TAp73 enhances the pentose phosphate pathway and supports cell proliferation

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TAp73 is a structural homologue of the pre-eminent tumour suppressor p53. However, unlike p53, TAp73 is rarely mutated, and instead is frequently overexpressed in human tumours. It remains unclear whether TAp73 affords an advantage to tumour cells and if so, what the underlying mechanism is. Here we show that TAp73 supports the proliferation of human and mouse tumour cells. TAp73 activates the expression of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP). By stimulating G6PD, TAp73 increases PPP flux and directs glucose to the production of NADPH and ribose, for the synthesis of macromolecules and detoxification of reactive oxygen species (ROS). The growth defect of TAp73-deficient cells can be rescued by either enforced G6PD expression or the presence of nucleosides plus an ROS scavenger. These findings establish a critical role for TAp73 in regulating metabolism, and connect TAp73 and the PPP to oncogenic cell growth.

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with the reducing equivalent NADPH for reductive biosynthesis (for example, the synthesis of lipids and deoxyriboses) and antioxidant defence (Supplementary Fig. S2a). The pacemaker of the PPP is G6PD, which catalyses the first committing step of this pathway. Here we investigate TAp73 in cell proliferation and identify a critical role for TAp73 in promoting biosynthesis and antioxidant defence through the induction of G6PD expression.

RESULTS
TAp73 supports tumour growth
To investigate the role of TAp73 in tumour cell proliferation, we used E1A/RasV12-transformed mouse embryonic fibroblast (MEF) cells with wild-type (+/+) or homozygous disruption of (−/−) TAp73 (ref. 14). Interestingly, TAp73−/− MEF cells proliferated significantly slower than TAp73+/+ MEFs (Fig. 1a and Supplementary Fig. S1b). To investigate the role of TAp73 in tumour formation, we injected TAp73−/− and TAp73+/+ MEF cells into immuno-compromised mice. TAp73−/− MEF cells gave rise to much smaller tumours than TAp73+/+ MEF cells did (Fig. 1b and Supplementary Fig. S1c). To extend these analyses to human tumour cells, we stably knocked down TAp73 in human osteosarcoma U2OS cells using small hairpin RNA (shRNA). This significantly reduced the ability of U2OS cells to proliferate in culture and to establish tumours in a xenograft mouse model (Fig. 1c,d and Supplementary Fig. S1d). These observations suggest that TAp73 is required for optimal cell proliferation and tumour formation.

For comparison, we also investigated the role of ΔNp73 in tumour cell proliferation using E1A/RasV12-transformed ΔNp73+/− MEFs and the matched ΔNp73+/+ MEFs (ref. 16). We observed that loss of ΔNp73 resulted in a decrease in cell proliferation (Supplementary Fig. S1e,f), consistent with a role for ΔNp73 in tumour growth16.

TAp73 enhances the PPP
We investigated the mechanism by which TAp73 promotes proliferation. Our recent studies have shown that p53 inhibits the PPP (ref. 24). We assessed whether TAp73 has any regulatory effects on the glucose flux through this pathway. TAp73+/+ and TAp73−/− MEF cells were cultured in medium containing [2-13C]glucose, and the PPP flux was measured on the basis of the rate of glucose consumption and 13C labelling patterns in lactate determined by nuclear magnetic resonance (NMR) spectroscopy24,25. Of note, TAp73 deficiency reduced the PPP flux by ~50%, whereas ΔNp73 deficiency had no effect (Fig. 2a,b), suggesting that TAp73, but not ΔNp73, enhances the PPP flux.

The rate-limiting enzyme of the PPP is G6PD, which catalyses the conversion of glucose-6-phosphate to 6-phosphate-gluconolactone (Supplementary Fig. S2a)26. We assayed G6PD activity in TAp73+/+ and TAp73−/− MEF cells. Importantly, TAp73−/− MEFs showed a strong reduction (~45–55%) in overall G6PD activity compared with TAp73+/+ MEFs. However, no significant difference in G6PD activity was observed between ΔNp73−/− and ΔNp73+/+ MEFs (Fig. 2c,d). Correlating with these results, deficiency of TAp73, but not of ΔNp73, led to strong reduction in G6PD messenger RNA levels (Fig. 2c,d).
This led to a noticeable decline in both the activity and expression (p21 target gene) of the specificity of the siRNA. Likewise, on an siRNA-resistant form of TAp73 in p73-siRNA-treated cells largely G6PD the corresponding wild-type MEFs. Bottom: G6PD and β-actin mRNA levels, with the relative ratios of G6PD to β-actin indicated. Data are means ± s.d. (n = 3 independent experiments). Western blots represent two independent experiments. (e–i) G6PD activity in U2OS cells (e), isogenic p53+/− (f), p53+/− (g) or p21+/− (h) HCT116 cells, or IMR90 cells (i) that were treated with either control (−) or p73 siRNA. The expression of G6PD, TAp73 and actin was detected using western blotting (e–h) or RT–PCR (i). Data are means ± s.d. (n = 3 independent experiments). Western blots represent three independent experiments. Uncropped images of blots are shown in Supplementary Fig. S8.

