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ARTICLES

p63 regulates an adhesion programme and cell survival in epithelial cells

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p63 is critical for epithelial development yet little is known about the transcriptional programmes it regulates. By characterising transcriptional changes and cellular effects following modulation of p63 expression, we have defined a vital role for p63 in cellular adhesion. Knockdown of p63 expression caused downregulation of cell adhesion-associated genes, cell detachment and anoikis in mammary epithelial cells and keratinocytes. Conversely, overexpression of the TAp63γ or ΔNp63α isoforms of p63 upregulated cell adhesion molecules, increased cellular adhesion and conferred resistance to anoikis. Apoptosis induced by loss of p63 was rescued by signalling downstream of β4 integrin. Our results implicate p63 as a key regulator of cellular adhesion and survival in basal cells of the mammary gland and other stratified epithelial tissues.

The formation of specialized epithelial tissues is regulated by the orchestration of complex transcriptional programmes[1]. p63, a member of the p53-family, has a pivotal role in epithelial development[2,3]. p63 function has been examined in genetic models where p63 expression is disrupted or overexpressed. *p63*$^{-/-}$ mice exhibit severe abnormalities in the development of stratified squamous epithelia and its derivatives[2,3]. Ectopic *p63* expression in skin is sufficient to drive crucial aspects of stratification and if unchecked, results in the induction of metaplasia[4]. Furthermore, in fibroblasts, ectopic p63 expression induces anchorage-independent growth and tumour formation in nude mice[5]. There is increasing evidence that p63 may function in human cancers[5,6], although its precise role remains to be fully clarified. Thus, p63 may function as a molecular switch that initiates epithelial stratification or cell fate determination in developing tissues[4], while regulating the proliferative potential of the basal cell compartment and/or stem cells in mature tissues[7].

Analysis of p63 function is complicated by the presence of at least six distinct isoforms[8] (Fig. 1a). Transactivating (TA) isoforms contain an amino-terminal exon that encodes a p53-like transactivation domain, whereas ΔN-isoforms lack this domain but contain the common DNA binding domain (DBD), suggesting that TAp63 and ΔNp63 isoforms may have opposing functions. Indeed, ΔNp63 isoforms can act as transcriptional repressors both *in vitro* and *in vivo* and strongly oppose p53-family-mediated reporter transactivation[4,8]. However ΔN-isoforms of p63 and p73 have been shown to act as positive regulators of transcription[9], due to a second transactivation domain[9,10]. Although several p63 targets have been identified[11–13], a more complete assessment of p63 target genes is critical to understanding its functions.

p63 function has been characterised primarily in the context of the epidermis, and little is known about its role in other tissues. *p63*$^{-/-}$ mice completely lack mammary glands, highlighting a critical role for p63 in this tissue[2,3]. In the mature mammary gland, p63 expression is restricted to the myoepithelial and/or basal cells[3,14], which mediate the interaction between luminal cells and the extracellular matrix. Cells of a basal epithelial phenotype are the earliest detected during the development of the mammary gland, and possibly indicate early mammary progenitor cells. ΔNp63α is the predominant isoform expressed in these and other epithelial cells — to the near exclusion of TAp63 isoforms[8,15,16] — suggesting that ΔNp63 isoforms have a major role in the biology of this cell type. The normal human breast epithelial cell line, MCF-10A, expresses markers commonly associated with a basal and/or myoepithelial phenotype, including high molecular weight cytokeratins and ΔNp63α[17], making this a relevant model system to dissect the physiological functions of p63 in mammary epithelial biology.

Here, the effects of loss or gain of p63 expression in MCF-10A cells, primary mammary epithelial cells and primary human keratinocytes has been examined. We demonstrate that p63 regulates the expression an array of proteins that mediate cell adhesion and, thus, has a central role in mammary epithelial integrity and survival. Our results provide an initial understanding of the subprogrammes downstream of p63 and define a role for p63 as a critical regulator of epithelial cell adhesion.

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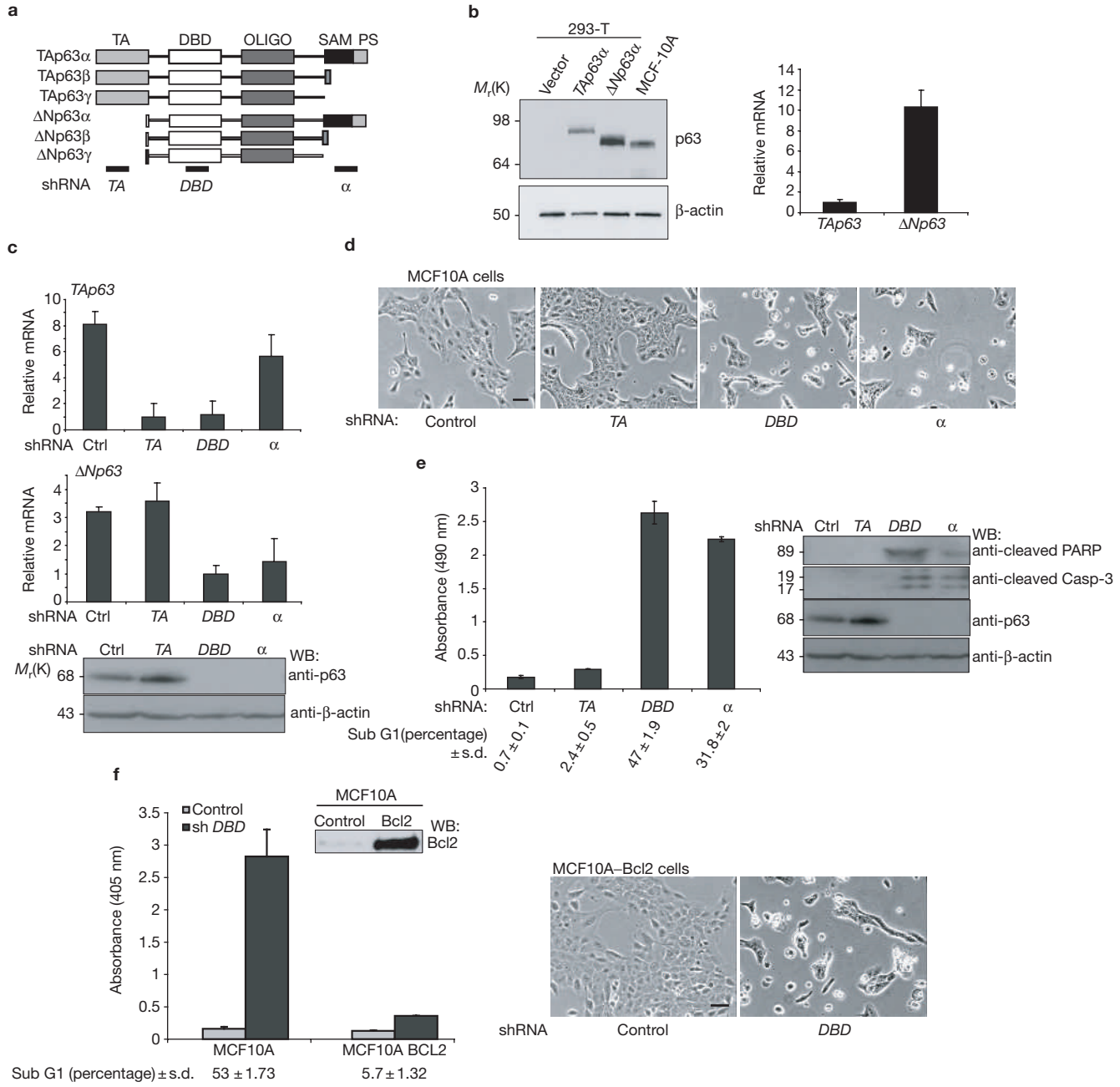


