Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence

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Cellular senescence both protects multicellular organisms from cancer and contributes to their ageing7. The pre- eminent tumour suppressor p53 has an important role in the induction and maintenance of senescence, but how it carries out this function remains poorly understood3-5. In addition, although increasing evidence supports the idea that metabolic changes underlie many cell-fate decisions and p53-mediated tumour suppression, few connections between metabolic enzymes and senescence have been established. Here we describe a new mechanism by which p53 links these functions. We show that p53 represses the expression of the tricarboxylic-acid-cycle-associated malic enzymes ME1 and ME2 in human and mouse cells. Both malic enzymes are important for NADPH production, lipogenesis and glutamine metabolism, but ME2 has a more profound effect. Through the inhibition of malic enzymes, p53 regulates cell metabolism and proliferation. Downregulation of ME1 and ME2 reciprocally activates p53 through distinct MDM2- and AMP-activated protein kinase-mediated mechanisms in a feed-forward manner, bolstering this pathway and enhancing p53 activation. Downregulation of ME1 and ME2 also modulates the outcome of p53 activation, leading to strong induction of senescence, but not apoptosis, whereas enforced expression of either malic enzyme suppresses senescence. Our findings define physiological functions of malic enzymes, demonstrate a positive-feedback mechanism that sustains p53 activation, and reveal a connection between metabolism and senescence mediated by p53.

We previously found that p53 inhibits the important NADPH producer glucose-6-phosphate dehydrogenase4. As this did not fully explain the effect of p53 on NADPH, we investigated whether p53 controls the expression of malic enzymes, which catalyse the oxidative decarboxylation of malate to generate pyruvate and either NADPH or NADH5,6 (Supplementary Fig. 1). In mammalian cells, three malic enzyme isoforms have been identified: a cytosolic NADPH-dependent isoform (ME1), a mitochondrial NAD(P)H-dependent isoform (ME2) and a mitochondrial NADP+-dependent isoform (ME3), of which ME1 and ME2 are the main isoforms (Supplementary Fig. 2a). By recycling the tricarboxylic acid (TCA) cycle intermediate malate into the common TCA cycle carbon source pyruvate, malic enzymes may have a regulatory role in matching TCA flux to cellular demand for energy, reducing equivalents and biosynthetic precursors (Supplementary Fig. 1).

We knocked down TP53 in human osteosarcoma U2OS cells and normal diploid fibroblast IMR90 cells using short hairpin RNA (shRNA). This led to a significant increase in messenger RNA levels of ME1 and ME2 (Fig. 1a, b and Supplementary Fig. 2b), accompanied by increased protein levels and total enzymatic activity of ME1 and ME2 (Fig. 1a, c and Supplementary Fig. 2c). Likewise, expression of ME1 and ME2 were substantially higher in TP53 knockout (TP53−/−) compared to p53-wild-type (TP53+/+) mouse embryonic fibroblasts (MEFs) (Fig. 1d). The normally short-lived p53 protein is stabilized by DNA-damage signals. Cells treated with the genotoxic agents etoposide and doxorubicin showed both time- and concentration-dependent reductions in the expression of ME1 and ME2 (Fig. 1e and Supplementary Fig. 2b, d–g). When TP53 was knocked down, the expression of ME1 and ME2 no longer responded to DNA damage (Fig. 1e). These results indicate that the expression of ME1 and ME2 is controlled by p53, both at basal levels and when p53 is stabilized by DNA-damage signals.

Figure 1 | p53 represses the expression of malic enzymes. a, Malic enzyme mRNA and protein expression in U2OS cells stably expressing TP53 shRNA or control (Ctrl) shRNA. Relative malic enzyme/actin ratios are given. b, c, mRNA expression (b), total activity and protein levels (c) of malic enzymes in TP53-depleted and control IMR90 cells. Data shown are mean ± s.d. (n = 3). d, Malic enzyme gene expression in Trp53+/+ and Trp53−/− MEFs. e, TP53-depleted and control U2OS cells were treated with increasing amounts of etoposide (Eto) and assayed for malic enzyme expression. f, TP53+/+ HCT116 cells treated with or without doxorubicin (Dox; 1 μg ml−1) were subjected to chromatin immunoprecipitation assay with anti-p53 DO-1 antibody, control mouse IgG (mIgG), or no antibody (– Ab). DMSO, dimethylsulfoxide; qRT–PCR, quantitative reverse-transcriptase PCR. **P < 0.01.