**G6PD is a target gene for TAp73**

To examine the p73 isoform-specific effect on G6PD expression, we knocked down either total p73 or ΔNp73 in HeLa cells, which express relatively high levels of ΔNp73, compared with other cell lines tested (Supplementary Fig. S2c). Silencing total p73, but not ΔNp73, led to a noticeable reduction in G6PD levels (Supplementary Fig. S2d). We also knocked down TAp73 using shRNA, which strongly decreased G6PD expression in U2OS, IMR90 and human lung cancer H1299 cells (Fig. 3a,b and Supplementary Fig. S2e,f). Conversely, enforced expression of a TAp73 isoform (TAp73ΔN), but not the corresponding ΔNp73 isoform (ΔNp73ΔN), augmented G6PD expression (Fig. 3c and Supplementary Fig. S2g). Unlike TAp73, neither overexpression nor knockdown of p53, or the other p53 family member

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**Figure 2** p73 regulates PPP flux, G6PD activity and expression. (a,b) TAp73+/+ and TAp73−/− MEFs (a), or ΔNp73+/+ and ΔNp73−/− MEFs (b), were cultured in medium containing [2-13C]glucose. Oxidative PPP flux was measured on the basis of the rate of glucose consumption and the ratio of 13C incorporated into carbon 3 (indicating PPP) and carbon 2 (indicating glycolysis) of lactate determined by NMR spectroscopy. Data are means ± s.d. (n = 3 independent experiments). **(c,d)** Top: G6PD activity in two independent clones of TAp73+/− MEFs (c), ΔNp73+/− MEFs (d), and the corresponding wild-type MEFs. Bottom: G6PD and β-actin mRNA levels, with the relative ratios of G6PD to β-actin indicated. Data are means ± s.d. (n = 3 independent experiments). Western blots represent two independent experiments. **(e–i)** G6PD activity in U2OS cells (e), isogenic p53+/− (f), p53+/− (g) or p21+/− (h) HCT116 cells, or IMR90 cells (i) that were treated with either control (−) or p73 siRNA. The expression of G6PD, TAp73 and actin was detected using western blotting (e–h) or RT–PCR (i). Data are means ± s.d. (n = 3 independent experiments). Western blots represent three independent experiments. Uncropped images of blots are shown in Supplementary Fig. S8.

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To ascertain the stimulatory effect of TAp73 on G6PD, we used small interfering RNA (siRNA) to knock down p73 in U2OS cells. This led to a noticeable decline in both the activity and expression levels of G6PD (Fig. 2e and Supplementary Fig. S2b). Introducing an siRNA-resistant form of TAp73 in p73-siRNA-treated cells largely restored G6PD expression (Supplementary Fig. S2b), underscoring the specificity of the siRNA. Likewise, on p73 knockdown, G6PD activity and expression were reduced in three isogenic human colorectal HCT116 cell lines, which were wild-type for p53 and its target gene p21 (p53+/+), deficient in p53 (p53−/−), or deficient in p21 (p21−/−) (Fig. 2f–h). A strong effect of p73 knockdown on G6PD was also observed in the normal human diploid fibroblast IMR90 cells (Fig. 2i).

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**Figure 3** p73 regulates G6PD transcription. (a) mRNA levels in U2OS cells that were stably infected with lentiviruses expressing a control (Ctrl) shRNA or shRNA against the indicated p53 family gene. (b) IMR90 cells were infected with lentiviruses expressing a control shRNA or a TAp73-specific shRNA for the indicated durations. Protein and mRNA expression was detected using RT–PCR and western blotting (WB), respectively. Data represent three independent experiments. (c) H1299 cells were transfected with increasing amounts of control plasmid or plasmid expressing the indicated p73 family gene. Western blots represent two independent experiments. (d) MEF cells were treated with the genotoxic agent etoposide (ETP), TAp73 was stabilized and G6PD expression was elevated (Fig. 3d). In contrast, p63, affected G6PD levels (Fig. 3a,c and Supplementary Fig. S2f,g). Together, these results indicate that TAp73 specifically enhances the expression of G6PD.
ETP reduced G6PD expression in TAp73<sup>−−</sup> MEFs (Fig. 3d). When IMR90 cells were treated with ETP, TAp73 was also stabilized, and both the G6PD mRNA and protein were significantly increased. However, in cells devoid of p73, G6PD failed to accumulate on DNA damage (Fig. 3e). Together, these results suggest that TAp73 stimulates the expression of G6PD both at basal levels in unstressed cells and when TAp73 is stabilized by DNA damage. In addition, a TAp73-independent mechanism might reduce G6PD expression on DNA damage, but TAp73 can override this effect.

To determine whether TAp73 is a transcriptional activator for the G6PD gene, we analysed the human G6PD gene sequence for potential p53 family protein response elements, which share the consensus sequence of 5′-RRCWWGYYY-(0–13 base pair (bp) spacer)-RRCWWG YYY-3′ (where R is a purine, Y a pyrimidine, and W an A or T; ref. 31). One potential response element was identified in the second intron of G6PD (Fig. 3f). To investigate the binding of p73 to this response element, we performed chromatin immunoprecipitation (ChIP) assays. In the p53-wild-type A549 cells, endogenous p73 bound to the response element region of the G6PD gene; the binding was strengthened when cells were treated with the DNA damaging agent doxorubicin (DOX), which stabilized the p53 family proteins (Fig. 3g), even though p53 bound strongly to its target gene Puma as expected (Fig. 3i). Similarly, when H1299 cells were transfected individually with green fluorescence protein (GFP) fusions of p53 family members, GFP–TAp73α, but not GFP–p53 or GFP–TAp63α, associated with the G6PD genomic DNA (Fig. 3h).