Figure 1 Loss of endogenous *p63* expression induces detachment and death in mammary epithelial cells. (**a**) Schematic representation of the p63 isoforms. The relative positions of shRNA sequences are shown. TA, transactivation domain; DBD, DNA binding domain; Oligo, oligomerization domain; SAM, Sterile alpha motif domain; and PS, post-SAM domain. (**b**) Lysates from parental MCF-10A cells and 293T cells transfected with cDNAs encoding Δ*Np63*α or *TAp63*α were immunoblotted with a pan-p63 antibody (left). QRT–PCR analysis of the relative levels of Δ*Np63* and *TAp63* isoform mRNAs in MCF-10As using *TA* or Δ*N p63* specific primers (right). (**c**) Expression levels of p63 isoforms in MCF-10A cells 48 h after isoform specific knockdown using shRNA (Ctrl, vector control; TA, TA specific shRNA targets α, β and γ *TAp63* isoforms; DBD, targets the core DNA binding domain present in all p63 isoforms; α, shRNA targets both Δ*Np63* and *TAp63*α-isoforms. Expression of *TAp63* and Δ*Np63* mRNA was assessed by QRT–PCR as above. The values represent the mean±s. d. of three replicate samples from one representative experiment ($n=3$). Expression of p63 protein was determined by immunoblotting (bottom). (**d**) Phase contrast micrographs show the morphology of MCF-10A cells 48 h after infection with control or *p63* isoform specific shRNAs. (**e**) Cells were harvested 48 h after infection with control or *p63* shRNAs and assayed for apoptosis by cell death ELISA (bar graph) and FACS analysis (percentage sub-G1 DNA content). Values represent the mean+s.d. of three replicate samples from one representative experiment ($n=3$). Cell lysates were analysed for proteins indicative of apoptosis by western blot analysis (right). (**f**) MCF-10A cells stably expressing Bcl2 were subjected to *p63* knockdown by shRNA as described in **c**. Cells were analysed at 48 h for cell death by cell death ELISA and FACS analysis (left). Values represent the mean±s.d. of three replicate samples from one representative experiment ($n=3$). Phase contrast micrographs show morphology of MCF-10A–Bcl2 cells 48 h after transduction with control or *p63 DBD* shRNAs (right). Scale bars represent 50 μM.

RESULTS

p63 loss induces detachment and death in mammary epithelial cells

Which of the six isoforms of p63 are expressed in MCF-10A cells was determined by immunoblotting and by quantitative real-time RT–PCR (QRT–PCR). ΔNp63α was the major species detected on immunoblots and *ΔNp63* mRNA was expressed at tenfold greater levels than *TAp63* (Fig. 1b). *ΔNp63* mRNA expression was reduced by short hairpin RNAs (shRNAs) that target α and not TAp63-isoforms (Fig. 1c).

To investigate the function and relative importance of individual p63 isoforms, the expression of specific p63 isoforms was disrupted using adenovirus-transduced shRNAs in MCF-10A cells. The specificity of the shRNA vectors was validated by transient cotransfection experiments (see Supplementary Information, Fig. S1). p63 knockdown was verified by immunoblotting and QRT–PCR 48 h following adenoviral transduction (Fig. 1c). Ablation of TAp63 isoforms had little effect on MCF-10A morphology relative to vector-infected cells (Fig. 1d). However, ablation of p63α or all isoforms using shRNAs targeted against the α-tail or the core DNA binding domain (DBD) had pronounced phenotypic effects. Cells lacking α or all isoforms of p63 displayed a rounded morphology, detached from the plate and underwent apoptosis as determined by fluorescence activated cell sorting (sub-G1 DNA content), a DNA fragmentation ELISA assay and immunoblotting for proteins cleaved by apoptotic caspases (PARP and caspase-3; Fig. 1e). The specificity of these shRNA-induced effects was addressed using *p63* variants resistant to the shRNA sequence. Expression of an shRNA-insensitive mutant of *ΔNp63α*, but not *TAp63γ*, blocked both cell detachment and death following p63 knockdown (see Supplementary Information, Fig. S2). Furthermore, expression of the anti-apoptotic protein Bcl2 blocked apoptosis induced by *p63* knockdown, but not the cell detachment (Fig. 1f), indicating that detachment induced by *p63* loss is independent of apoptosis. Together, these data indicate that ΔNp63α is essential for MCF-10A cell survival.

p63 regulates an adhesion subprogramme

Transcriptional profiling was used to identify possible mechanisms whereby p63 loss causes cell detachment. As a complementary approach, the effects of ectopic ΔNp63α and TAp63γ expression was analysed using retroviral transduction in MCF-10A cells[8,18]. Both isoforms were expressed at levels approximately fourfold greater than the respective endogenous isoforms (Fig. 2a). We were unable to detect TAp63γ protein, most likely due to its short half-life.

The changes in gene expression profiles 48 h following either loss (control, TA and DBD) or gain (control, ΔNp63α and TAp63γ) of p63 function were compared (Fig. 2b and see Supplementary Information, Tables S1, S2) using Affymetrix U133A2.0 arrays. Downregulation of *p63* by the *DBD* shRNA reduced the expression of 734 genes and upregulated 549, whereas the *TA*-specific shRNA downregulated 204 genes and upregulated 269. Ectopic expression of ΔNp63α upregulated 610 genes and downregulated 439, whereas TAp63γ induced more than three-times as many genes (2136) and downregulated more than six-times as many (2711). Of the 734 genes that were downregulated by the *DBD* shRNA, 56.1% were upregulated by ectopic expression of ΔNp63α, suggesting that ΔNp63α may contribute to a substantial proportion of the genes regulated by p63.

Genes encoding many aspects of cell adhesion were regulated by modulation of p63 (see Supplementary Information, Table S1). A strong bias towards downregulation of the cell adhesion genes by the *DBD* shRNA (57 of 924 adhesion genes showed altered regulation versus 734 of 22277 total regulated genes; $P = 0.0000675$), particularly in the cell-matrix adhesion group (18 of 124; $P = 0.00005.61$), was observed. There was no enrichment in the *TA* shRNA subset, suggesting that ΔNp63 isoforms are responsible for the regulation of adhesion genes. This is consistent with the failure of the *TA* shRNA to induce cell detachment. Although many genes involved in cell adhesion were regulated by overexpression of ΔNp63α there was no statistically significant enrichment. This lack of enrichment is likely to reflect the greater specificity of the shRNA approach for identifying endogenous p63 target genes.

Many adhesion genes that displayed reduced levels of expression when *p63* was downregulated with *p63* shRNAs showed elevated levels of expression in the context of p63 overexpression (Fig. 2b). Within this group, multiple genes were preferentially downregulated by the *DBD* shRNA and upregulated by one or both *p63* cDNAs (including integrins, extracellular matrix (ECM) components, cadherins–catenins, other adhesion receptors and intracellular adhesion molecules). These genes may be regulated by ΔNp63; the evidence that many genes were upregulated by overexpression of both p63 isoforms is likely to reflect the ability of either isoform to induce their transcription when overexpressed. The finding that p63 regulates the expression of many key adhesion genes suggests that it may be regulating, either directly or indirectly, a whole axis of cellular adhesion.

QRT–PCR validated changes in the expression of several cell-matrix adhesion genes identified in both loss- and gain-of-function experiments (Fig. 2c). These included β1, β4 and α6-integrins, as well as laminin-γ2 and fibronectin. Interestingly, fibronectin provides an example of the small number of adhesion genes regulated predominantly by TAp63 rather than the ΔNp63 isoforms.

Many of the regulated genes contain putative p53-family response elements within their upstream regulatory sequences, raising the possibility that they may be direct targets of p63. Chromatin immunoprecipitation (ChIP) assays revealed that p63 binds to p53-motif containing regulatory regions adjacent to five of the six validated genes, including integrins α3, β4, α5 and α6 and laminin-γ2 *in vivo* (Fig. 2d). p63 association was not observed with the upstream region of integrin β1, suggesting that the regulatory region for this gene may be distal to the assessed site, or that p63 does not directly regulate integrin β1.

Specific sequence analysis of essential elements in the *ITGB4* promoter was examined by cloning the promoter into a luciferase reporter vector, followed by p63 induction. It was found that ΔNp63α robustly transactivated the *ITGB4* reporter construct and transactivation by TAp63γ was also observed but at fourfold lower levels. Deletion of several putative p53/p63 consensus elements within this region (151–403 bp upstream of the transcriptional start site) completely abrogated p63-mediated luciferase induction (Fig. 2e), confirming a direct role for p63 in the regulation of *ITGB4* transcription.

Regulation of cell adhesion proteins by p63

Alterations in gene expression caused by loss or gain of p63 strongly correlated with protein levels for integrins and ECM proteins (Fig. 3a–d). Furthermore, shRNA-mediated knockdown of *p63α* isoforms caused a reduction in β-integrins identical to that observed with complete *p63* ablation using the *DBD* shRNA (data not shown).