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By analysing the malic enzyme gene sequences, we identified a putative p53 response element in the first intron of the ME1 gene (ME1-RE) and three putative response elements in the first intron of the ME2 gene (ME2-RE1, ME2-RE2 and ME2-RE3) (Supplementary Fig. 3a). Chromatin immunoprecipitation assays in HCT116 cells revealed that p53 bound to the genomic regions of ME1-RE, ME2-RE1 and ME2-RE3, but not ME2-RE2. This binding was further enhanced when p53 was stabilized by treatment with doxorubicin (Fig. 1). In addition, p53 repressed the expression of a luciferase gene driven by the genomic fragment containing ME1-RE, ME2-RE1 or ME2-RE3, but not ME2-RE2 (Supplementary Fig. 3b). p53-mediated repression of certain target genes involves histone deacetylases.

Treatment with trichostatin A, an inhibitor of histone deacetylases, reversed through the silencing of TP53, an inhibitor of histone deacetylases, abrogated p53-mediated repression of ME1 and ME2 genes (Supplementary Fig. 2g).

TP53 deficiency also led to a strong increase in the ME3 transcript ( Supplementary Fig. 4a). A putative p53 response element is present in the first intron of the ME3 gene (ME3-RE) ( Supplementary Fig. 4b). p53 bound to the genomic region of ME3-RE in cells ( Supplementary Fig. 4c) and reduced the expression of a luciferase reporter driven by this response element ( Supplementary Fig. 4d). Given the low abundance of ME3 expression in cell lines that have been tested ( Supplementary Fig. 2a), we focused on ME1 and ME2 in subsequent analyses.

Although ME1 and ME2 have been extensively characterized in vitro, there is a paucity of information on their functions in cells. Silencing ME1 and ME2—in particular ME2—with short interfering RNA (siRNA) reduced cellular NADPH levels in IMR90 and U2OS cells (Fig. 2a and Supplementary Fig. 5a). This effect was also observed when a separate set of malic enzyme siRNAs, as well as malic enzyme shRNAs, were used (Supplementary Fig. 5b, c). By contrast, forced expression of ME1 or ME2—in particular ME2—or the addition of a malic enzyme substrate (dimethyl L-malate) increased cellular NADPH levels (Fig. 2b, lanes 1–3, and Supplementary Fig. 5d). To determine whether the effect of malic enzymes is due to their enzymatic activity, we generated two ME1 mutations (ME1mut1 and ME1mut2) and three ME2 mutations (ME2mut1, ME2mut2 and ME2mut3), each of which exhibited little or no enzymatic activity in vitro as well as in vivo (Supplementary Fig. 6). None of these mutants were able to increase cellular NADPH levels (Fig. 2b, lanes 4–10). Thus, both ME1 and ME2 are required for maintaining cellular NADPH levels through their enzymatic activity, with ME2 having a more profound effect. As previously observed, knockdown of TP53 led to a significant increase in NADPH levels. This increase was partially reversed through the silencing of ME1 and near-completely reversed through the silencing of ME2 (Fig. 2a and Supplementary Fig. 5a, b). These results indicate that p53 regulates NADPH metabolism through the suppression of both malic enzymes, particularly ME2.

As NADPH provides reducing equivalents for reductive biosynthesis, we examined the role of malic enzymes in lipid production. MEF cells and murine-derived 3T3-L1 pre-adipocytes were cultured with a cocktail that stimulated their differentiation into adipocytes. Triglycerides and total lipid levels in these cells declined when Me1 or Me2—particularly Me2—was depleted in these cells (Fig. 2c and Supplementary Fig. 7). By contrast, overexpression of both enzymes, particularly ME2, but none of the ME1 or ME2 mutants, increased lipid abundance (Fig. 2d). Concordant with previously published data, we observed a marked increase in lipid levels in Trp53-deficient cells compared to Trp53-proficient cells. Me1 knockdown partially reversed this increase, whereas Me2 knockdown prevented it entirely, correlating with its greater influence on cellular NADPH levels (Fig. 2c and Supplementary Fig. 7). These results indicate that the enhanced lipid accumulation in p53-deficient cells is dependent on the malic enzymes, especially ME2.

