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TAp63 induces senescence and suppresses tumorigenesis *in vivo*

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Abstract

p63 is distinct from its homologue p53 in that its role as a tumour suppressor is controversial, an issue complicated by the existence of two classes of p63 isoforms¹. Here we show that TAp63 isoforms are robust mediators of senescence that inhibit tumorigenesis *in vivo*. Whereas gain of TAp63 induces senescence, loss of p63 enhances sarcoma development in mice lacking p53. Using a new TAp63-specific conditional mouse model, we demonstrate that TAp63 isoforms are essential for Ras-induced senescence, and that TAp63 deficiency increases proliferation and enhances Ras-mediated oncogenesis in the context of p53 deficiency *in vivo*. TAp63 induces senescence independently of p53, p19^{Arf} and p16^{Ink4a}, but requires p21^{Waf/Cip1} and Rb. TAp63-mediated senescence overrides Ras-driven transformation of p53-deficient cells, preventing tumour initiation, and doxycycline-regulated expression of TAp63 activates p21^{Waf/Cip1}, induces senescence and inhibits progression of established tumours *in vivo*. Our findings demonstrate that TAp63 isoforms function as tumour suppressors by regulating senescence through p53-independent pathways. The ability of TAp63 to trigger senescence and halt tumorigenesis irrespective of p53 status identifies TAp63 as a potential target of anti-cancer therapy for human malignancies with compromised p53.

Determining the role of p63 in cancer has been challenging, largely because *p63* encodes six different proteins. Isoforms containing or lacking an amino-terminal p53-like transactivation domain are referred to as TA and ΔN isoforms, respectively, and they have distinct, even opposing, functions^{1,2}. ΔNp63 isoforms enhance proliferation^{3,4} and inhibit apoptosis⁵, whereas TAp63 isoforms induce apoptosis^{6,7} and inhibit cell-cycle progression⁸. Although these findings are in line with ΔNp63 and TAp63 proteins promoting and suppressing tumorigenesis, respectively, this has not been directly demonstrated.

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AUTHOR CONTRIBUTIONS

X.G. and A.A.M. designed and performed experiments, analysed data and prepared the manuscript. W.M.K. performed BrdU immunohistochemistry and immunofluorescent staining; C.P. and W.L. performed western blotting analyses; H.V. performed histopathology; J.Z. and S.W.L. designed and constructed the Tet-on system, which formed the basis of the TAp63-specific inducible expression system.

COMPETING FINANCIAL INTERESTS

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Cellular senescence is a tumour-suppressive mechanism that prevents progression of pre-malignant lesions *in vivo*⁹ and its activation by p53 induction leads to tumour regression and clearance^{10,11}. We previously reported that p63 deficiency induces cellular senescence in epidermal keratinocytes¹². This former study ablated all p63 isoforms and therefore did not address which p63 isoform(s) regulates senescence. Here, we asked whether TAp63 isoforms, which are most similar to p53 in structure and transactivation capabilities¹, could induce senescence in mouse embryonic fibroblasts (MEFs). We chose these cells as TAp63 is expressed within the dermis, the tissue from which MEFs are derived (Supplementary Information, Fig. S1a). Expression of TAp63 α , TAp63 β and TAp63 γ in wild-type MEFs induced morphological characteristics of cellular senescence, increased senescence-associated β -galactosidase (SA- β -Gal) activity, enhanced expression of Rb and decreased proliferation (Fig. 1a; Supplementary Information, Fig. S1b, c). TAp63 β and TAp63 γ were more potent than TAp63 α in each of these assays. As TAp63 had been associated with apoptosis in transformed cells^{1,6} as well as in oocytes⁷, we investigated whether apoptosis was occurring by analysing the sub-G1 population. Consistent with the absence of microscopically visible apoptotic cells, TAp63 expression did not alter the percentage of apoptotic cells (0.7%, 1.1%, 0.8% for vector control, TAp63 β and TAp63 γ , respectively). We asked whether TAp63 isoforms also induced senescence in human cells, and indeed found that this was the case (Fig. 1b). TAp63 isoforms also induced senescence in p63^{-/-} MEFs, however Δ Np63 α and Δ Np63 γ could not induce senescence in either p63^{-/-} or wild-type MEFs (Supplementary Information, Fig. S1d–g). Furthermore, Δ Np63 α , but not Δ Np63 γ , blocked TAp63 γ -induced senescence (Supplementary Information, Fig. S1e, g). These findings indicate that expression of TAp63 is sufficient to induce cellular senescence in both mouse and human cells.