To evaluate whether the response element within G6PD confers TAp73-dependent transcriptional activation, we cloned DNA fragments containing the wild-type response element or a mutant response element into the promoter region of a firefly luciferase reporter plasmid. Both TAp73α and TAp73β were able to induce luciferase expression from the wild-type reporter plasmid but not from the mutant reporter plasmid (Fig. 3j,k). Consistent with previous observations<sup>32,33</sup>, TAp73β had higher transcriptional activity when compared with TAp73α (Fig. 3j). In contrast, p53, TAp63α and ΔNp73 failed to activate the wild-type response-element-responsive luciferase (Fig. 3j and Supplementary Fig. S2i). These results suggest that TAp73, not p53 or TAp63, binds to the consensus p53 family response element within the second intron of G6PD and activates G6PD expression.

Tumour-derived p53 mutants may impair the function of TAp73 (ref. 34). We investigated two common p53 mutants, R175H and R273H. Overexpression of mutant p53 failed to suppress G6PD expression regardless of whether TAp73 was co-expressed (Supplementary Fig. S2j). Also, knocking down these mutant forms of p53 did not affect the expression of G6PD (Fig. 3l). Thus, mutant p53 proteins may not impede the ability of TAp73 to stimulate G6PD expression.

### p73 regulates NADPH homeostasis through G6PD

The PPP provides NADPH and ribose, both of which are important synthetic precursors for nucleic acids. We compared DNA synthesis in TAp73<sup>+/+</sup> and TAp73<sup>−−</sup> MEFs using 5-bromo-2′-deoxyuridine (BrdU) incorporation assays. DNA synthesis was sharply reduced in TAp73<sup>−−</sup> MEFs, when compared with TAp73<sup>+/+</sup> MEFs (Fig. 6a). Likewise, DNA synthesis slowed down when p73 was silenced in U2OS and various HCT116 cells (Fig. 6b,c and Supplementary Fig. S5a,b). In each cell type, treatment with DHEA or G6PD siRNA inhibited DNA synthesis in TAp73-depleted cells especially in control cells, lessening the difference between them (Fig. 6a–c and Supplementary Fig. S5a,b).

A role for p73 in antioxidative defence

G6PD plays an important role in antioxidative metabolism by producing NADPH to regenerate reduced glutathione<sup>36,37</sup>. Treatment of TAp73<sup>+/+</sup> MEFs with a G6PD-specific siRNA or the G6PD inhibitor dehydroepiandrosterone (DHEA) led to the accumulation of ROS (Fig. 5a and Supplementary Fig. S4a,b). Similarly, when G6PD was depleted in U2OS and IMR90 cells, ROS levels markedly rose (Fig. 5b and Supplementary Fig. S4c). Importantly, TAp73<sup>−−</sup> MEFs also exhibited high ROS levels, similar to G6PD siRNA or DHEA-treated TAp73<sup>+/+</sup> MEFs. In TAp73<sup>−−</sup> MEFs, treatment with DHEA or G6PD siRNA only slightly increased ROS (Fig. 5a and Supplementary Fig. S4a,b). Similarly, U2OS and IMR90 cells devoid of p73 contained abundant ROS, and knockdown of G6PD in these cells raised ROS only slightly higher (Fig. 5b and Supplementary Fig. S4c).

To ascertain that the sensitivity of TAp73-deficient cells to oxidative stresses is due to the reduction in G6PD expression, we investigated the effect of G6PD expression in these cells. Forced expression of G6PD by siRNA or DHEA reduced the survival of TAp73<sup>−−</sup> cells and especially TAp73<sup>−−</sup> cells, minimizing the difference between these two cell types (Fig. 5c and Supplementary Fig. S4d). Knockdown of p73 in U2OS also sensitized these cells to H₂O₂ treatment, and silencing G6PD diminished the difference between these cells and the control cells (Supplementary Fig. S4e).

### p73 regulates DNA biosynthesis and cell senescence

The PPP provides NADPH and ribose, both of which are important synthetic precursors for nucleic acids. We compared DNA synthesis in TAp73<sup>+/+</sup> and TAp73<sup>−−</sup> MEFs using 5-bromo-2′-deoxyuridine (BrdU) incorporation assays. DNA synthesis was sharply reduced in TAp73<sup>−−</sup> MEFs, when compared with TAp73<sup>+/+</sup> MEFs (Fig. 6a). Likewise, DNA synthesis slowed down when p73 was silenced in U2OS and various HCT116 cells (Fig. 6b,c and Supplementary Fig. S5a,b). In each cell type, treatment with DHEA or G6PD siRNA inhibited DNA synthesis in TAp73-depleted cells especially in control cells, lessening the difference between them (Fig. 6a–c and Supplementary Fig. S5a,b).

We also examined the effect of p73 on cell-cycle progression. Knockdown of p73 led to a reduction in the number of cells in...
We investigated the role of G6PD in this process using human A R T I C L E S

Figure 4 p73 regulates NADPH homeostasis. (a,b) NADPH levels (a) and NADP+/NADPH ratios (b) in two independent clones of TAp73+/- and TAp73-/- MEF cells are shown. Data are means ± s.d. (n = 3 independent experiments). (c,d) NADPH levels (c) and NADP+/NADPH ratios (d) in U2OS cells transfected with control (-) or p73 siRNA. Data are means ± s.d. (n = 3 independent experiments). (e,f) G6PD activity (e) and NADP+/NADPH ratios (f) in TAp73+/- and TAp73-/- MEF cells stably overexpressing G6PD or vector control are shown. Protein expression was analysed (e). Data are means ± s.d. (n = 3 independent experiments). Western blots represent three independent experiments. Uncropped images of blots are shown in Supplementary Fig. S8.