Silencing of ME2, as well as of ME1, did not significantly alter NADH levels or the NADP+/NADH ratio in IMR90 cells (Supplementary Fig. 8a, b), despite the fact that ME2 is characterized as either NADP+ or NAD+ dependent. NADH is the main electron donor for the electron transport chain that drives ATP production. Silencing of either malic enzyme did not significantly alter the abundance of cellular ATP or ADP in IMR90 cells (Supplementary Fig. 8c). In U2OS cells silencing of ME1, but not ME2, reduced NADH levels and increased the NAD+/NADH ratio (Supplementary Fig. 8d, e). These results are consistent with a cell-type-specific role of ME1 and a minimal role of ME2 in maintaining cellular NADH and ATP levels.

Next we investigated the role of ME1 and ME2 in the metabolism of glucose and glutamine. In TP53−/− HCT116 cells silencing of either malic enzyme, but especially ME2, strongly reduced glutamine consumption (Fig. 2e, f), with ME2 depletion achieving a greater effect than ME1 (Fig. 2f). The metabolic effect of ME1 and ME2 silencing was observed in IMR90 cells transfected with shRNA against TP53 (Fig. 2g, h), with ME2 silencing having a more profound effect. As previously observed, knockdown of TP53 prevented the increase in NADP+/NADH ratio (Supplementary Fig. 8d, e).

Figure 2 | ME1 and ME2 influence NADPH production, lipid production and glutaminolysis. a, b, NADPH levels in TP53-depleted and control IMR90 cells transfected with control, ME1 or ME2 siRNA (a), or in IMR90 cells stably overexpressing wild-type malic enzymes, mutant malic enzymes or vector control (b). Protein expression is shown below. Stable protein expression was achieved using the pBabe retroviral expression system. c, d, Triglyceride contents in Trp53-depleted and control 3T3-L1 cells transfected with control, Me1 or Me2 siRNA (c), or 3T3-L1 cells stably expressing wild-type malic enzymes, mutant malic enzymes or vector control (d). mRNA (c) and protein (d) expression is shown below. e, f, Effect of ME1 and ME2 knockdown in TP53−/− HCT116 cells on glucose and glutamine consumption (e) and glutaminolytic flux (f). Protein expression is shown below. All error bars represent mean ± s.d. (n = 3). *P < 0.05; **P < 0.01.

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consumption (Fig. 2e), whereas silencing of either malic enzyme had a moderate effect on glucose consumption. We extended this analysis by evaluating the rate of glutaminolysis. Depletion of either ME1 or ME2 noticeably slowed down glutaminolytic flux (Fig. 2f). These results indicate that both ME1 and ME2 have a key role in glutamine metabolism but a relatively minor role in glucose metabolism.

p53 is critical for the induction and maintenance of senescence1–3. We noticed that in IMR90 cells, a well-established senescence model, silencing of each malic enzyme by either siRNA or shRNA caused a profound increase in cells expressing senescence-associated β-galactosidase, stopping growth (Fig. 3a, b and Supplementary Fig. 9a–f). The induction of senescence in malic enzyme-knockdown cells was also indicated by the marked accumulation of the promyelocytic leukaemia protein nuclear bodies (PML-NBs) (Fig. 3c and Supplementary Fig. 9g). Notably, even a moderate reduction (20–30%) in either ME1 or ME2 strongly elicited senescence (Supplementary Fig. 10a). Malic enzyme-loss-induced senescence also occurred in U2OS and TP53−/− HCT116 tumour cell lines (Supplementary Fig. 10b, c). In TP53-deficient primary and tumour cell lines senescence decreased markedly and malic enzyme depletion lost its ability to induce this phenotype (Fig. 3b, c and Supplementary Figs 9b, f, g and 10c). By contrast, malic enzyme depletion did not cause cell death (Supplementary Fig. 11a); it induced the expression of p53 target genes implicated in senescence1,3 but not apoptosis (Supplementary Fig. 11b). These data indicate that downregulation of malic enzymes induces senescence through p53.