To determine whether TAp63 functions through p21^{Waf1/Cip1} (p21), p16^{Ink4a} (p16) or Rb, we used short hairpin RNAs (shRNAs) to knock down expression of these senescence mediators, and assessed whether TAp63 isoforms could still induce senescence. Focusing on TAp63 β and TAp63 γ , because they were the most robust senescence inducers, we found that whereas MEFs expressing TAp63 β or TAp63 γ became senescent, the morphological changes of senescence normally induced by TAp63 did not occur when p21 was simultaneously knocked down (Supplementary Information, Fig. S2a). Whereas 33% of empty vector-expressing control cells were proliferating, expression of TAp63 β and TAp63 γ decreased the BrdU (5-bromo-2-deoxyuridine)-positive populations to 18.3% and 13.7%, respectively (Fig. 1c). However, knockdown of p21 restored proliferation to levels comparable to those of empty vector-expressing control cells (Fig. 1c, d), indicating that p21 is required for TAp63-induced cell-cycle arrest. Consistent with it being essential for TAp63-induced senescence, p21 was induced upon TAp63 γ expression (Supplementary Information, Fig. S2b). We also found that knockdown of Rb, but not p16, inhibited the ability of TAp63 to induce senescence (Fig. 1c, e, f; Supplementary Information, Fig. S2a), although the effect was more dramatic when p21 was depleted. As the level of p16 knockdown was not extensive, we tested whether p16 (as well as p19^{Arf}, p19) was required for TAp63-induced senescence by expressing TAp63 isoforms in NIH 3T3 cells, which lack the *Ink4a/Arf* locus that encodes both p16 and p19 (Supplementary Information, Fig. S2c)¹³. TAp63 induced senescence in NIH 3T3 cells (Supplementary Information, Fig. S2d), indicating that p16 and p19 are dispensable for this process. These findings indicate that TAp63-induced cellular senescence is p21- and Rb-dependent, consistent with endogenous p21 and Rb being induced by TAp63.

To determine whether p63 is required for oncogene-induced senescence (OIS), a tumour-suppressive program that has been reported to require an intact p53 pathway¹⁴, we generated MEFs from p63-deficient mice¹⁵ and assessed their ability to undergo OIS. Whereas H-Ras^{V12} induced senescence in wild-type MEFs, OIS was dramatically compromised in p63-

deficient MEFs, and these cells had enhanced proliferation (Fig. 2a, b). Ras-mediated p21 expression was compromised in p63-deficient MEFs (Fig. 2c), a finding consistent with our observation that TAp63 induces p21 during senescence. Even though $p63^{-/-}$ cells failed to senesce in response to Ras, $p63^{-/-}$ Ras-expressing MEFs were distinct from $p53^{-/-}$ Ras-expressing MEFs in that they did not form colonies in soft agar or tumours *in vivo* (Supplementary Information, Fig. S3; X.G. and A.A.M., personal observation). These findings indicate that although p63 deficiency compromises Ras-induced senescence, it is distinct from p53 deficiency. This is probably due to the fact that other barriers to oncogenesis can compensate in this context.

We previously conducted a spontaneous tumour study in p63-compromised mice¹⁶. Analysis of tumorigenesis in the p53-deficient cohort indicated that heterozygosity of $p63$ significantly enhanced sarcoma development (Fig. 2d). Importantly, p63 was not detectable in sarcomas that developed in $p63^{+/-}$; $p53^{-/-}$ mice (Fig. 2e, upper panels). We also assessed whether human sarcomas were similarly devoid of p63 expression. Whereas p63 expression was robust in non-neoplastic epithelial tissue in these sections, it was not detectable in tumours (Fig. 2e, lower panels). This absence of p63 expression in both mouse and human sarcomas is consistent with results from a previous study where 375 out of 385 human sarcomas analysed were p63-negative¹⁷, supporting the idea that p63 loss facilitates sarcomagenesis.