S phase and an increase in the number of cells in G1 and G2/M phases. This effect was independent of the status of p53 and the p53/p73-inducible cell-cycle regulator p21. Knockdown of G6PD diminished the difference in DNA synthesis between p73-depleted cells and control cells (Fig. 6d and Supplementary Fig. S5c,d). Conversely, forced expression of G6PD almost completely restored DNA synthesis in p73-depleted U2OS cells (Fig. 6e). These results suggest that TAp73 stimulates DNA synthesis through G6PD.

The loss TAp73 in mouse cells accelerates cellular senescence⁶⁹. We investigated the role of G6PD in this process using human IMR90 cells, a well-established model for senescence. When compared with cultures treated with control siRNA, IMR90 cultures treated with p73 siRNA showed a noticeable increase in the number of cells with senescence phenotypes, including flat morphology and the expression of the senescence-associated β-galactosidase (SA-β-gal; Fig. 6f and Supplementary Fig. S5e). Knockdown of G6PD increased the number of senescent cells strongly in control cultures, but relatively moderately in p73-depleted cultures, reducing the difference between these two groups (Supplementary Fig. S5e). More importantly, overexpression of G6PD effectively reversed the induction of senescence in p73-depleted cultures (Fig. 6f). These data suggest that the lack of TAp73 induces senescence in IMR90 cells at least partially due to the downregulation of G6PD.

p73 enhances cell proliferation through G6PD

Silencing p73 strongly impeded cell growth in various cell lines, including MEFs, U2OS and isogenic HCT116 cell lines (Figs 1a,c and 7a, and Supplementary Figs S1b,d and S6a–c). Growth delay due to p73 downregulation also occurred in p53-/- HCT116 cells (Supplementary Fig. S6b), indicating a requirement for p73 in cell growth regardless of the status of p53. Depletion of G6PD reduced the growth rate in control cells and especially in TAp73 knockdown cells (Fig. 7a and Supplementary Fig. S6a–c). Conversely, enforced expression of G6PD partially restored cell growth in p73 knockdown U2OS cells and TAp73-/- MEF cells but had a minimal effect on control cells (Fig. 7b,c and Supplementary Fig. S6d–h).
We examined whether the growth-stimulatory effect of TAp73 is related to both a decrease in ROS and an increase in intracellular nucleotides, two main outcomes of the PPP flux. We cultured TAp73+/+ and TAp73−/− MEF cells in medium containing the ROS scavenger N-acetylimidazole (NAC), four ribonucleosides (A, G, U and C) plus four deoxynucleosides (da, dG, dT and dc), or both. Nucleosides in culture medium can be taken up by cells and converted to the corresponding nucleotides, thus bypassing the requirement for de novo ribose synthesis. Importantly, in the presence of both NAC and nucleotides, but neither individually, TAp73+/− MEFs grew nearly as well as TAp73+/+ MEF cells (Fig. 7d). Moreover, combination treatment with NAC and nucleotides rescued the growth of TAp73+/− MEFs stably overexpressing G6PD or vector control, but to lesser extents when compared with TAp73+/− MEFs (Supplementary Fig. S6e–h). These results suggest that TAp73 promotes cell proliferation at least partially through upregulating G6PD, which leads to simultaneous reduction of ROS and enhancement of ribose synthesis.

Analysis of G6PD expression and metastasis status of a large number of primary breast tumors revealed that increasing G6PD expression was significantly correlated with the risk of metastasis (Supplementary Fig. S7a,b). To evaluate the role of G6PD in tumour formation, we injected immuno-compromised mice with U2OS cells or TAp73+/+ MEF cells expressing G6PD siRNA or control siRNA. G6PD-siRNA-expressing cells generated much smaller tumours when compared with control cells (Fig. 7e,g and Supplementary Fig. S7d). Conversely, reintroduction of G6PD partially restored tumour formation by TAp73−/− MEFs (Fig. 7f and Supplementary Fig. S7c). We also tested the effect of NAC on the growth of TAp73- and G6PD-deficient tumour cells. In agreement with cell proliferation results, NAC alone failed to restore the growth of tumours derived from either TAp73−/− MEFs or G6PD-depleted MEFs (Fig. 7g and Supplementary Fig. S7d).

**DISCUSSION**

The present study indicates that TAp73 promotes G6PD expression and PPP flux, enhancing biosynthesis and ROS detoxification. Metabolic reprogramming in tumour cells is altered to meet the demand for producing macromolecules and minimizing oxidative damage. Both require NADPH, and it has been proposed that NADPH production may be a rate-limiting step in cell proliferation. The production of NADPH seems to be tightly controlled by oncogenes and tumour suppressors. For instance, the oncogene K-Ras stimulates G6PD production in pancreatic cancer cells by enhancing the flux through malic enzyme 1, a cytoplasmic isoform of the NADPH-generating malic enzymes. In contrast, p53 inhibits the expression of all three malic enzymes that are localized either in the cytoplasm or in the mitochondria. The PPP is not only a major source for NADPH but also highly responsive to oxidative stress. G6PD is a common target for directly adjusting PPP flux. It is allosterically activated by its substrate NADP+ and inhibited by its product NADPH. This homeostatic regulation ensures that the PPP is upregulated when more NADPH is needed. The stimulation of G6PD expression by TAp73 may represent a major mechanism that modulates G6PD expression. The TAp73–G6PD pathway is engaged by DNA damage signals, probably increasing the supply of NADPH and ribose for DNA repair. We found that G6PD overexpression partially restores the growth of TAp73−/− MEF cells and p73-depleted U2OS cells.
This suggests that, at least in these cells, other TAp73 targets may contribute to its proliferative function. A recent study revealed a role for TAp73 in regulating cytochrome c oxidase subunit 4 (Cox4i1), a subunit of the complex IV of the mitochondrial electron transport chain. By maintaining levels of G6PD as well as Cox4i1, TAp73 likely plays a pre-eminent role in antioxidant response in both unstressed and stressed cells.