We next examined the role of malic enzymes in replicative senescence of normal human cells, a p53-regulated process1–3. IMR90 cells were serially passaged in culture until a substantial number of them (~50%) entered senescence. The expression of ME1 remained at comparable levels at different passages, whereas the expression of ME2, which stayed unchanged initially, noticeably declined at the late stage (Fig. 3d). To test whether the decline in ME2 contributes to senescence in this setting, we evaluated the replicative capacity of IMR90 cells forced to express ME2. Compared with control cells, ME2-overexpressing cells could be cultured for extended passages with a greatly delayed onset of senescence (Fig. 3e and Supplementary Fig. 12a). As ME1 expression was maintained during replicative senescence, we were surprised to observe a delay in senescence when ME1 expression was forced (Fig. 3e and Supplementary Fig. 12a).

By contrast, forced expression of any of the malic enzyme mutants did not delay senescence and instead moderately promoted senescence (Supplementary Fig. 12b–d), possibly through a dominant-negative effect on the endogenous malic enzymes. These results indicate that both enzymes—particularly ME2—are capable of suppressing senescence and suggest that the decline in ME2 may contribute to replicative senescence.

To examine the effect of malic enzymes on other scenarios of p53-regulated senescence, we found that culturing IMR90 cells in medium containing no or low levels of glutamine resulted in p53-dependent senescence (Supplementary Fig. 13a, b). This senescence could be delayed by overexpression of either ME1 or ME2 (Supplementary Fig. 13b), or the addition of the malic enzyme substrate malate (Supplementary Fig. 13c). By contrast, exogenous malic enzyme expression did not influence premature senescence of IMR90 cells induced by the oncogene HrasV12 (Supplementary Fig. 13d), which is not dependent on p53 (refs 14, 15). These results indicate that ME1 and ME2 expression suppress the specific way in which p53 induces senescence.

We investigated the mechanism for senescence induced by malic enzyme downregulation. In IMR90 and U2OS cells in which the expression of either ME1 or ME2 was silenced by siRNA, even moderately, p53 levels were increased, accompanied by enhanced phosphorylation of p53 and induction of its target gene p21 (also known as CDKN1A) (Figs 2a and 4a and Supplementary Figs 5a, b and 10a).

By contrast, overexpression of ME1 or ME2 in IMR90 cells substantially reduced p53 levels and activity in late passages (Fig. 3f). Overexpression of ME1 or ME2 in U2OS cells also diminished DNA-damage-induced p53 activation (Supplementary Fig. 14). These observations suggest a strong role for malic enzymes in the suppression of p53. They also indicate the existence of a positive-feedback loop for the p53–malic enzyme pathway: a higher p53 level leads to less malic enzyme expression, which alleviates the inhibition of malic enzymes on p53, leading to even higher p53 activation.

We next examined the mechanism for the regulation of p53 by ME1 and ME2. In unstressed cells, MDM2-mediated ubiquitination maintains a low basal level of p53 (ref. 2). When ME1 was knocked down in both IMR90 and U2OS cells, the abundance of the MDM2 protein and mRNA declined markedly (Fig. 4a and Supplementary Fig. 15a, b), suggesting that ME1 downregulation activates p53 through a reduction in MDM2 expression. ME2 knockdown did not significantly affect MDM2 levels. Instead, it turned on AMP-activated protein kinase (AMPK) (Fig. 4a and Supplementary Fig. 15a, b), an intracellular energy gauge that activates p53 through phosphorylation18. We tested whether AMPK is required for induction of p53 by ME2 by knocking it down in IMR90 and U2OS cells, and by comparing AMPK null and wild-type MEFs. In both situations, loss of AMPK expression prevented ME2 knockdown from activating p53 (Fig. 4b and Supplementary Fig. 15c). Because silencing of ME2 did not influence...
Figure 4 | Mechanisms of p53 activation induced by malic enzyme downregulation and a role of malic enzymes in tumour growth. a, Effect of ME1 and ME2 knockdown on p53 and AMPK activation and MDM2 expression. ACC, acetyl-CoA carboxylase; p, phosphorylated; p53, and AMPK activation in Ampk−/− and Ampk+/− ME cells transfected with control or Me2 siRNA. b, ROS levels, determined by 2′,7′-dichlorodihydrofluorescein diacetate (DCF), in IMR90 cells transfected with ME1, ME2 or control siRNA. d, Effect of N-acetyl-l-cysteine (NAC) on AMPK and p53 activation in IMR90 cells transfected with control or ME2 siRNA. 