As OIS was compromised in the absence of p63 and TAp63 isoforms mediate senescence, we investigated whether TAp63 isoforms were essential for OIS by examining Ras-mediated senescence in cells specifically lacking TAp63. Using chromosomal engineering¹⁸, we generated a TAp63-conditional mouse model in which TAp63-specific exons were flanked by *loxP* sites (Supplementary Information, Fig. S4). In this model, TAp63 isoforms (but not $\Delta Np63$ isoforms) were ablated by Cre-mediated recombination in both cultured cells and *in vivo* (Supplementary Information, Fig. S4d–f). Whereas MEFs prepared from both wild-type and $TAp63^{F/F}$ siblings were susceptible to OIS, and tamoxifen-induced Cre expression did not prevent OIS in wild-type MEFs, Cre activation in $TAp63^{F/F}$ MEFs compromised OIS (Fig. 3a–c). When cultured for an extended period, Ras-expressing TAp63-deficient MEFs grew to high confluency, whereas Ras-expressing wild-type MEFs remained senescent (Fig. 3a, lower panels). We converted the $TAp63$ floxed conditional allele to a $TAp63$ null allele by crossing $TAp63^{F/+}$ mice with *CMV-Cre* mice and generated MEFs from $TAp63^{-/-}$ embryos (which did not have an overt phenotype) and sibling controls, allowing us to demonstrate that $TAp63^{-/-}$ MEFs had enhanced proliferation and compromised OIS (Fig. 3d, e). Furthermore, endogenous TAp63 was induced by Ras at the transcript level, and TAp63 γ was the predominant p63 protein detectable (Fig. 3f). To directly assess the tumour-suppressive activity of TAp63 *in vivo*, we investigated whether TAp63 deficiency exacerbated tumorigenesis of p53-compromised, Ras-driven cells. Indeed, $TAp63^{-/-}$ MEFs in which p53 was knocked down in the context of oncogenic Ras had increased tumour size and decreased latency relative to control cells (Fig. 3g). These findings indicate that TAp63 inhibits proliferation, facilitates OIS and prevents tumorigenesis *in vivo*.

The finding that TAp63 inhibits tumorigenesis in Ras-expressing cells in which p53 has been knocked down suggests that p53 is dispensable for the tumour-suppressive function of TAp63. To demonstrate this more rigorously, we showed that TAp63 α , TAp63 β and TAp63 γ inhibit proliferation of $p53^{-/-}$ MEFs (Fig. 4a). As we found previously, TAp63 α was less efficient at inhibiting proliferation than the other TAp63 isoforms, which was most likely due to this isoform having a carboxy-terminal inhibitory domain¹⁹. The G1/S population was increased, whereas the S-phase population was decreased, in TAp63 isoform-expressing $p53^{-/-}$ MEFs, indicating that TAp63 isoforms promote cell-cycle arrest at the G1/S transition (Fig. 4b; Supplementary Information, Fig. S5a). As in wild-type

MEFs, TAp63 isoforms induced senescence in $p53^{-/-}$ MEFs (Fig. 4c; Supplementary Information, Fig. S5b). These observations demonstrate that TAp63 induces senescence independently of p53.

Aberrant mitogenic signals cooperate with tumour-suppressor loss to cause cellular transformation and tumour formation⁹; however, senescence can override this process. To evaluate the ability of TAp63 to induce senescence in the context of both p53 loss and oncogenic Ras expression, we co-expressed H-Ras^{V12} and TAp63 isoforms in $p53^{-/-}$ MEFs. Whereas H-Ras^{V12} transforms $p53^{-/-}$ MEFs, causing these cells to form rapidly growing tumours when injected subcutaneously in nude mice^{14,20}, we found that co-expression of TAp63 isoforms and Ras^{V12} in $p53^{-/-}$ MEFs led to senescence (Fig. 4d; Supplementary Information, S5). These findings are in agreement with our observations that TAp63 is essential for regulating proliferation, senescence and tumorigenesis *in vivo*.