Nevertheless, ROS scavengers alone cannot rescue the proliferative defects of TAp73-deficient cells. Rather, both ROS scavengers and nucleosides are needed (Fig. 7). These observations suggest that TAp73 promotes nucleotide synthesis as well as antioxidant defence, and that the PPP may be a main proliferative target of TAp73. Previous studies showed that on overexpression, TAp73 could activate certain p53 target genes involved in apoptosis or cell-cycle arrest. Yet, the physiological function of TAp73 is probably determined by its endogenous levels of expression, its interplay with the ΔN isoforms, and its regulation by upstream factors. Despite high similarity among p53 family members in the DNA-binding domain, TAp73 alone is able to induce G6PD expression, although the mechanism underlying this selectivity remains to be determined (Fig. 3). Moreover, ΔNp73 and tumour-associated p53 mutants do not seem to inhibit this function of TAp73 (Fig. 3). These observations support the notion that G6PD is a high-affinity, physiologically relevant target of TAp73, and that tumour cells benefit from TAp73 upregulation even in the presence of elevated ΔNp73 or mutated p53 proteins.

The critical role of TAp73 in cell proliferation raises the question of why TAp73-deficient mice show increased tumour formation. An explanation may be that extensive genomic instability associated with congenital loss of TAp73 outweighs any proliferative defects in these mice by facilitating the accumulation of oncogenic mutations. It is noteworthy that the function of TAp73 in maintaining genomic stability seems to be cell type specific, as it operates in lung fibroblasts but...
not in thymic cells, and that TAp73+/− mice primarily develop lung adenocarcinomas14. The role of TAp73 in G6PD expression, on the other hand, is observed in different cell types that have been tested in independent experiments. Representative images of cells stained with crystal violet at day 4 (right). (e) U2OS cells expressing G6PD siRNA or control siRNA were individually injected subcutaneously into the dorsal flanks of the three nude mice. Tumour weights (mean ± s.d., n = 3 mice in each group) were measured 3 weeks after inoculation. (f) TAp73+/+ and TAp73+/– MEF cells (2 × 10^6) stably overexpressing G6PD or vector control were individually injected subcutaneously into the dorsal flanks of the four nude mice. Tumour weights (mean ± s.d., n = 4 mice in each group) were measured 3 weeks after inoculation. (g) TAp73+/+ and TAp73+/– MEF cells (1 × 10^7) expressing G6pd siRNA or control siRNA were individually injected subcutaneously into the dorsal flanks of the eight nude mice. Half were given water without NAC and the other half were given water with 40 mM NAC throughout the experiment. Tumour weights (mean ± s.d., n = 8 mice in each group) were measured 3 weeks after inoculation.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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ARTICLES

AUTHOR CONTRIBUTIONS
W.D., P.J., X.Y. and M.W. designed the experiments and interpreted results. W.D. and P.J. performed all the experiments except those mentioned below. A.M. and P.J. analysed the oxidative PPP flux. A.S. and M.D.B. helped with the FACS analysis and xenograft study, respectively. A.J.M. performed the breast cancer data analysis. T.W.M. supplied the MEF cells deficient for TAp73 and ΔNp73. W.D., P.J. and X.Y. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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**METHODS**

**Antibodies and reagents.** Antibodies against the following proteins/epitopes were used in this study with the company, catalogue number, and dilution or concentration indicated: G6P (Clontech Laboratories, Mountain View, 632381; 1:4,000), actin (Sigma-Aldrich; A2066; 1:4,000), Flag (Sigma, F3165; 1:4,000), p53 (DO-1) (Santa Cruz Biotechnology; sc-126 HRP; 1:1,000), p73 (Imagenex; IMG-259A; 1:1,000) (Bethyl Laboratories; A300-126A; 1:100) (Sigma, SABA430054; 1:1,000), mouse p73 (Santa Cruz, sc-7238; 1:500) (Cell Signaling Technology; 4300354; 1:1,000). G6PD (Sigma, HPA000834; 1:3,000), p21 (Cell Signaling, 2947; 1:1,000) and fluorescein isothiocyanate (FITC)-labelled anti-Brdu antibody (BD Bioscience Pharmingen; 556208; 10 μM). Anti-p63 antibody was provided by C. Chen.

The following reagents were purchased from Sigma: glucose-6-phosphate (G6P), 6-phosphogluconate (6PG), β-nicotinamide adenine dinucleotide 2′-phosphate (NADP+), β-nicotinamide adenine dinucleotide 2′-phosphate, reduced (NADPH), etoposide (ETO), doxorubicin (DOX), N-acetylcyesteine (NAC), propidium iodide (PI), dihydroxipiedrostonone (DHEA), crystal violet (CV), hydrogen peroxide (H2O2), 2′, 7′-dichlorofluorescein diacetate (DCF) and bromooueaxycyanid (Brdu).

