panel: wound-healing assay was performed with MDCK or Np63-KD MDCK cells. Bottom

panel: the time required for wound closure was measured and presented as mean $\pm$ s.d. from three separate experiments. (G–J) Western blots were prepared using extracts from MDCK (lane 1) and Np63-KD MDCK cells (lanes 2–3). The blots were probed with antibodies against p21 (G), PUMA (G), MIC-1 (G), Bax (H), 14-3-3 σ (H), laminin V (I), E-cadherin (I), β -catenin (I), Snail (J), Slug (J), Twist (J) and actin (G–J), respectively. (K) The mRNA levels of E-cadherin, Cadherin-6, Snail, Twist and actin were determined by RT–PCR by using total RNAs from MDCK cells (lane 1) and Np63-KD MDCK cells (lanes 2–3).

Figure 5.

Np63 fine-tunes tubular formation in 3-D culture. **(a)** Representative images of MDCK or Np63-KD MDCK cells in 3-D culture for 8 or 10 days plus treatment with 10 ng/ml of HGF for 24–72 h. Scale bar: 1 μ M. **(b)** Proposed model of p63 in tubulogenesis.