To examine whether TAp63 isoforms prevent tumour development *in vivo*, we injected $p53^{-/-}$ cells expressing Ras^{V12} plus GFP (control) or Ras^{V12} plus TAp63 isoforms subcutaneously into nude mice and monitored for tumorigenesis. The constructs containing cDNAs encoding TAp63 proteins also expressed GFP, providing a marker for TAp63 expression that also facilitated *in vivo* imaging. Whereas $p53^{-/-}$ cells expressing Ras^{V12} plus GFP formed rapidly growing tumours, the formation of tumours was dramatically inhibited in $p53^{-/-}$ cells expressing Ras^{V12} in combination with TAp63 α , TAp63 β or TAp63 γ ; again, TAp63 β and TAp63 γ were the most effective (Fig. 4e). As we did not drug select for infected cells, TAp63-non-expressing cells were probably present in the injected pool. Indeed, the small tumours that eventually developed in mice injected with cells transduced with Ras^{V12} plus TAp63 α (5 out of 6) or TAp63 β (6 out of 6) were GFP-negative, indicating that they were derived from Ras^{V12}-expressing $p53^{-/-}$ cells in which TAp63 was not expressed. These observations indicate that exogenous expression of TAp63 isoforms prevents oncogene-mediated tumour formation of p53-deficient cells.

Given our findings that TAp63 induces senescence and blocks tumour initiation, we investigated whether TAp63 γ was potent enough to induce senescence in cells that were already transformed and tumorigenic. We constructed a tetracycline-inducible system where TAp63 γ was expressed under the control of the TRE_{tight} promoter, and coupled it to an IRES-GFP cassette, which allows the monitoring of TAp63 γ expression in cultured cells as well as *in vivo* (Supplementary Information, Fig. S6a). $p53^{-/-}$ MEFs were co-transduced with a bicistronic retrovirus expressing N-Ras^{G12D} along with the tetracycline reverse transactivator protein (rtTA), as well as a retrovirus expressing either TRE-GFP or TRE-TAp63 γ /GFP. In the absence of the inducer, doxycycline (Dox), TAp63 γ /GFP expression was not detectable and $p53^{-/-}$ cells harbouring either TRE-GFP control or TRE-TAp63 γ /GFP showed a spindle-shaped morphology characteristic of transformed cells. However, in the presence of Dox, TAp63 γ , GFP and p21 were induced (Supplementary Information, Fig. S6b, c). In response to Dox, cells infected with TRE-GFP continued to proliferate, whereas cells infected with TRE-TAp63 γ /GFP became senescent in a dose-dependent manner (Supplementary Information, Fig. S6b, d–f). Regulated expression of TAp63 γ also induced senescence in human BJ (Supplementary Information, Fig. S7a), IMR90 and LFS041 cells (X.G. and A.A.M., personal observation). These findings indicate that TAp63 γ induces cellular senescence and inhibits proliferation of transformed cells.

To test whether TAp63 could cease the progression of tumours *in vivo*, we injected $p53^{-/-}$ cells expressing rtTA/N-Ras plus TRE-TAp63 γ /GFP into nude mice. Whereas these cells formed large tumours in untreated mice, tumours did not progress in Dox-treated mice (Fig. 5a). Dox administration caused robust GFP expression 24 h after treatment, with the GFP intensity declining rapidly during the following days (Fig. 5a, middle panels). Whereas

tumours in Dox-treated mice stopped growing and did not progress during the 4 days following Dox treatment, tumours eventually formed, which was most likely due to the outgrowth of cells with low or absent TAp63 expression. Indeed, as we described above, these escapers were GFP-negative (Fig. 5a, middle panels). Thus, regulated expression of TAp63 γ effectively halts the progression of established tumours *in vivo*.