**Cell culture and gene knockdown with shRNA and siRNA.** HCT116 cells and MEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies), and when indicated, in minimum essential medium Eagle with ribonucleosides and deoxyribonucleosides (Sigma, M8042). TAp73+/−, ∆Np73−/−, and the corresponding wild-type MEFs have been previously described. U2OS cells were maintained in McCoy’s 5A Medium, IMR90 cells in MEM, and H1299 cells in RPMI1640 medium (Life Technologies). All media, if not specifically described, were supplemented with 10% fetal bovine serum (FBS).

**Expression plasmids for shRNAs were made in a PLKO.1-puro vector.** The target sequences were: p53, 5′-GATCCAGTGGTAACTCACC-3′ (ref. 46); p63, 5′-GGGTTACGCGTGTATATTGCT-3′ (ref. 47); and TAp73, 5′-GGATCCAGCTATGGCAGCTT-3′ (ref. 47; all of human orig- in). The following siRNAs were purchased from Invitrogen (Carlsbad) with the catalogue number and sequences indicated: human G6PD, HS013893, 5′-AACACACUCUCUUCUACGCGCU-3′; human p21, HS381596, 5′-GACCGGAGGGAAGACAAGGAGG-3′; p53, HS202515, 5′-CCACCAAGCAGAUAAACACAUUU-3′; human ∆Np73, 5′-UAGGUUGGGACACCCGACGG-3′. siRNAs were transfected into cells using Lipofectamine RNAmax transfection agent (Invitrogen) following the manufacturer’s instruction. Stable shRNA transfection was achieved by selecting in 1 μg ml−1 puromycin (Calbiochem, catalogue number 540222) as previously described.

**Semiquantitative PCR with reverse transcription and quantitative real-time PCR.** Total RNA was isolated from cells by TRIzol reagent (Invitro- gen, catalogue number 15590618) and 2 μg RNA of each sample was reversered to cDNA by the First-strand cDNA Synthesis System (Marligen Biosciences, catalogue number 118001). cDNA (0.2 μg) of each sample was used as a template to perform PCR. The primer pairs for human genes were: G6PD, 5′-ATGGCAGACGGTTGTCGCT-3′ and 5′-TCATGGAGGCTGCTAC-3′; β-actin, 5′-GACTCTAGACTCCTCATGAATG-3′ and 5′-GTACATCTTACCTGATGGTT-3′; and rat/mouse G6PD, R802151, 5′-CAACAAAGCAGUACACAAUCUU-3′; human ∆Np73, 5′-UAGGUUGGGACACCCGACGG-3′. siRNA transfections were selected in medium containing 1 μg ml−1 puromycin (Calbiochem, catalogue number 540222) as previously described.

**NDPH and ROS levels.** NADPH and NADP+/NADPH ratios were determined using the NADPH/NADP Quantification kit (BioVision, catalogue number K347). MEP cells were analysed as described previously. Briefly, cells were incubated at 37 °C for 30 min in phosphate buffered saline (PBS) containing 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Cells were then washed twice with PBS, treated with trypsin, and re-suspended in PBS. Fluorescence signal was immediately measured using a FACScan Flow Cytometer (Becton Dickinson).

**PPP fluxes.** The flux of the oxidative PPP was measured on the basis of the rate of glucose consumption and the ratio of 13C incorporated into carbon 2 (generated by glycolysis) and carbon 3 (generated by the PPP) of lactate determined by NMR spectroscopy as described previously.

**Analysis of cell-cycle disruptions.** Cells were washed with PBS twice and fixed with 75% ethanol overnight at −20 °C. Cells were then treated with 0.1% Triton X-100 and 50 μg ml−1 RNase A for 30 min at 37 °C followed by PI staining. Cell-cycle distribution was analysed using a FACScan flow cytometer (BD Biosciences). The data were analysed using FlowJo software (Tree Star).

**Brdu incorporation assay.** Cells were treated with G6PD siRNA or control siRNA. After the indicated times, cells were pulse-labelled with Brdu (10 μM final concentration) for 1 h and analysed by anti-Brdu immunostaining as described previously. In brief, Brdu-labelled cells were washed with PBS and fixed sequentially with 2% paraformaldehyde and 70% ethanol at 4 °C. Afterwards, cells were permeabilized with 0.2% Triton X-100 and washed with PBS. DNA was denatured in 4N HCl for 20 min and incubated with an excess of biotin-DTP (approximately 100 μM) followed by sequential staining with anti-Brdu antibody and FITC-labelled BrdU-specific antibody. The images were acquired with an Olympus DPT1X microscope (Olympus) and Brdu-positive cells were counted. The FITC signal was converted to a red colour for better visualization.

**Xenograft tumour models.** The xenograft study was performed as described previously. Briefly, cells were injected subcutaneously into the flanks of 4- to 5-week-old athymic Balb/c nu/nu male mice (Taconic Farms). Three mice in total were used for each treatment in Figs 1b,d and Fig. 7c. Four mice (Fig. 7c) and eight mice (Fig. 7g) were used for each treatment. Tumour growth was evaluated at 2 or 3 weeks post-injection as indicated. All animal experiments were performed in accordance with relevant guidelines and regulations and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

**Senescence-associated SA-β-gal activity assay.** The SA-β-gal activity in cultured cells was determined using a Senescence Detection Kit (BioVision, catalog No: K320) following the manufacturer’s instructions. Percentages of SA-β-gal positive cells were calculated by counting more than 1,000 cells in random fields per cell line.