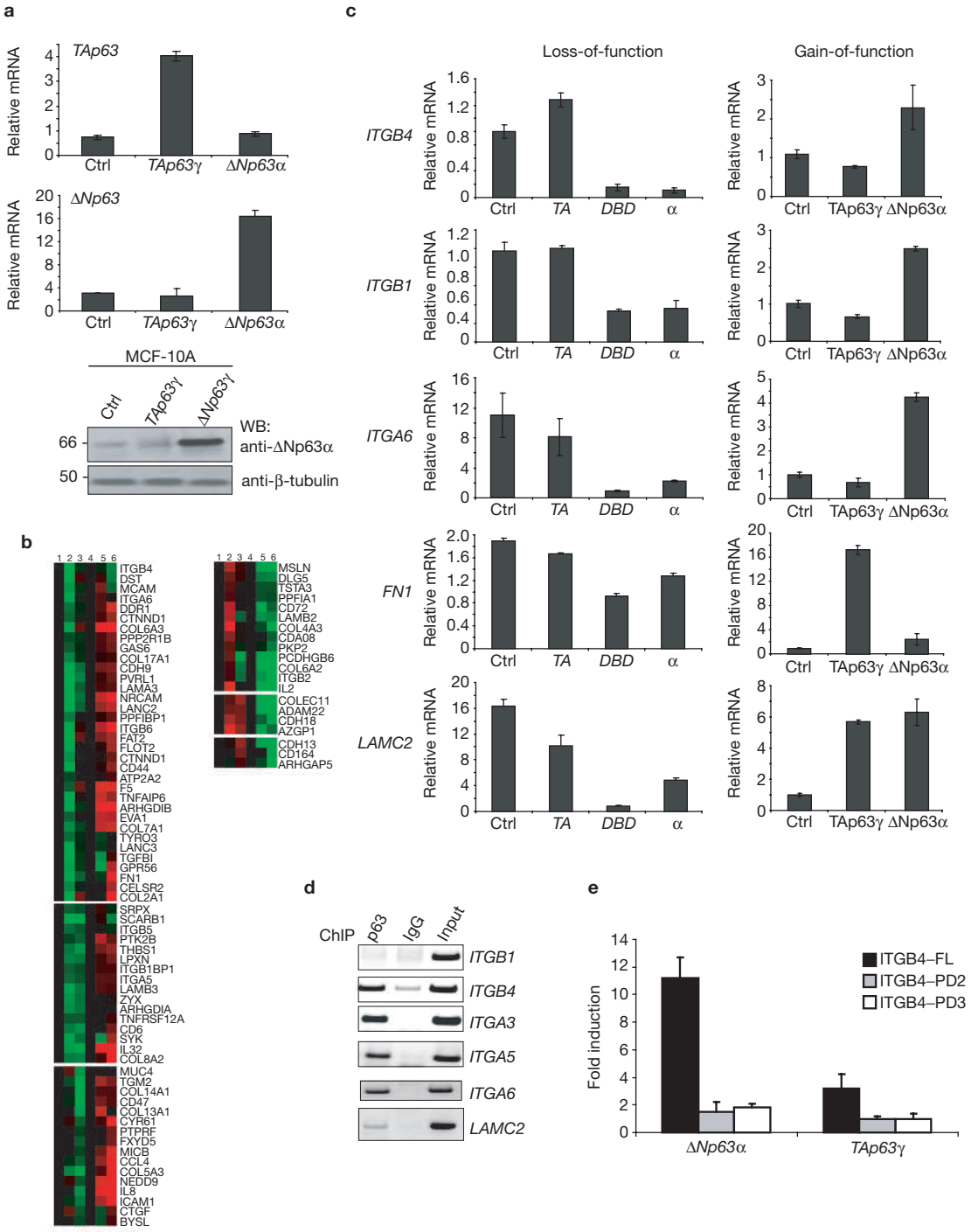


Figure 2 Identification of an adhesion subprogramme regulated by p63. (**a**) p63 expression in cells expressing TAp63γ, ΔNp63α or vector control (Ctrl) was determined by QRT–PCR using *TA* or Δ*Np63* specific primers (bar graphs) or by western blotting using a pan-p63 antibody (bottom). (**b**) Heatmaps of cell adhesion genes significantly regulated by p63 (downregulation, green; insignificant change, black; upregulation, red). Columns 1–3: loss-of-function using isoform specific shRNA (1, Vector control; 2, *DBD* shRNA; 3, *TA* shRNA). Columns 4–6: gain-of-function using isoform specific cDNA (4, Vector control; 5, TAp63γ; 6, ΔNp63α). Genes that were downregulated by *p63* loss and upregulated by *p63* overexpression (left), and genes upregulated by *p63* shRNAs and downregulated by *p63* gain (right). Separated sections demonstrate more specific expression patterns: the upper left genes showed downregulation with the *DBD* shRNA; the middle left genes showed downregulation with both *p63* shRNAs, and the lower left genes showed downregulation with *TA* shRNA. Similarly, the right panel was divided into three corresponding upregulation patterns. (**c**) Validation of microarray data by QRT–PCR 48 h after isoform-specific knockdown (loss-of-function) or following infection with retroviruses encoding p63 isoforms (gain-of-function). Gene targets selected include: β1-integrin (ITGB1), β4-integrin (ITGB4), α6-integrin (ITGA6), fibronectin (FN1) and laminin γ2 (LAMC2). Values represent the mean±s.d. of three replicate samples from one representative experiment ($n=3$). (**d**) *In vivo* binding of p63 to regulatory regions of selected gene targets. Crosslinked chromatin from MCF-10A cells was immunoprecipitated with antibodies against p63 or IgG control and analysed by PCR with primer pairs spanning regulatory regions upstream of indicated genes. (**e**) Saos2 cells were cotransfected with the full length (FL) *ITGB4* promoter reporter construct or deletion mutants (PD2, Δ151–403 base pairs upstream of the start site: PD3, Δ151–616 base pairs), and indicated p63 isoforms. The graph shows fold activation of the reporter by the p63 isoform relative to the empty vector and represents the average of four experiments±s.d.