To determine whether this abrupt shutdown of tumour progression was due to cellular senescence, we performed SA- β -Gal assays on tumour sections. We found that senescent cells were much more prevalent in tumours from Dox-treated mice than in tumours from untreated mice (Fig. 5b). Immunofluorescent analysis revealed that at day 2 both TAp63 γ and its target, p21, were significantly induced in tumours from Dox-treated mice (Fig. 5c). This enhanced p21 expression observed at the protein level is consistent with our finding that *p21* transcript is induced and that p63 is bound to the *p21* promoter in TAp63 γ -expressing *p53*^{-/-} MEFs (Supplementary Information, Fig. S7b). By day 4, TAp63 γ -expressing cells were virtually undetectable in the tumour (Fig. 5c, d), suggesting that TAp63 γ -expressing tumour cells are rapidly lost. This clearance of cells that had become senescent in response to TAp63 γ induction could be through the innate immune response¹⁰. These findings indicate that regulated expression of TAp63 γ induces p21 and cellular senescence, which halts tumour progression *in vivo*.

Our observations demonstrate that TAp63 isoforms are potent inducers of senescence that prevent cancer. The findings that p53 is dispensable for TAp63-mediated senescence and that TAp63 deficiency increases the tumorigenicity of p53-deficient Ras-expressing cells highlight the potent tumour-suppressive function of TAp63. Unlike p53, which induces senescence in response to oncogenic stimuli²¹, TAp63 can induce senescence on its own. TAp63 isoforms induce senescence in wild-type cells, as well as in cells that have lost nodal tumour-suppressive pathways, such as p16, p19 and p53. Notably, TAp63 triggers cellular senescence in *p53*^{-/-} cells both before and after they have been transformed by oncogenic Ras, halting tumour progression. Thus, expression of TAp63 is tumour protective at both the initiation and progression stages.

We reported previously that p63 deficiency causes cellular senescence in proliferating keratinocytes of the skin, leading to accelerated ageing *in vivo*¹². Although the role of different p63 isoforms was not addressed, we found recently that Δ Np63 α — the predominant isoform expressed in keratinocytes^{1,22}, which has a pro-proliferative function^{3,4,23} — regulates senescence in this setting (W.M.K. and A.A.M., unpublished data). Here, we identify TAp63 γ as the isoform induced in MEFs in response to oncogenic Ras and define TAp63 isoforms as essential mediators of senescence. In yet another setting — skin-derived progenitor cells of the hair follicle, which are distinct from both keratinocytes and MEFs — specific loss of TAp63 isoforms has been reported to induce hyperproliferation that culminates in senescence²⁴. Whereas loss of TAp63 in both of these settings enhances proliferation, it remains to be determined whether TAp63 is an essential mediator for OIS in skin-derived progenitor cells, as we found in MEFs. Thus, the finding that TAp63 induces senescence in fibroblasts complements and augments our previous discovery in keratinocytes, adding to the growing list of tissue-specific settings in which senescence occurs, and indicating isoform-specific roles of p63 proteins in senescence regulation.

Since senescence is a barrier against tumour progression *in vivo*, activation of this program in tumour cells^{10,11}, especially in those that are resistant to chemotherapy-induced cell death due to genetic lesions such as p53 loss, provides a rationale for anti-cancer therapy. Our study suggests that robust induction of TAp63 is one such therapeutic approach. This strategy is especially promising in light of the p53- and p16-independence of senescence