**Cell proliferation assay and CV staining of cells.** Cell proliferation assay were performed as described previously. Briefly, cells were transfected with siRNAs for 24 h and seeded in 6-well cell culture dishes in triplicate at a density of 5,000 or 20,000 cells as indicated per well in 2 ml of medium supplemented with 10% FBS. The medium was changed every day. Cell number at the indicated time points was determined using a dual Luciferase Assay System (Promega, catalogue number: E1910). Transfection efficiency was normalized on the basis of the Renilla luciferase activity.

**Promoter Reporter Program (Genomatix, software, http://www.genomatix.de).** For chromatin immunoprecipitation assays, cells were crosslinked with 1% formalde- hyde for 15 min at room temperature and crosslinking was stopped by the addition of 125 mM glycine (final concentration). Cells lysates were sonicated to generate DNA fragments with an average size below 1,000 bp and immunoprecipitated with the indicated antibodies. Bound DNA fragments were eluted and amplified by PCR. Primer pairs were: gpdβ, 5′-CTCTAGCTCCTCTCTTATGCT-3′ (+6164 + 6185) and 5′-CTCTAGCTCCTCTCTTATGCT-3′ (+6643 + 6663); puma, 5′-TGGCTCAGTGCTGCTGAGT-3′ and 5′-GGACAGTACGGTACGAG-3′ (ref. 47).

For reporter assay, the G6PD genomic fragments (+6164+6663) contain- ing either the wild-type (5′-GGTCTAGGACCAATCGACTG-3′) or mutant (GGTTATACGAAATGATAC) form, with mutated nucleotides underlined) p37- binding region were cloned into pGL3-basic vector (Promega, catalog No: E1751). Luciferase reporter assays were performed as described previously. Briefly, the reporter plasmids were transfected into p35-null H1299 cells together with a Renilla luciferase plasmid and increasing amounts of plasmids expressing p53 family proteins. Twenty-four hours after transfection, the luciferase activity was determined using a dual Luciferase Assay System (Promega, catalogue number: E1910). Transfection efficiency was normalized on the basis of the Renilla luciferase activity.
determined by counting using a haemocytometer. For CV staining, cells were fixed with 10% formalin for 5 min and stained with 0.05% CV for 30 min. After being washed with distilled water, the cells were photographed.

**Statistical analysis.** Statistical significance was analysed by Student’s t-test and expressed as a P value.

**Western blotting.** Whole-cell lysates were made in modified RIPA lysis buffer (10 mM Tris–HCl at pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.025% SDS and complete protease cocktail) for 15 min on ice, and boiled in 2× loading buffer. Protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose membrane, which was blocked in 5% skimmed milk in TBST and probed with the indicated antibodies.

**Univariable and multivariable survival analysis.** We used a combined series of 871 breast cancer patients available through the GEO (accession numbers: GSE1456, GSE2990, GSE3494, GSE7390, GSE11121). All samples were profiled using the Affymetrix U133a microarray platform. Raw data were downloaded, processed using the RMA method, z-score transformed, and median centred. Probes mapping to the same gene were combined by using the probe with the highest variance. For Cox multivariable regression, G6PD and age were used as continuous variables, and other clinical and pathological factors were used as binary variables. Missing data were imputed. For Kaplan–Meier survival analysis, G6PD levels were discretized by using a mean cutoff. All survival analysis was performed using the R package survival version 2.37 and the R language and environment for statistical computing version 2.13.