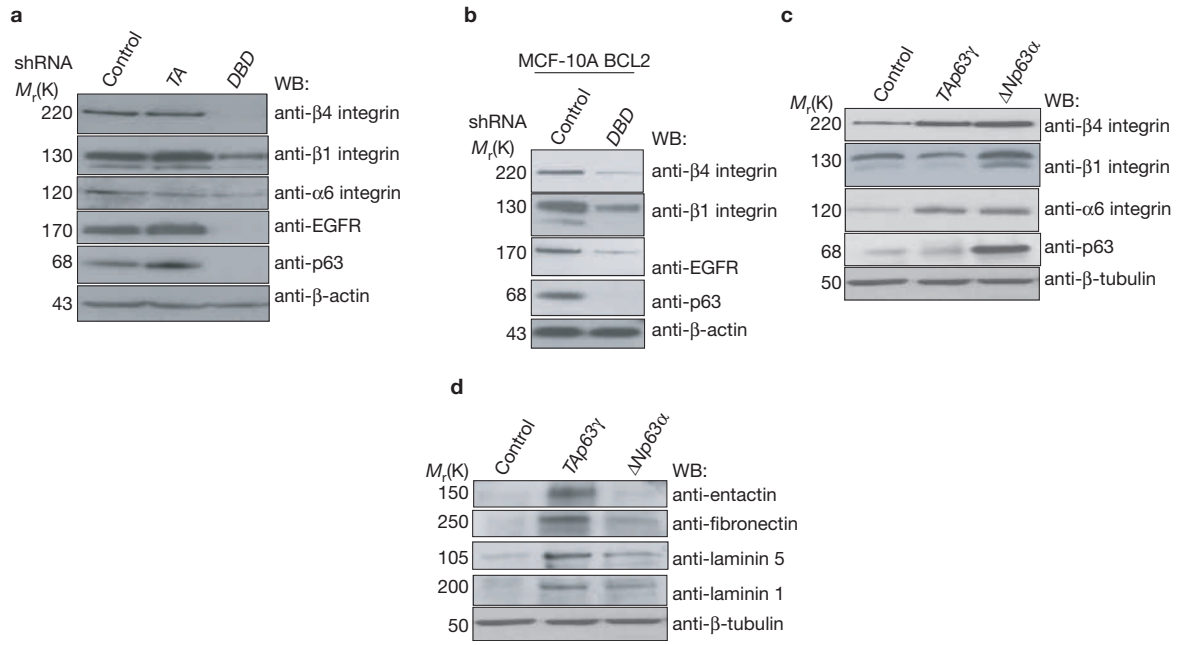


Figure 3 Regulation of cellular adhesion factors by p63. (**a**) Loss of all isoforms, but not TAp63 isoforms causes a marked reduction in cell adhesion proteins. Lysates from MCF-10A cells transduced with control or isoform-specific *p63* shRNAs were analysed by western blotting with the indicated antibodies 48 hs after infection. Knockdown of TAp63 isoforms was confirmed by QRT–PCR (data not shown). (**b**) Reduction of cellular adhesion proteins mediated by p63 loss is independent of cell death. Lysates from cells stably expressing Bcl2 were infected with control or *DBD p63* shRNAs and were analysed 48 h after infection by western blotting with the indicated antibodies. (**c**) Ectopic p63 expression increases integrin expression levels. Cell lysates from MCF-10A cells 48 h after infection with retroviruses encoding either TAp63γ or ΔNp63α isoforms or vector control were analysed by western blotting with the indicated antibodies. TAp63γ expression was verified by QRT–PCR (data not shown). (**d**) p63 augments cellular levels of ECM components in MCF-10A cells, determined by western blotting with indicated antibodies 48 h after transduction with retroviruses encoding either TAp63γ or ΔNp63α isoforms or vector control.

Interestingly, downregulation of all isoforms, but not TAp63 isoforms, caused a marked reduction in EGFR (Fig. 3A), a known transcriptional target of p63 (ref. 19), that is lost following MCF-10A cell detachment[20]. Importantly, the reduction of β1 and β4 integrins or EGFR levels caused by p63 loss was not affected by Bcl2 expression (Fig. 3b), suggesting that these events are independent of cell death.

As endogenous levels of ECM components were undetectable in parental cells, the loss of expression was not detectable at a protein level following shRNA-mediated *p63* reduction (data not shown). However, ectopic expression of p63 elevated expression of several ECM components (fibronectin, laminin-1 and -5) in cells expressing either isoform of p63, although to a greater extent in cells expressing TAp63γ (Fig. 3d). Furthermore, the matrix component entactin/nidogen was only upregulated in cells expressing TAp63γ (Fig. 3d) confirming the specific increase in mRNA expression observed in the microarray analysis. These data strongly support a role for p63 in the regulation of cell adhesion programmes, particularly those involved in cell–matrix adhesion.

Cell adhesion is regulated by p63 levels

As alterations in p63 levels markedly changed ECM/integrin expression levels, we examined whether downstream signalling pathways were activated in p63-expressing cells. A marked increase in tyrosine phosphorylation of Cas, FAK and paxillin was detected (Fig. 4a). Unlike FAK and paxillin, the total level of Pyk2 was increased several-fold following p63 expression (Fig. 4a). These data indicate that ectopic expression of p63 can alter integrin-mediated cell adhesion signalling, supporting the notion that p63 regulates cell adhesion.

The functional consequences of alterations in p63 expression levels on cell adhesion were examined by assessing the ability of cells expressing either *p63* cDNAs or *p63* shRNAs to adhere to a variety of exogenous matrix proteins (laminin-1, basement membrane complex (BMC), fibronectin and collagen IV). Increased expression of either p63 isoforms enhanced adhesion to laminin 1 (two- and fivefold, respectively), BMC (2.6- and 3.6-fold), fibronectin (19- and 17-fold) and collagen (two- and threefold), relative to control cells (Fig. 4b).

Reciprocal effects on cell adhesion were observed when adhesion to matrix proteins was examined 24 h after transduction of shRNAs targeting all isoforms or *p63α* isoforms, but not the *TA*-specific isoforms (Fig. 4c). Reduction in adhesion to exogenous matrix following *p63* knockdown was unaffected by stable Bcl2 expression; thus, functional loss of adhesion to exogenous matrix precedes cell death (Fig. 4d). The evidence that modulation of ΔNp63α specifically affects cell adhesion is consistent with our model that ΔNp63α is the major p63 isoform regulating the cellular adhesion programme in MCF-10A cells.

p63 mediates suppression of anoikis

As loss of p63 function was sufficient to induce anoikis, we investigated whether increased expression of p63 could protect MCF-10A cells from apoptosis following detachment of cells from matrix[21–23]. High levels of cell death can be detected 48 h after MCF-10A-cell detachment and is accompanied by loss of β1 integrin and EGFR expression[24]. Interestingly, endogenous levels of p63 also decrease dramatically following cell detachment (see Supplementary Information, Fig. S4a). Cells expressing either TAp63γ or ΔNp63α displayed a 2–3-fold reduction in cell death