induction by TAp63 and given the fact that most human cancers have defective p53 (ref. ²⁵) or frequent loss of *INK4a* (ref. ²⁶), but retain the *p63* locus^{27–29}. Additional strategies that stabilize TAp63 (refs ^{8, 30}) or to inhibit pathways that negatively regulate TAp63 activity, such as the common negative regulators of p53 family members, iASPP (refs ^{31–33}), could provide effective approaches for cancer therapy.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Yang A, et al. p63, a p53 homolog at 3q27–29, encodes multiple products with trans-activating, death-inducing, and dominant-negative activities. *Mol Cell* 1998;2:305–316. [PubMed: 9774969]
2. Wu G, et al. Δ Np63 α and TAp63 α regulate transcription of genes with distinct biological functions in cancer and development. *Cancer Res* 2003;63:2351–2357. [PubMed: 12750249]
3. Hibi K, et al. AIS is an oncogene amplified in squamous cell carcinoma. *Proc Natl Acad Sci USA* 2000;97:5462–5467. [PubMed: 10805802]
4. Patturajan M, et al. Δ Np63 induces β -catenin nuclear accumulation and signaling. *Cancer Cell* 2002;1:369–379. [PubMed: 12086851]
5. Rocco JW, Leong CO, Kuperwasser N, DeYoung MP, Ellisen LW. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* 2006;9:45–56. [PubMed: 16413471]
6. Gressner O, et al. TAp63 α induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J* 2005;24:2458–2471. [PubMed: 15944736]
7. Suh EK, et al. p63 protects the female germ line during meiotic arrest. *Nature* 2006;444:624–628. [PubMed: 17122775]
8. Gallegos JR, et al. SCF TrCP1 activates and ubiquitylates TAp63 γ . *J Biol Chem* 2008;283:66–75. [PubMed: 17965458]
9. Narita M, Lowe SW. Senescence comes of age. *Nature Med* 2005;11:920–922. [PubMed: 16145569]
10. Xue W, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 2007;445:656–660. [PubMed: 17251933]
11. Ventura A, et al. Restoration of p53 function leads to tumour regression in vivo. *Nature* 2007;445:661–665. [PubMed: 17251932]
12. Keyes WM, et al. p63 deficiency activates a program of cellular senescence and leads to accelerated aging. *Genes Dev* 2005;19:1986–1999. [PubMed: 16107615]
13. Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;83:993–1000. [PubMed: 8521522]
14. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997;88:593–602. [PubMed: 9054499]

15. Mills AA, et al. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 1999;398:708–713. [PubMed: 10227293]
16. Keyes WM, et al. p63 heterozygous mutant mice are not prone to spontaneous or chemically induced tumors. *Proc Natl Acad Sci USA* 2006;103:8435–8440. [PubMed: 16714381]
17. Lee CH, et al. *Gene* expression profiling identifies p63 as a diagnostic marker for giant cell tumor of the bone. *Mod Pathol* 2008;21:531–539. [PubMed: 18192965]
18. Zheng B, Mills AA, Bradley A. A system for rapid generation of coat color-tagged knockouts and defined chromosomal rearrangements in mice. *Nucleic Acids Res* 1999;27:2354–2360. [PubMed: 10325425]
19. Serber Z, et al. A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol* 2002;22:8601–8611. [PubMed: 12446779]
20. Tanaka N, et al. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 1994;77:829–839. [PubMed: 8004672]
21. Ferbeyre G, et al. Oncogenic ras and p53 cooperate to induce cellular senescence. *Mol Cell Biol* 2002;22:3497–3508. [PubMed: 11971980]
22. Liefer KM, et al. Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* 2000;60:4016–4020. [PubMed: 10945600]
23. Westfall MD, Mays DJ, Sniezek JC, Pietenpol JA. The Δ Np63 α phospho-protein binds the p21 and 14-3-3 σ 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]
24. Su X, et al. TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell* 2009;5:64–75. [PubMed: 19570515]
25. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* 1991;253:49–53. [PubMed: 1905840]
26. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell* 2002;2:103–112. [PubMed: 12204530]
27. Osada M, et al. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nature Med* 1998;4:839–843. [PubMed: 9662378]
28. Sunahara M, et al. Mutational analysis of p51A/TAp63 γ , a p53 homolog, in non-small cell lung cancer and breast cancer. *Oncogene* 1999;18:3761–3765. [PubMed: 10391684]
29. Moll UM, Slade N. p63 and p73: roles in development and tumor formation. *Mol Cancer Res* 2004;2:371–386. [PubMed: 15280445]
30. MacPartlin M, Zeng SX, Lu H. Phosphorylation and stabilization of TAp63 γ by IkappaB kinase- β . *J Biol Chem* 2008;283:15754–15761. [PubMed: 18411264]
31. Bergamaschi D, et al. iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nature Genet* 2003;33:162–167. [PubMed: 12524540]
32. Robinson RA, Lu X, Jones EY, Siebold C. Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73. *Structure* 2008;16:259–268. [PubMed: 18275817]
33. Bell HS, et al. A p53-derived apoptotic peptide derepresses p73 to cause tumor regression *in vivo*. *J Clin Invest* 2007;117:1008–1018. [PubMed: 17347683]

Figure 1.