e, f, Average weights of xenograft tumours (mean ± s.d., n = 6) generated by TP53+/+ and TP53−/− HCT116 cells transfected with ME1, ME2 or control siRNA (e), or TP53+/+ HCT116 cells stably overexpressing wild-type or mutant malic enzymes (f). *P < 0.05; **P < 0.01; ***P < 0.001.

Our studies on limited tumour samples suggest that the activity of ME2 is highly increased in these tumours and correlates with tumour progression18-20. A survey of public gene-expression databases (http://www.oncomine.org) showed that both ME1 and ME2 expression was significantly upregulated in a variety of human cancers (Supplementary Fig. 18). We investigated whether malic enzymes could influence tumour cell growth. Depletion of ME1 or ME2 in U2OS and HCT116 cells, regardless of p53 status, strongly impaired their growth (Supplementary Fig. 19), and reduced the number of cells at the S phase of the cell cycle (Supplementary Fig. 20). By contrast, overexpression of ME1 or ME2, but none of the malic enzyme mutants, enhanced tumour cell growth (Supplementary Fig. 21). In a soft agar assay, tumour cells deprived of malic enzyme gene expression, unlike their control counterparts, failed to form anchorage-independent colonies (Supplementary Fig. 22a, b), whereas cells transduced with wild-type malic enzymes, but not any of the mutants, showed enhanced anchorage-independent growth (Supplementary Fig. 22c, d).

To analyse the function of malic enzymes in the tumour xenograft model, we injected immunocompromised mice with TP53+/+ and TP53−/− HCT116 cells treated with ME1, ME2 or control siRNA. TP53−/− HCT116 cells gave rise to tumours that were twice the weight of tumours generated by TP53+/+ HCT116 cells. When ME1 or ME2—particularly ME2—was silenced in these cells, the tumour sizes were markedly reduced (Fig. 4e and Supplementary Fig. 23a, b). TP53−/− HCT116 tumours devoid of malic enzymes showed extensive senescence and were substantially smaller compared to the corresponding TP53+/+ HCT116 tumours (Supplementary Fig. 23c).

Conversely, overexpression of wild-type ME1 or ME2, but not mutant malic enzymes, accelerated the growth of TP53+/+ HCT116 tumours (Fig. 4f and Supplementary Fig. 23d). These observations indicate that malic enzymes are essential for tumour growth through both p53-dependent and -independent mechanisms.

Although p53 is able to induce a range of anti-proliferative responses, emerging evidence indicates that senescence induction and metabolic regulation are central to its function as a tumour suppressor18-20. Our results demonstrate a positive-feedback loop comprising p53 and malic enzymes that influences p53 activation and links metabolism with the onset of senescence (Supplementary Fig. 24). p53 suppresses all malic enzyme expression by directly binding to response elements on the genome, and this binding is strongly downregulated upon depletion of ME1 or ME2. The marked stabilization of p53 upon ME1 or ME2 downregulation is achieved through different mechanisms. The p53–malic enzyme positive-feedback loop is likely important to alleviate the negative-feedback regulation (for example, the p53–MDM2 feedback loop) that restrains its activity4. The p53–malic enzyme positive-feedback loop is likely important to alleviate the negative-feedback regulation (for example, the p53–MDM2 feedback loop) that restrains its activity4.
of senescence. The involvement of the p53–malic enzyme pathway in senescence demonstrates a close link between metabolism and this irreversible fate of the cell.