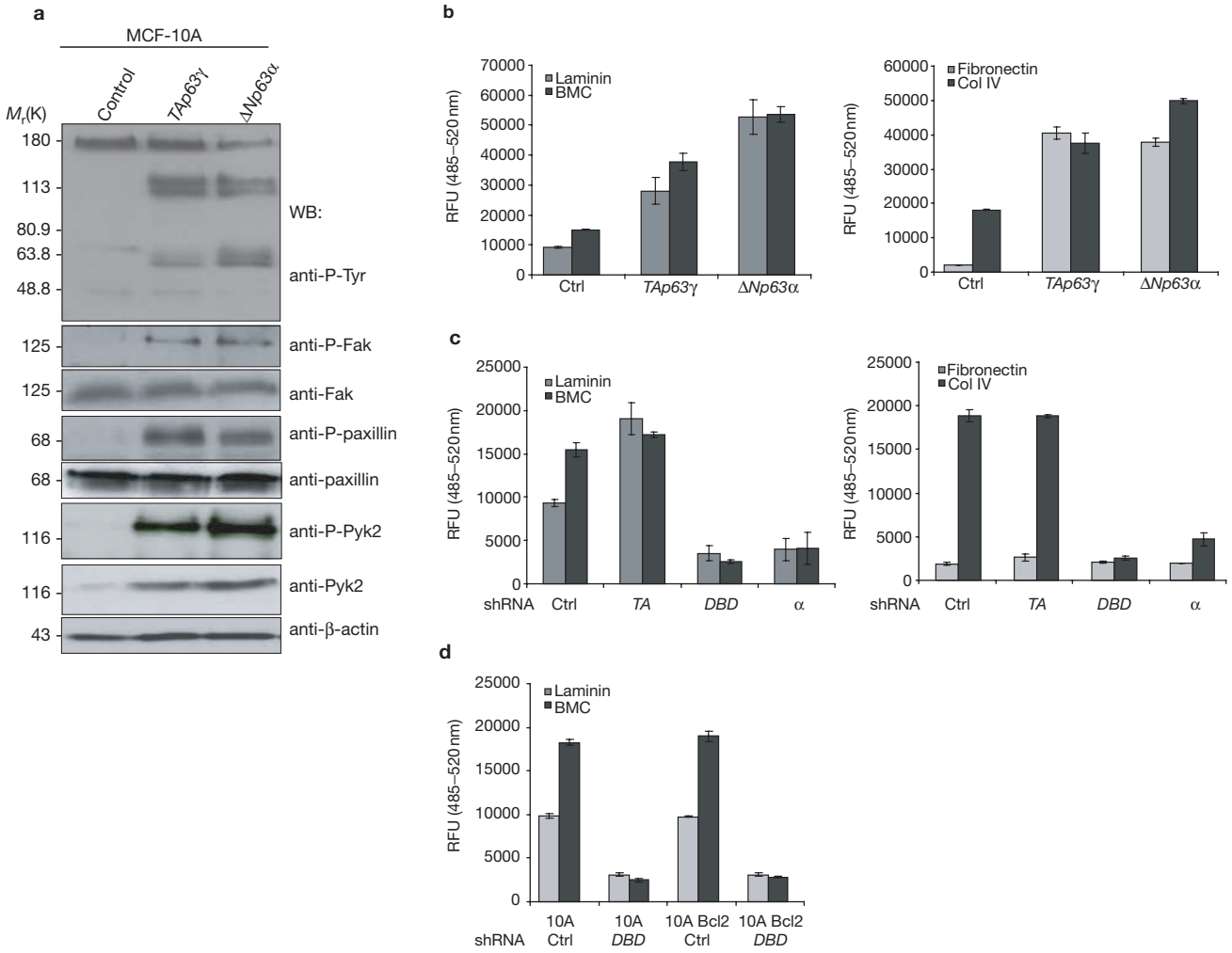


Figure 4 p63 activates adhesion–integrin signalling and promotes cell adhesion. (**a**) p63 expression enhances phosphorylation of integrin-regulated focal adhesion proteins. MCF-10A cells infected with control, TAp63γ or ΔNp63α retroviruses were lysed 48 h after infection and analysed by western blot with indicated antibodies. (**b–d**) Effect of loss or gain of p63 on adhesion to basement membrane proteins. Cells were infected with viral vectors and after the indicated time were plated on dishes coated with the indicated basement membrane proteins for 1 h and then adherent cells were quantified as described in Methods. Col IV, collagen IV. Values represent the mean ± s.d. of three replicate samples from one representative experiment ($n = 3$). Adhesion was measured 48 h after infection with control or p63 isoform-encoding retroviruses (**b**). Adhesion was measured 24 h after infection with control or *p63* isoform-specific shRNAs (**c**). Adhesion was monitored 48h following infection of control or Bcl2 expressing cells with control or p63 DBD shRNA encoding adenoviruses (**d**).

relative to control cells, with ΔNp63α providing the greater protection (Fig. 5). Expression of ΔNp63α in suspended cells prevents loss of expression of β1 integrin and EGFR (see Supplementary Information, Fig. S4), two events critically linked to anoikis[20], indicating that p63 is able to induce the expression of these proteins even under detached conditions. Furthermore, reduction in *β1* or *β4 integrin* levels, individually or in combination, by siRNA, blocks the anoikis protection conferred by p63 (see Supplementary Information, Fig. S4c), suggesting that increased expression of these adhesion proteins is partially responsible for this protective effect.

Detachment-induced apoptosis following p63 downregulation can be rescued by β4 integrin signalling

As p63 modulation altered expression of genes that are involved in matrix-induced survival, we examined whether constitutive expression of these genes was sufficient to block or delay detachment and apoptosis induced by p63 loss. *p63* expression was downregulated by *DBD* shRNA transduction in MCF-10A cells overexpressing either EGFR, β1 or β4 integrins, and cells were monitored for apoptosis 48 h after adenoviral shRNA infection by a DNA fragmentation cell-death ELISA. Expression of either EGFR, or β1 or β4 integrin, partially (40% relative to control cells) rescued the defect in adhesion to exogenous laminin or BMC caused by p63 knockdown (data not shown). However, only β4 integrin, not β1 or EGFR, was sufficient to significantly reduce the apoptosis caused by downregulation of total p63 (Fig. 6a).

A dominant-negative mutant form of β4 integrin that lacked the cytoplasmic tail was used to evaluate whether signalling downstream of β4 integrin is required for this cell survival activity. Unlike the protective effect observed with wild-type β4 integrin, the truncated mutant was unable to reduce cell death caused by p63 knockdown, as determined by cell death ELISA or immunoblotting for apoptotic markers (Fig. 6b, c). A distinct function of β4 integrin is to link the cytoskeleton

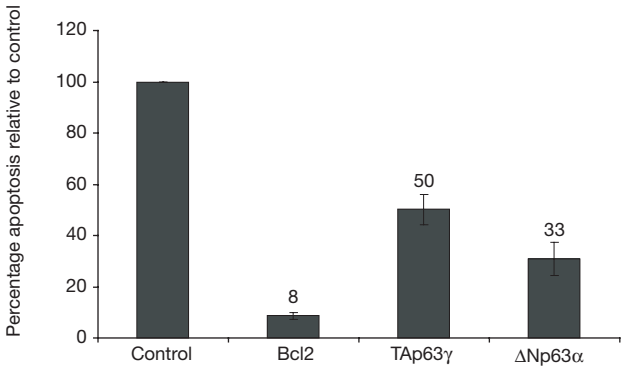


Figure 5 Elevated p63 expression suppresses apoptosis following cell detachment. MCF-10A cells stably expressing p63 isoforms, or Bcl2 as a positive control, were placed in suspension for 48 h and then assayed for apoptosis using DNA fragmentation cell-death ELISA. Values represent the mean ± s.d. of three independent experiments.

to hemidesmosomes and function in basal membrane-directed tissue polarity and resistance to apoptosis[25]. To address the importance of hemidesmosome integrity in these effects, a smaller directed-deletion mutant with several amino acids in the connecting segment (Δ1314–1486) critical for targeting β4 integrin to hemidesmosomes, was examined. This mutant was also unable to prevent apoptosis due to loss of p63 (data not shown). These observations establish a critical role for β4 hemidesmosome signalling downstream of p63.

Cell adhesion is regulated by p63 in primary mammary cells and keratinocytes

To determine whether p63 is required to regulate cell adhesion and survival in primary mouse cells, the effects of acute conditional loss of p63 on cell adhesion was examined in primary mouse mammary epithelial cells (MMECs) isolated from mice engineered to contain flox sites flanking the core domain within the *p63* gene[26]. Conditional ablation of *p63* by Cre-mediated gene disruption offers a genetic alternative to RNA interference. Wild-type or $p63^{flox/flox}$ MMECs were infected with control or Cre-recombinase expressing adenoviral vectors 24 h after plating and the effects on integrin expression, adhesion to exogenous matrix and cell death were monitored (Fig. 7). Loss of *p63* induced a significant reduction in the expression of proteins involved in cell adhesion and was associated with a marked reduction in the ability of the cells to adhere to exogenous laminin-1 and BMC relative to wild-type or $p63^{flox/flox}$ control cells (Fig. 7c). Furthermore, the induction of apoptosis (by DNA fragmentation cell death ELISA) was observed following the conditional ablation of *p63* (Fig. 7d). Similar results were obtained using *p63*-directed shRNA in wild-type mouse and human primary MECs. Downregulation of all, but not TAp63 isoforms, resulted in a marked decrease in integrin expression, cell adhesion to exogenous substrates and cell death (data not shown).

Finally, a similar induction of apoptosis (Fig. 8a) and reduction in β1 integrin, β4 integrin and EGFR expression was observed following *p63* knockdown using the *DBD* shRNA in another primary epithelial cell type (Fig. 8b), human foreskin keratinocytes (HFKs), suggesting that p63 regulates cell adhesion in other epithelial cells.

DISCUSSION

Using a loss- and gain-of-expression approach coupled with transcriptional profiling, we have defined a critical role for p63 in the