TAp63 isoforms mediate senescence in mouse and human cells. **(a, b)** TAp63 isoforms induce cellular senescence and inhibit proliferation. **(a)** Wild-type MEFs infected with retroviral vectors expressing cDNA encoding TAp63 α , TAp63 β , TAp63 γ were assayed for SA- β -Gal activity 4 days after infection (upper panels), or pulsed with BrdU 6 days after infection (lower panels). The percentage of SA- β -Gal or BrdU positive cells is shown (right). Values represent mean $\pm$ s.d. ($n = 3$ fields). **(b)** Human BJ cells transduced with TAp63 α , TAp63 β or TAp63 γ were assayed for SA- β -Gal activity and BrdU incorporation as in **a**. Values represent mean $\pm$ s.d. ($n = 3$ fields). **(c)** TAp63-induced senescence requires p21 and Rb. Wild-type MEFs were co-infected with MSCV vectors expressing a TAp63 isoform with a vector control or with an shRNA specific for *p21*, *Rb* or *p16*. Infected cells were subjected to BrdU incorporation and flow cytometric analysis. The x-axis shows DNA content and the y-axis shows BrdU incorporation. The percentage of BrdU-positive cells is indicated. **(d–f)** The knockdown efficiencies of the shRNA constructs are shown. Values represent mean $\pm$ s.d. from a triplicate experiment. Scale bars in **a** and **b**, 100 μ m. Vector, empty control vector.

Figure 2. p63 deficiency compromises Ras-induced senescence and enhances sarcoma development *in vivo*. **(a)** p63 is required for Ras-induced senescence. Wild-type (left) or *p63*^{-/-} (middle) MEFs expressing oncogenic H-Ras^{V12} were assayed for SA-β-Gal activity, which was quantified (right). Values represent mean ± s.d. (*n* = 6 fields). **(b)** p63 deficiency confers a growth advantage in Ras-expressing MEFs. Colony formation assays were performed by plating cells at the indicated numbers and staining with crystal violet. **(c)** Immunoblot analysis for p53 and p21 in wild-type, *p53*^{-/-} and *p63*^{-/-} cells expressing Ras, relative to wild-type control cells. The band depicted by an asterisk is detectable in Ras-expressing MEFs. **(d)** *p63*^{+/-}; *p53*^{-/-} mice have increased sarcoma development. Tumour data is expressed as the percentage of tumours that are sarcomas (upper panels) as well as the percentage of the cohort that developed sarcomas (*n* = 78 and 28 for the *p53*^{-/-} and *p63*^{+/-}; *p53*^{-/-} cohorts, respectively). **(e)** p63 is not expressed in mouse or human sarcomas. Histology (left) and immunohistochemical analysis for p63 expression (right) are presented, showing nuclear immunostaining for p63 in non-neoplastic squamous epithelium overlying the subcutaneous sarcomas. H&E, hematoxylin and eosin; WT, wild-type; T, tumour; N, non-neoplastic. Scale bars in **a** and **e**, 100 μm. A full scan of the blot in **c** is shown in Supplementary Information, Fig. S8.

Figure 3.