METHODS SUMMARY
Malic-enzyme-dependent glutaminolytic flux was determined by labelling the malate pool with $^{13}$C from $[^{15}$C]glutamine and monitoring the conversion of $[^{13}$C]malate to pyruvate. $[^{13}$C] enrichments were determined with gas chromatography–mass spectrometry. Glucose and glutamine consumption was determined using a YSI 7100 Multiparameter Bioanalytical System. Detailed experimental procedures are presented in Methods.

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

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**METHODS**

**Antibodies and reagents.** The antibodies against the following proteins/epitopes were purchased from the indicated sources: ME2, actin and β-tubulin (Sigma); AMPK, phospho-AMPK (Thr172), phospho-p53 (Ser15) and phospho-acetyl-coenzyme A carboxylase (Ser79) (Cell Signaling Technology); ME1, p21 and PML (Santa Cruz Biotechnology); prohibitin (Thermo Scientific); GAPDH (Novus Biologicals); p53 (DO-1), Oncogene, and Santa Cruz Biotechnology); and MDM2 (Calbiochem, and Santa Cruz Biotechnology). The following reagents were purchased from Sigma: dimethyl 1-malate, NADPH, NAD+, doxorubicin, etoposide, trichostatin A, NAC, insulin, troglitazone, dexamethasone and isobutylmethylxanthine.

**Cell culture and gene knockdown with shRNA and siRNA.** Cells were maintained in standard culture conditions without any antibiotic. Expression plasmids for TP53, ME1 and ME2 shRNA were made in a PLOK-1 puro vector. The target sequences for human TP53 and mouse TP53 were 5′-GACAGTGTTTTCCTA-3′ (ref. 26) and 5′-GTTCGGTCCCTTCTCAAT-3′ (ref. 27), respectively. The target sequences for human ME1 and ME2 are 5′-GGCGAT ATTGCTTCCAGTT-3′ and 5′-GCAAGCGTCAAGGAAGGAT-3′, respectively. Stable shRNA cell lines were established as previously described29. siRNAs for ME1, ME2 and AMPK were purchased from Invitrogen. siRNA sequences were 5′-UAACUAUCAGGUAACAUUGGCUA-3′ (human ME1), 5′-UUAUGGUACCAGAGAUGAUUACAC-3′ (human ME2), 5′-CCUCUGGUGAACUUGGCUCAUAU-3′ (human ME2) no. 2), 5′-CUUGUAAAAUGGCAUUAUAAA (mouse Me1), 5′-GGGCCACUGGUAACGUGCAUAUAU-3′ (mouse Me2) and 5′-ACCAUGAUAUGAUGAGGCUAUAU-3′ (human AMPK). siRNAs were transfected into cells using Lipofectamine RNAiMax Transfection Agent (Invitrogen).

**Quantitative RT–PCR**

**Semi-quantitative RT–PCR and quantitative RT–PCR.** Total RNA was isolated from cells by TRIzol Reagent (Invitrogen). Two micrograms of RNA for each sample were reversed to complementary DNA by First-strand cDNA Synthesis System (Marlin Biociences), and 0.2 µg cDNA was used as a template to perform PCR. The primer pairs for human genes were: ME1-5′-ACAGCATATAATTTTCTCTACATG-3′ and 5′-CTACTGTTGCACTTCTTGGT-3′; ME2- 5′-ATTAGTGACGACCTTTTCTTCA-3′ and 5′-CTATCTTGTTATACAGCAGG-3′; p21-5′-CCGGGAGCCCGGATGAG-3′ and 5′-CTTCCTCCTGGGAGAGGAT-3′; ActB-5′-GACCAGTACGACTACCTCATGAAGAT-3′ and 5′-GTCACTGATTGAGTGGTGAAGG-3′; TP53-5′-CACAGCTGCTGCCACCCGG-3′ and 5′-TCTCGGAGCTGCTGAGT-3′. Primer pairs for mouse genes were: Me1, 5′-GATGATAGAAGTCTCCCTGACC-5′ and 5′-TACTGCTTGTGCTTCTGCTG-3′; Me2, 5′-TCTTTAGAACTCTGGAAAAGC-3′ and 5′-TCATGGGTGGGGACTGTCT-3′; p21, 5′-AATCTCGTCTTGCCAGGCAG-3′ and 5′-TCAAGGGTTTCTCTGAGA-3′; ActB, 5′-ACCTCATTACATCTCCTT-3′ and 5′-CTAGAAGACCTTGCGGTTG-3′; TP53, 5′-GAAGTCTCCTTGGCTGCAACC-3′ and 5′-CTACGAGTTTGCGCTCTC-3′. All RT–PCR reactions were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions were: 50 °C for 2 min followed by an initial de-naturation step at 95 °C for 10 min, 45 cycles at 95 °C for 15 s, 60 °C for 1 min, and a dissociation curve at 95 °C for 15 s. The experiments were carried out in triplicate for each data point. Using this method, we obtained the fold changes in gene expression normalized to an internal control gene.