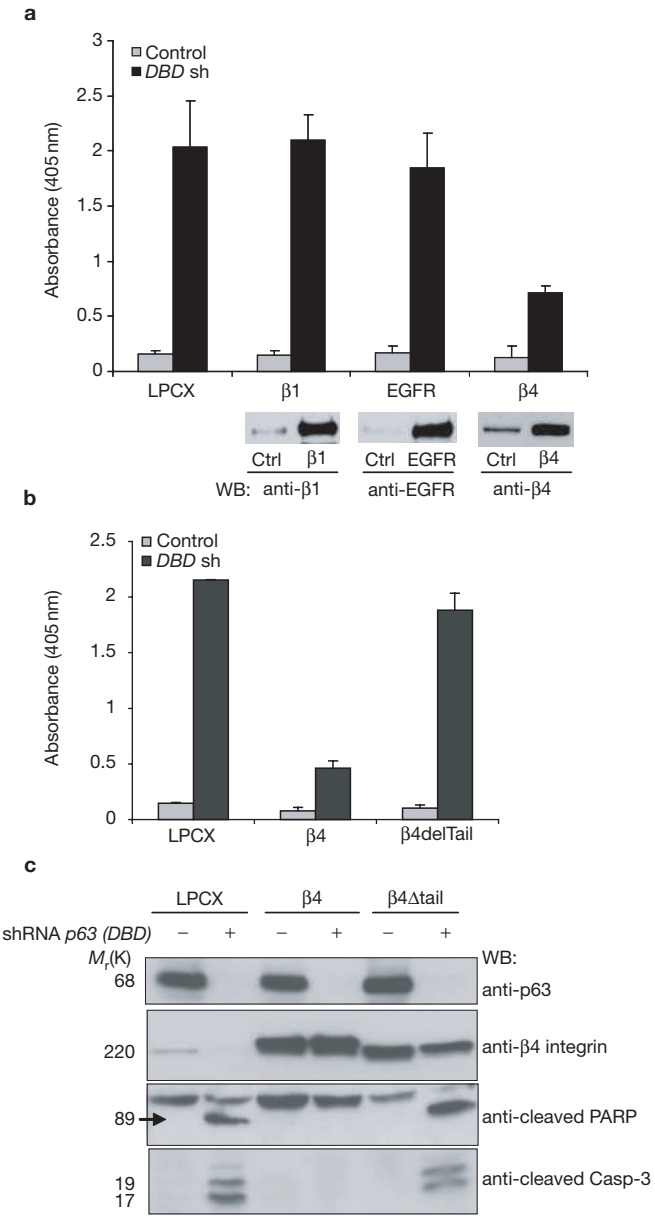
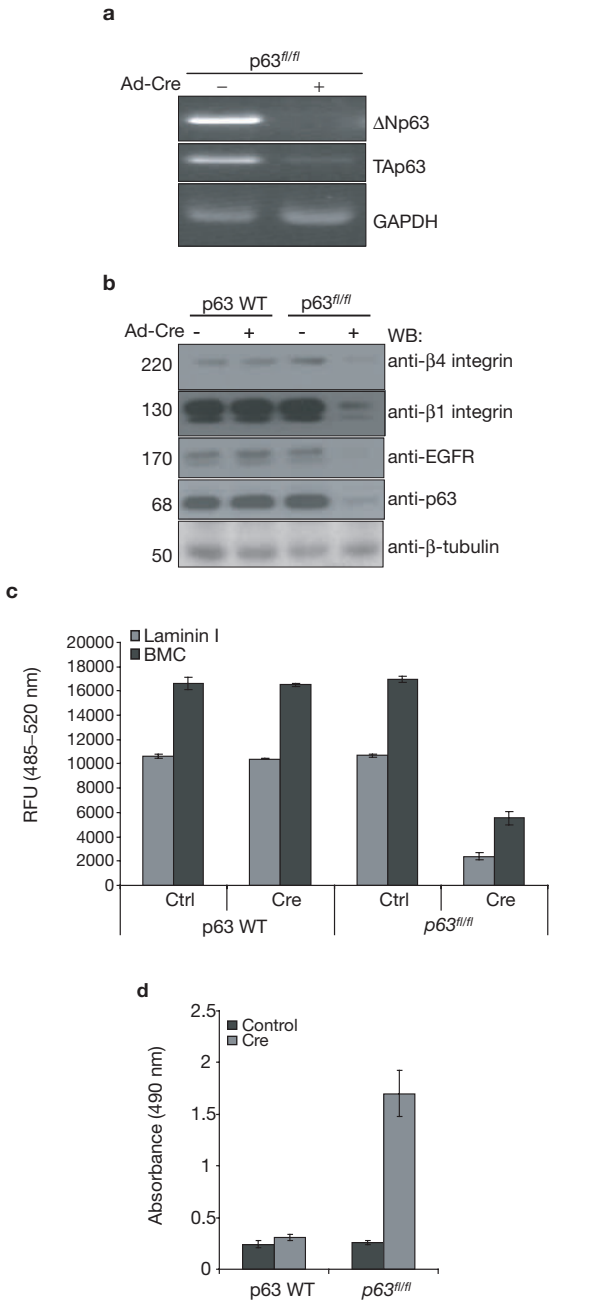


Figure 6 β4 integrin partially protects from anoikis induced by p63 loss. (**a**) MCF-10A cells stably expressing β1 integrin, EGFR or β4 integrin were subjected to *p63* knockdown by shRNA. Cells were analysed 48 h after infection for cell death by DNA fragmentation cell-death ELISA. Inserts below the bar graph show western blots of each of the overexpressed proteins. (**b**, **c**) β4 integrin signalling is required for partial protection from cell death induced by p63 loss. MCF-10A cells stably expressing vector control (LPCX), β4 integrin or a mutant form β4 integrin lacking its cytoplasmic tail, were subjected to *p63* knockdown by shRNA. Cells were analysed 48 h later for cell death by DNA fragmentation cell-death ELISA (**b**) or by western blotting (**c**) with the indicated antibodies. Values for the ELISA represent the mean ± s.d. of three independent experiments.

regulation of cellular adhesion. Knockdown of endogenous ΔNp63 induced downregulation of cell adhesion-associated genes, cell detachment and anoikis. These findings were supported by gain-of-function studies in which increased expression of either TAp63γ or ΔNp63α upregulated genes encoding key cell adhesion molecules that were downregulated by *p63* directed shRNAs, increased cellular adhesion to exogenous ECM and conferred resistance to anoikis. Furthermore, cell



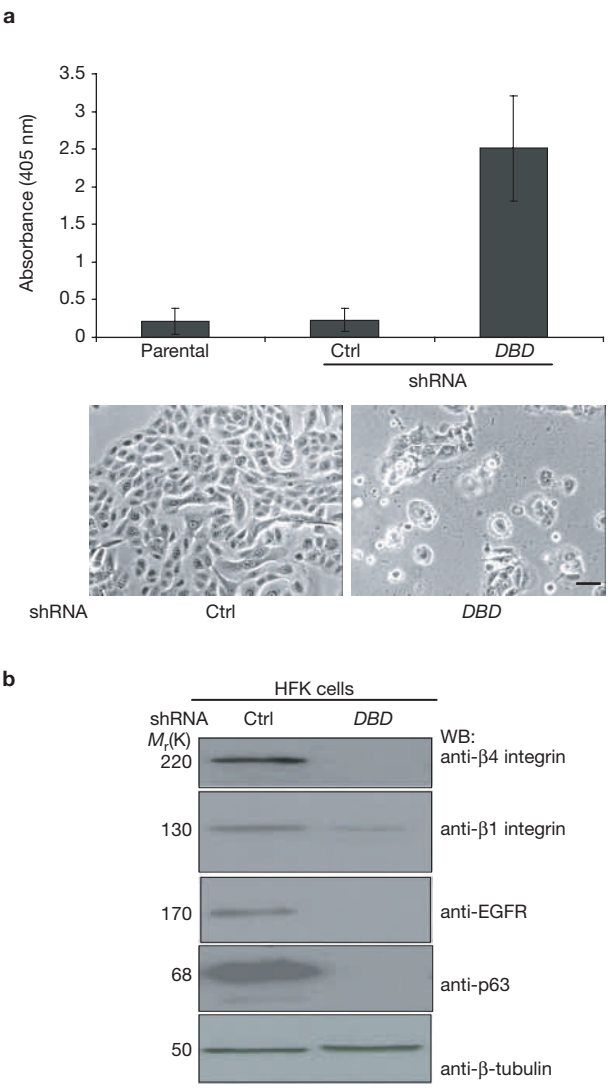


Figure 8 p63 regulates cellular adhesion and survival in other epithelial tissues. (**a**) Primary HFKs were infected with *p63* shRNAs and cells were analysed 48 h later for cell death by DNA fragmentation cell-death ELISA (top). Phase micrographs of infected cells are also shown. (**b**) Expression levels of cellular adhesion proteins was analysed by western blotting with indicated antibodies 48 h after infection with control or *DBD* shRNA. The scale bars represent 50 μM.

Figure 7 p63 controls a cellular adhesion programme in primary mammary epithelial cells. (**a**) MMECs from $p63^{flox/flox}$ ($p63^{fl/fl}$) mice were infected with either a control adenoviral vector (–) or one encoding Cre-recombinase (+), and *p63* mRNA expression levels were analysed 24 h after infection by semi-quantitative RT-PCR using primers specific for either the *TAp63* (35 cycles) or Δ*Np63* isoforms (25 cycles). (**b**) Acute genetic ablation of *p63* causes a marked reduction in cell adhesion proteins. Lysates from wild-type (WT) or $p63^{fl/fl}$ MMECs infected with control (–) or Cre-recombinase expressing (+) adenovirus were analysed by western blotting with the indicated antibodies 24 h after infection. (**c**) *p63* ablation causes a reduction in cell adhesion. After infection with control or Cre-recombinase expressing adenovirus (24 h), wild-type $p63^{fl/fl}$ MMECs were plated on dishes coated with laminin 1 or BMC for 1 h and adherent cells were quantified as described in Methods. Values represent the mean±s.d. of three replicate samples from one representative experiment ($n=3$). (**d**) *p63* ablation causes cell death. Wild-type or $p63^{fl/fl}$ MMECs were analysed 24 h after infection with control or Cre-recombinase expressing adenoviral vectors for cell death by DNA fragmentation cell-death ELISA. Values represent the mean±s.d. of three independent experiments.

death induced by p63 loss was rescued by signalling downstream of β4 integrin. Lastly, similar effects following loss of p63 were observed in primary mammary epithelial cells and keratinocytes, thus implicating p63 as a key regulator of the integrity and survival of basal cells in the mammary gland and other stratified epithelial tissues.