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Figure S1 Requirement of TAp73 for cell proliferation and tumor growth. (a) Schematic representation of p53 and the TAp73 and ΔNp73 isoform classes. Each p73 isoform class comprises various splicing variants (α, β, γ, etc.) that differ in their C-terminal regions. TA: transactivation domain. DBD: DNA-binding domain. OD: oligomerization domain. SAM: sterile alpha motif. (b) TAp73+/+ and TAp73−/− MEF cells cultured for 4 days were stained with crystal violet. (c) Representative images of animals two weeks after injected with TAp73+/+ and TAp73−/− MEF cells. (d) U2OS cells stably expressing control or TAp73 shRNA were stained with crystal violet at day 6. (e,f) Proliferation of two independent clones of ΔNp73−/− and ΔNp73+/+ MEFs in culture and images of crystal violet staining at day 6. Data are means ± SD (n = 3 independent experiments).
Figure S2  p73 enhances G6PD expression. (a) The pentose phosphate pathway and its link with glycolysis. FBP: fructose 1,6-biphosphate. PEP: phosphoenolpyruvate. TCA cycle: tricarboxylic acid cycle. (b) Expression of G6PD and TAp73 proteins in U2OS cells transfected with control siRNA or p73 siRNA, plus control vector or siRNA-resistant plasmid encoding Flag-TAp73. Western blots represent two independent experiments. (c) Semiquantitative RT-PCR analysis of ΔNp73 expression in HeLa, U2OS, H1299, and p53+/+ HCT116 cells. (d) Comparison of effect of p73 and ΔNp73 on G6PD expression in p73, ΔNp73 or control siRNA in HeLa cells by RT-PCR. (e) U2OS cells were infected with lentiviruses expressing a TAp73 shRNA or a control shRNA and cultured for the indicated time. The expression of G6PD, TAp73, and actin was analyzed by Western blot. The data represents three independent experiments. (f) mRNA expression in H1299 cells stably expressing control shRNA, p63 shRNA, or TAp73 shRNA was assayed by RT-PCR. (g) Quantitative RT-PCR analysis of G6PD expression in H1299 cells transfected with increasing amounts of control plasmid or plasmid expressing the indicated p53 family proteins. Data are means ± SD (n = 3 independent experiments). (h) A549 cells were treated with 1 µg/ml doxorubicin for 24 hour and protein expression was analyzed by Western blot. (i) Luciferase reporter assay of 293T cells transfected with p73 RE-luciferase construct or control construct, plus TAp73 or ΔNp73. Data are means ± SD (n = 3 independent experiments). (j) H1299 cells were transfected with increasing amounts of Flag-p53R175H, Flag-p53R273H, or Flag-TAp73 as indicated. Protein expression was examined by Western blot. The data represents two independent experiments.
Figure S3 TAp73 regulates NADPH metabolism. (a,b) NADPH levels (a) and relative NADP⁺/NADPH ratios (b) in ΔNp73⁺⁺⁺ and ΔNp73⁻⁻⁻ MEF cells. Data are means ± SD (n = 3 independent experiments). (c) Relative NADPH levels in IMR90 cells transfected with control (-) or p73 siRNA. Data are means ± SD (n = 3 independent experiments). (d) NADP⁺/NADPH ratios of IMR90 cells treated with p73 or control siRNA. Data are means ± SD (n = 3 independent experiments). (e) Relative NADPH levels in p53⁺⁺⁺ and p53⁻⁻⁻ HCT116 cells transfected with control (-) or p73 siRNA. Data are means ± SD (n = 3 independent experiments).
Figure S4 A role of TAp73 in anti-oxidant defense. (a) TAp73<sup>+/+</sup> and TAp73<sup>−/−</sup> MEF cells treated with control or G6pd siRNA. ROS was measured by 2′,7′-di-chlorofluorescein (DCF) staining and flow cytometry (left), and protein expression by Western blot (Right). Western blots represent three independent experiments. (b) ROS levels in TAp73<sup>+/+</sup> and TAp73<sup>−/−</sup> MEF cells that were treated with or without DHEA. (c) IMR90 cells were transfected with p73, G6PD, and control siRNA as indicated. ROS was measured. (d) TAp73<sup>+/+</sup> and TAp73<sup>−/−</sup> MEF cells were treated with or without 50 mM H<sub>2</sub>O<sub>2</sub> for 30 min and then cultured for 24 h in the presence or absence of DHEA. Cell viability was analyzed. Data are means ± SD (n = 3 independent experiments). (e) U2OS cells were transfected with control siRNA (-), p73 siRNA, and G6PD siRNA as indicated. Cells were treated with or without 250 µM H<sub>2</sub>O<sub>2</sub> for 24 h and cell viability was analyzed by trypan blue staining. Data are means ± SD (n = 3 independent experiments).
Figure S5 p73 regulates DNA synthesis and cell senescence via G6PD.  
(a,b) p53−/− and p21−/− HCT116 cells were transfected with control (-), p73, and G6PD siRNA as indicated. Cells were assayed for BrdU incorporation. Data are means ± SD (n = 3 independent experiments). 
(c,d) Cell-cycle profile of p53−/− (c) and p21−/− (d) HCT116 cells transfected with p73, G6PD, and control siRNA as indicated. Protein expression is shown below. Western blots represent three independent experiments. 
(e) Percentage of SA-β-gal positive cells in IMR90 culture that were transfected with control, p73, or G6PD siRNA, and cultured for 72 hours. Protein expression is shown below. Data are means ± SD (n = 3 independent experiments). Western blots represent three independent experiments.
**SUPPLEMENTARY INFORMATION**

Figure S6 p73 promotes cell proliferation through G6PD. (a–c) Proliferation of p53+/− (a), p53−/− (b), and p21−/− (c) HCT116 cells transfected with the control, G6PD, and p73 siRNAs as indicated. Data are means ± SD (n = 3 independent experiments). (d) Growth of U2OS cells transfected with control siRNA or p73 siRNA, plus control vector or Flag-G6PD. Data are means ± SD (n = 3 independent experiments).

Protein expression is shown on the Right. Western blots represent three independent experiments. (e–h) Growth of TAp73+/− and TAp73−/− MEF cells stably overexpressing G6PD or vector control. Cells were cultured in medium containing vehicle (e), NAC (f), four ribonucleosides and four deoxyribonucleosides (Nuc) (g), or both NAC and Nuc (h). Data are means ± SD (n = 3 independent experiments).
**Figure S7** G6PD levels are associated with breast cancer metastasis and a role for G6PD and TAp73 in tumor growth. (a,b) G6PD levels are associated with breast cancer metastasis. (a) Cox multivariable regression for metastasis risk using the indicated variables was performed on a cohort of 871 breast cancer patients. Shown are the hazard ratio (HR) and p-values. (b) Kaplan-Meier survival curves for metastasis-free survival. The patients were stratified by mean G6PD levels (“hi” is greater than mean, “lo” is less than or equal to mean). P-value was determined by the log-rank test. (c) Representative images of animals three weeks after injected with TAp73+/- and TAp73-/- MEF cells stably overexpressing G6PD or vector control. (d) Mice were injected with TAp73+/- and TAp73-/- MEF cells that were treated with control or G6pd siRNA, and were fed with water containing no NAC or 40 mM NAC. Representative images of animals at three weeks are shown.
Figure S8  Full scans of immunoblots used in main figures
Figure 3i

Figure 4e

Figure 4g

Figure 6b

Figure 6d

Figure 6f

Figure 6e

Figure S8 continued