**Cell lysis fractionation and malic enzyme activity.** Cell fractionation was carried out as described29. Cells were homogenized in 20 mM HEPES-KOH buffer, pH 7.5, 10 mM KCl, 1.5 mM sodium EDTA buffer, 1 mM sodium pyruvate enriched in all four carbons. The reactions were started by adding either cytosolic and mitochondrial extracts, and were monitored by absorbance at 340 nm every 5 s for up to 10 min. Background control was run without malic acid as substrate. Enzyme activity was determined by subtracting the activity of the background control to each sample. The resulting changes of absorbance versus time were normalized to protein contents, which were determined using the Bio-Rad protein assay.

**Analysis of malic enzyme gene sequence and chromatin immunoprecipitation (ChIP) assay.** We used the Genomatix Promoter Inspector software (http://www.genomatix.de) to search in malic enzyme genes for potential p53 response elements with the consensus sequence 5′-RRRCCWGGYYY- (0–13 base pair spacer)-RRRCCWGGYYY-3′, in which R is a purine, Y a pyrimidine, and W either A or T. The sequences for the putative p53 response elements in malic enzyme genes were: ME1-5′-TTCATCCTGTAATCAGCAGTGCC-3′; ME2-5′-AAGCAGTTGACAGCATGCCC-3′; ME2-5′-AGGACCTGCAAAACATGTC-3′; ME2-5′-GGCGATATTGCTCAGTT-3′; and ME2-5′-TGACCTGTGGCTCCTTGTC-3′.

**ChIP assay.** For ChIP assays, cells were washed with PBS and crosslinked with a 1% form- aldehyde solution for 15 min at room temperature (25 °C). The crosslinking reaction was stopped by the addition of glycine to 125 mM final concentration. Cell lysates were sonicated to generate DNA fragments with the average size below 1,000 base pairs and followed by immunoprecipitation with indicated antibodies. Bounded DNA fragments were eluted and amplified by PCR. The primer pairs were: ME1-5′-GCCCTAGTATGTTGAGTC-3′ and 5′-GAAGAAGGTTAGGGAAGAAGG-3′; ME2-5′-CTTGACCTTGTGGGAGG-3′ and 5′-CTACGAGCTGCTTCTGAG-3′; ME2-5′-TCTCGGAGCTGCTGAGT-3′ and 5′-ACCGAGGTCAGCTGAC-3′; ME2-5′-CTGAGGTCCGCTTGTC-3′ and 5′-TGCTGCTGAGTTGGCTTTCG-3′.

**Reporter assay.** The DNA fragment containing the potential p53-binding region was amplified by PCR with primers used in the ChIP assay and was cloned into a pGL3-promoter vector (Promega). 293T cells were plated 18 h before transfection in 24-well plates and transiently transfected with 450 ng of the reporter plasmid and/or 100 ng of the p53 plasmid using Lipofectamine 2000 (Invitrogen). The luciferase activity was determined according to the manufacturer’s instructions (Promega). Transfection efficiency was normalized on the basis of the Renilla luciferase activity.