MCF-10A cells express ΔNp63α to the near exclusion of other isoforms. Thus, it is not surprising that downregulation of all p63 isoforms and αp63 isoforms caused similar phenotypic effects. In contrast, loss of TAp63 isoforms had little or no effect on cellular morphology, survival and ability to adhere to exogenous matrix. However, following selective loss of TAp63 isoforms, an increase in endogenous ΔNp63α protein was observed (Fig. 1c), suggesting that TAp63 isoforms may act to regulate ΔNp63 expression levels[27,28]. As the reported ablations of p63 in mice involved deletion of exons common to all p63 isoforms[2,3], it was not possible to conclude which deficient isoform(s) of p63 accounted for

the complete absence of mammary epithelium. Our results point to a predominant role for ΔNp63α in regulating adhesion and survival, two processes that could significantly affect progenitor cell maintenance and/or differentiation. The results here demonstrate a role for ΔNp63α in regulating cell adhesion and support recent studies that implicate two specific adhesion-related genes as p63 transcriptional targets, *α3-integrin*[13] and *Perp*[29]. Interestingly, Perp functions to stabilize desmosomal adhesive complexes[29]. In addition, p63 is required for epithelial stratification and differentiation — processes that have been linked to asymmetric cell division[30]. Integrins and cadherins are required for this process, which fails, together with stratification, in *p63*-null epithelia[30]. These studies are consistent with our findings, which strongly argue for a primary role for p63 in regulating a programme of cellular adhesion.

Although earlier studies indicated that ΔNp63 isoforms function as dominant negatives to inhibit p53-family target gene activation[8], our results, and others[11–13,29], argue that ΔNp63 isoforms can promote transcription. Indeed, seven times as many adhesion-related genes were downregulated following knockdown of all p63 isoforms compared with knockdown of TAp63 isoforms, suggesting that ΔNp63 isoforms positively regulate the transcription and/or expression of large sets of genes. Many genes that were downregulated by *DBD* shRNA relative to *TA* shRNA, were upregulated by ΔNp63α overexpression, supporting this conclusion. We present multiple lines of evidence indicating that p63 directly regulates the transcription of several genes that were affected by loss or gain of p63 expression, including *in vivo* binding and transcriptional reporter analyses (Fig. 2). Additionally, we found that the expression of integrins β4, α6 and α3, as well as laminin γ2 and fibronectin, was increased as early as eight hours after infection with p63-encoding adenoviral vectors (see Supplementary Information, Fig. S3). Together, these data provide compelling evidence that there is direct transcriptional regulation of several of the identified target genes by p63.

It is difficult to extrapolate whether loss of p63 in the context of a normal tissue would lead to a similar induction of cell death as many types of adhesive interactions are functional in a tissue context that are not replicated *in vitro*. Recently, p63 loss was shown to induce cell senescence in the skin[31] and loss of cell adhesion proteins could contribute to this phenotype *in vivo*. Although loss of p63 induces detachment and apoptosis, ectopic expression of either ΔNp63α or TAp63γ can protect cells from apoptosis following forced physical detachment, possibly due to p63-induced upregulation of adhesion proteins and enhanced integrin-mediated adhesion and/or growth factor signalling (see Supplementary Information, Fig. S4). Moreover, p63 expression decreases within 24 h after MCF-10A cell detachment (see Supplementary Information, Fig. S4), suggesting that p63 levels are regulated by cell adhesion signalling and raising the possibility that there is a reciprocal relationship between adhesion strength and p63 expression. Indeed, signalling from, and attachment to, the underlying mesenchyme may be required for *in vivo* regulation of p63 expression in primitive ectodermal cells before commitment toward a stratified epithelial lineage.

Exogenous EGFR expression is sufficient to block anoikis in MCF-10A cells[20] and in human keratinocytes[32]. However, expression of EGFR or β1 integrin was unable to protect against anoikis induced by p63 loss. It is possible that EGFR was not sufficient to compensate for the extensive loss of adhesion proteins caused by p63 loss. β4 integrin is not lost during anoikis of parental cells, whereas it is lost with downregulation of p63. Interestingly, ectopic expression of β4 integrin significantly reduced apoptosis following p63 knockdown and this rescue required signalling downstream of β4 and hemidesmosome integrity. A distinct function of β4-integrin is to physically link the cytoskeleton to hemidesmosomes, which are essential for cell adhesion and survival, as well as for basal membrane-directed tissue polarity and Rac and NF-κB activation[25,33,34].

The formation of squamous epithelial derived tissues is a complex processes involving ectoderm–mesenchyme crosstalk, secreted factors, as well as cell–cell and cell–matrix interactions[1]. Regulation of cell adhesion is a general feature underlying early morphogenesis of several ectoderm-derived organs including the mammary gland[35,36]. Commitment of specialized progenitor or stem cells requires extensive signalling, and interactions with non-stem cells and basal lamina, within a specialized niche[37,38]. Adhesion proteins, such as cadherins–catenins through adherens junctions and integrins through interactions with the extracellular matrix, are thought to play a major role within these specialized microenvironments. Loss-of-function studies in mice have revealed that both integrins and adherens junctions have critical roles in maintaining the location, adhesiveness and proliferative status of epithelial stem cells within tissues[37]. Transcriptional profiling of these specialized cells has highlighted the importance of integrins, their ligands, and other cell-adhesion and polarity proteins, and increased levels of expression of integrins are often characteristic of stem cells[39]. Alterations in integrin expression allows departure from the stem-cell niche though differentiation or apoptosis, modulation of basement membrane composition and the local concentration of secreted factors available within the stem cell niche[37]. Given that p63 can regulate many of these same cell adhesion-associated genes, it is tempting to speculate that p63 may have a major role in stem cell and progenitor cell biology and/or the regulation of adhesion involved in epithelial morphogenesis. In the mammary gland the basal and/or myoepithelial cells are the earliest cells detected during embryonic mammary gland development and possibly mark early mammary progenitor cells. This cell type mediates the interaction between ductal luminal cells and the secreted extracellular matrix. These cells are characterised by their high level expression of integrins and ECM proteins not seen within the luminal cells, further supporting a fundamental role for p63 in the biology of these cells.

In conclusion, we have shown that p63 is critical for basal epithelial cell adhesion and survival and that this regulation is mediated by transcription of a cell adhesion subprogramme. The precise mechanisms by which p63 exerts these functions remain poorly defined and are the focus of current investigations. □

METHODS

Cell culture and treatments. MCF10-A cells were maintained as previously described[20]. Primary human mammary epithelial cells (HMEC) (Clonectics, Cambrex, Rockland, ME) were maintained in MEGM supplemented with bovine pituitary extract. Primary human epidermal keratinocytes (HFK) were cultured as previously described[12]. 293T cells were maintained in DMEM with 10% v/v FCS. Primary mouse mammary epithelial cells (MMEC) were obtained from Balb/C, *p63* floxed mice or wild-type littermates[26] and maintained as previously described[20]. Generation of VSV-G pseudotyped retrovirus and retroviral infection of MCF-10A cells was carried out as previously described[17]. To determine the effect of p63 isoform expression on cell growth, stably infected MCF-10A cells (4,000 cells per well, 24-well plate) were plated and grown in assay media[17] in the absence of EGF, and cells were counted (triplicate wells per timepoint) on days 2, 4, 6, 8 and 10 after plating. Cell death was measured by propidium iodide staining followed by flow cytometric analysis or using the cell death detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Each experiment was performed, at least, in triplicate.