TAp63 deficiency compromises Ras-induced senescence, enhances proliferation and increases tumorigenesis. (a–c) TAp63 is essential for Ras-induced senescence. (a) MEFs from wild-type (left) or TAp63 conditional (*TAp63^{F/F}*, right) embryos were co-infected with CreER and H-Ras^{V12} and assayed for senescence (a, b) and proliferation (c) in the absence or presence of the inducer 4-OHT. Values in b and c represent mean ± s.d. (*n* = 3 fields). (d) *TAp63^{-/-}* cells have enhanced proliferation. Values represent mean ± s.d. (*n* = 3 wells). (e) *TAp63^{-/-}* cells bypass Ras-induced senescence. MEFs expressing Ras were subjected to a foci formation assay. (f) Endogenous TAp63 is induced by oncogenic Ras at both the transcript and the protein level. Wild-type MEFs expressing either vector control or H-Ras^{V12} were subjected to qRT-PCR analysis for TAp63 expression (left panel). Values represent mean ± s.d. (*n* = 3 independent experiments). Wild-type, *p53^{-/-}* or *p63^{-/-}* MEFs expressing Ras, or MEFs expressing exogenous TAp63α, TAp63β or TAp63γ were subjected to western blotting analysis (right panel). (g) TAp63 deficiency enhances tumorigenesis. MEFs from *TAp63^{-/-}* or wild-type sibling control mice that had been simultaneously infected with H-Ras^{V12} and shRNA against *p53* were subcutaneously injected in nude mice and tumour growth was monitored. *P* value determined using two-way ANOVA test is shown (*n* = 10 sites injected). Data represent mean ± s.e.m. Scale bar in a, 100 μm. A full scan of the blot in f is shown in Supplementary Information, Fig. S8. WT, wild-type; Vector, empty control vector.

Figure 4.

Tap63 blocks Ras-driven transformation and tumour formation of $p53^{-/-}$ cells *in vivo*. (**a**–**c**) Tap63 isoforms induce senescence independently of p53. (**a**) Tap63 isoforms inhibit the proliferation of $p53^{-/-}$ MEFs. 5×10^4 cells were plated in duplicate in 6-well plates and infected with retroviral vectors expressing Tap63 isoforms, and cell numbers were counted at the indicated time points. Values represent mean $\pm$ s.d. ($n = 2$ wells). (**b**) Tap63 induces cell-cycle arrest. DNA content analysis was performed in $p53^{-/-}$ MEFs expressing Tap63 isoforms, and the percentage of cells in each phase of the cell cycle was plotted. (**c**) In $p53^{-/-}$ MEFs, Tap63 increases SA- β -Gal activity. $p53^{-/-}$ MEFs infected with MSCV vectors expressing Tap63 isoforms were assayed and SA- β -Gal activity was quantified. Values represent mean $\pm$ s.d. ($n = 3$ fields). (**d**) Tap63 isoforms induce cellular senescence and block Ras-driven transformation. $p53^{-/-}$ MEFs co-infected with retroviral vectors expressing H-Ras^{V12} and either empty vector or Tap63-IRES-GFP were viewed using phase contrast (upper panels) and fluorescence (middle panels) before being assayed for SA- β -Gal activity (bottom panels). (**e**) Tap63 isoforms inhibit tumour formation *in vivo*. $p53^{-/-}$ MEFs expressing Ras plus empty MSCV-IRES-GFP (MIG) vector or Ras plus MIG expressing Tap63 isoforms were injected subcutaneously in nude mice and tumour growth was monitored. Representative mice were imaged at day 23. Tumour volume (bottom panel) was calculated as length $\times$ width² $\times$ $\pi/6$. Values represent mean $\pm$ s.d. ($n = 6$ sites). Scale bar in **d**, 100 μ m. Vector, empty control vector.

Figure 5.

Regulated expression of TAp63 γ induces senescence and halts tumour progression *in vivo*. **(a)** TAp63 expression halts tumour progression. *p53*^{-/-} MEFs expressing N-Ras^{G12D}/rtTA and TRE-TAp63 γ /GFP were injected subcutaneously into nude mice, and tumour growth was monitored in untreated mice (upper panels) or mice treated with Dox (middle panels) by measuring and *in vivo* imaging. Representative tumour growth in untreated (red) and Dox-treated (black) mice is shown (lower panel). **(b)** Hematoxylin and eosin (H&E) staining (upper panels) and SA- β -Gal assay (lower panels) of tumour sections from untreated mice or mice treated with Dox for 2 days. **(c)** Immunofluorescence for p63 and p21 expression in tumours. **(d)** Immunoblotting analysis for p63 and β -actin. Scale bars in **b** and **c**, 50 μ m. A full scan of the blot in **d** is shown in Supplementary Information, Fig. S8.