**Measurements of metabolites and lipid accumulation.** The levels of NADPH, NADH, NAD+, ATP and ADP in cultured cells were determined using a NADPH Quantitation Kit, NAD+ /NADH Quantitation Kit, ATP assay kit, and ADP assay kit (all from BioVision) respectively, following the manufacturer’s instructions. Glucose and glutamine consumption was determined using YSI7100 Multiparameter Bioanalytical System (YSI Life Sciences). Triglyceride was measured using a Triglyceride Assay Kit (BioVision). Total lipids were measured using Oil Red O staining28. For this, confluent cells were grown in medium with 10% FBS supplemented with insulin (5 µg ml−1), dexamethasone (1 µM), troglitazone (5 µM) and isobutylmethylxanthine (0.5 mM) for 2 days, and in medium supplemented with insulin and rosiglitazone for an additional 5 days. The medium was changed 2 days after. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature, washed with distilled H2O and 60% isopropanol, and stained with a filtered Oil Red O work solution at room temperature. Stain was then removed and cells were washed four times in distilled H2O.

**Measurement of glutaminolitic flux.** Malic enzyme-dependent glutaminolitic flux was determined by labelling the malate pool with 13C from [U-13C5]glutamine and monitoring the conversion of malate to pyruvate indirectly by detecting 13C-labelled lactate. Carbon-13 enrichments were determined with gas chromatography–mass spectrometry. We observed that this approach results in approximately 50% 13C enrichment and allows for the determination of glutaminolitic flux through malic enzyme with high sensitivity. Cells cultured on 10-cm plates with a round-bottom confluence were transfected with pCAGGME2. ME2 cDNA was subcloned into pBLCMV expression vector (Promega). 293T cells were plated 18 h before transfection in 24-well plates with a round-bottom confluence and transfected with pCAGGME2. The levels of ME1 and ME2 were determined with the YSI 7100 Multiparameter Bioanalytical System. The glutaminolitic flux through malic enzymes was calculated from the equation: Fm = Fm/(I + (I + I) + (I + I)), in which Fm is malic enzyme flux, Fm is total lactate flux, I = fraction of lactate enriched in all three carbons, and I = fraction of lactate enriched in all four carbons.

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Measurements of ROS. ROS levels were determined as described\textsuperscript{22}. Cells were incubated at 37°C for 30 min in PBS containing 10 μM 2',7'-dichlorodihydro-fluorescein diacetate (H2-DCFDA, Sigma). Afterwards, the cells were washed twice in PBS, treated with trypsin, and re-suspended in PBS. Fluorescence was immediately measured using a FACScan Flow Cytometer (Becton Dickinson).

Senescence-associated SA-β-gal activity. The SA-β-gal activity in cultured cells was determined using a Senescence Detection Kit (BioVision) following the manufacturer’s instructions. Percentages of cells that stained positive were calculated by counting 1,000 cells in random fields per cell line.

Immunofluorescence. Cells treated with siRNA for 48 h were washed with 1xPBS and fixed in 4% paraformaldehyde. After being treated with 0.1% Triton X-100, cells were stained using anti-PML antibody followed by Texas-red conjugated anti-mouse IgG antibody, and mounted with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). The images were acquired with a confocal microscope. A total of 200 nuclei were selected randomly and promyelocytic leukaemia nuclear bodies within each nucleus were counted.

Cell proliferation assay. Cells were treated with siRNAs for 24 h and seeded in 6-well cell culture dishes in triplicates at a density of 20,000 cells per well in 2 ml of medium containing 10% FBS. The medium was changed everyday. Cells were counted and cell number at the indicated time points was determined.

Soft agar assay and xenograft tumour models. For the soft agar assay, cells were suspended in 1 ml of 10% FBS DMEM medium containing a 0.3% agarose and plated on a firm 0.6% agarose base in 6-well plates (5,000 cells per well) as described previously\textsuperscript{23}. Cells were then cultured in a 37°C and 5% CO₂ incubator for 2 weeks. Images were obtained and colonies were counted under a microscope. Each experiment was done in triplicate. For the mouse xenograft experiment, cells (2 × 10^6) were injected subcutaneously into the flanks of 4- to 5-week-old athymic Balb-c nu/nu male mice (Taconic Farms). Tumour growth was evaluated at 2 weeks post-injection. 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).

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