Reagents, antibodies and DNA constructs. Commercial antibodies were obtained from the following sources: integrin β1 (clone 18), integrin β4 (7), EGFR (18), FAK (77), paxillin (349), Pyk2 (11) and phospho-tyrosine (4G10) from BD Biosciences (San Jose, CA); p63 (4A4), fibronectin (IST9), β-actin, and β-tubulin from Abcam (Cambridge, MA); integrin α6 (GoH3), nidogen–entactin and laminin 5 (D4B5) from Chemicon (Temecula, CA); laminin 1 from Sigma (St Louis, MO); collagen IV and I from Calbiochem (San Diego, CA); phospho-FAK (p-Y397) and phospho-paxillin (p-Y118) from Biosource International (Carlsbad, CA); cleaved PARP, cleaved caspase 3 (CASP-3), Erk, phospho-Erk, PKB, phospho-PKB and phospho-Pyk2 (p-Y402) from Cell Signaling (Beverly, MA); and Bcl2, p73 and p53 from Santa Cruz Biotechnology (Santa Cruz, CA).

Human *TAp63γ* and *ΔNp63α* cDNAs and shRNA rescue mutants were subcloned as *BamH*I–*Xho*I fragments into the retroviral vector pBabe puro. shRNA rescue mutants were constructed by introducing three or four silent nucleotide changes using site directed mutagenesis on human *TAp63γ* and *ΔNp63α* cDNAs in pcDNA3. Correct incorporation of mutations was confirmed by DNA sequence analysis.

Adenoviral infection and gene silencing with shRNAs. Cassettes containing the U6 RNA polymerase III promoter and shRNA sequences were subcloned into the pAD Shuttle plasmid (Stratagene, La Jolla, CA) followed by creation of replication-deficient adenovirus by homologous recombination in bacteria as previously described[12]. Purified adenoviral stocks were titred using standard viral plaque assays and were used at an MOI of 50 for all experiments. Cells were grown in full medium and infected with adenovirus expressing vector control or *p63* isoform shRNAs for 2 h. Cells were harvested for FACS, cell death ELISA, and protein and RNA extraction 48 h after infection. shRNA target sequences were as follows: *p63 TA* isoform specific, 5′-GGGATTTTCTGGAACAGCCTAT-3′; *DBD*–All *p63* isoform specific, 5′-GGGAACAGCCATGCCCAGTATG-3′; *αp63* isoform specific, 5′-GGGTGAGCGTGTTATTGATGCT-3′. *p63* gene ablation was performed *in vitro* by Cre recombinase-mediated excision of floxed *p63* alleles in primary MMECs. $p63^{fl/fl}$ and wild-type littermate MMECs were plated for 24 h following isolation. Cells were trypsinized, allowed to adhere and were then infected with Ad5–CMV–Cre–GFP or Ad5–CMV–GFP (Vector Development Lab, Baylor College of Medicine, Houston, TX) for 2 h. Cells were harvested for protein and RNA extraction at 24 and 48 h after infection.

Microarray and statistical analysis. Total RNA was isolated 48 h after retroviral or adenoviral infection and was subjected to reverse transcription, labelling and hybridization to U133Av2.0 gene chip arrays (Affymetrix, Santa Clara, CA) containing 14,500 human genes. The shRNA knockdown experiment was performed in duplicate and the cDNA overexpression experiment was performed in triplicate. Background correction and normalization of microarray data used the MAS5 function in the Bioconductor Affy package[40]. Normalised intensity data was log2 transformed before statistical tests. The differential expression was assessed using the empirical Bayes method implemented in Bioconductor Limma package, and the *P* values were adjusted by false discovery rates. The significance level was set at a false discovery rate 0.05 for overexpression experiments, which were performed in triplicate, whereas an ANOVA P value <0.01 was used as the significance cutoff in shRNA knockdown experiments, which were performed in duplicate, to avoid depletion of significant genes by an over-restricted statistical cutoff. Gene expression heatmaps were generated with the Treeview program[41].

Enrichment analysis. Differentially expressed genes were assessed for significant enrichment of gene ontology for cell adhesion category and all its subcategories. The enrichment analysis computes the probability that a set of genes that are annotated as within a specific category would occur by chance and the results are given in the form of *P* values. The values given in the text indicate the number of adhesion probe-sets regulated/total number of adhesion probe-sets and the total number of regulated probe-sets/total number of probe-sets.

Chromatin immunoprecipitation(ChIP) assays. ChIP assays were performed as previously described[42]. Chromatin was prepared from MCF10A cells and immunoprecipitated with a combination of monoclonal (4A4) and polyclonal (H-129, Santa Cruz) anti-p63 antibodies. The associated DNA was used for PCR with primers spanning regions containing p53 binding motifs within the upstream regulatory regions. Primer sequences are as follows: *ITGB1* (–1812 to 1603) Fw, ACAAGTAGACTCGTATTCTTGACCAATGAC, Rev, C AAGGACAGATAATAGGGGCTCAGATAATA; *ITGA3* (–2474 to 2144) Fwd, ATTTGTAAACTGTAAAGCTAGGCCAGGAC, Rev, ACTA GGATTTTCATCCTGTGTTATCTGACC; *ITGB4* (–1476 to 1256) Fwd, CATGGTGCTAGGTGCTAGAGAGTAGCTG, Rev, AGTTTATCCTTCTTGTCCTTGAAGACGTTG; *ITGA5* (–2878 to 2660) Fwd, GAACTCCTCTGTGGAAATTCACTCTTGT, Rev, CTTAA ACCTGACCTCTTTGGTACCCTCT; *ITGA6* (–632 to 475) Fwd, GCAAAAAGAAACACCTACCTCATAGGAC, Rev, GGGACTACAGCTGTGTAGACTGTTCTG; *LAMC2* (–583 to 364) Fwd, GGTTACTTTATGAGTTGCTAACCCTGGTG, Rev, CCCAATCTTAAGAGC GCTAACTCAGAAA.

Reporter assays. Saos2 cells (5×10^4 per well) were plated 24-well plates. The next day, cells were transiently transfected using Fugene 6 (Roche, Indian, IN) with 250 ng of either ΔNp63α or TAp63γ expression plasmids, as well as 25 ng pRLnul (Promega, Madiosn, WI) or pRL-B4 constructs and 25 ng SV40 Renilla. After transfection (48 h), cells were harvested and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase values were divided by Renilla luciferase values to control for transfection efficiency. Each experiment was performed four times, with duplicate samples in each experiment.

Protein preparation and immunoblotting. Protein lysates were prepared as previously described[20]. Protein concentration was determined using a Bradford dye-based assay (Biorad, Hercules, CA). Total protein (20 μg) was subjected to SDS–PAGE followed by immunoblotting with appropriate antibodies at the recommended dilutions. The blots were then incubated with peroxidase linked secondary antibodies followed by enhanced-chemiluminescent detection.

RNA isolation and RT–PCR. Total RNA was isolated from cells grown in 10-cm dishes using Tri-Reagent (Sigma) according to the manufacturers' instructions. Semi-quantitative RT–PCR was performed using 0.5 μg total DNAse-treated RNA, which was then amplified using gene specific primers with the One-Step RT–PCR kit (Qiagen, Chatsworth, CA) according to the manufacturers' instructions. All PCR products were analysed by gel electrophoresis. QRT-PCR was performed using Quantitect SYBR Green RT-PCR kit (Qiagen) according to the manufacturers' instructions. Primer sequences for *p63* isoforms are as follows: ΔNp63fwd, 5′-GGAAAACAATGCCCAGACTC-3′; ΔNp63rev, 5′-GTGGAATACGTCCAGGTGGC-3′; TAp63fwd, 5′-AAGATGGTGCGACAAACAAG-3′; TAp63rev, 5′-AGAGAGCATCGAAGGTGGAG-3′. All primers spanned at least one intron and control amplification was performed on RNA samples not subjected to the reverse transcription, in parallel, to ensure no contaminating genomic DNA was present.

Cell-adhesion assays. Cell adhesion to laminin 1, collagen IV, fibronectin or basement membrane complex was performed using the Innocyte ECM cell adhesion assay (Calbiochem) according to manufacturer's instructions. Briefly, cells (1×10^4) were allowed to adhere to exogenous matrix for 1 h at 37 °C in a 96-well plate. Wells were subsequently washed thoroughly to remove non-adherent cells and acetoxymethyl–calcein dye added to allow quantification of adherent cells. Following 1 h incubation with calcein AM at 37 °C, fluorescence was measured at 485–520nm.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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