Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells

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Cancer cells acquire distinct metabolic adaptations to survive stress associated with tumour growth and to satisfy the anabolic demands of proliferation. The tumour suppressor protein p53 (also known as TP53) influences a range of cellular metabolic processes, including glycolysis\(^1\), oxidative phosphorylation\(^2\)\(^,\)\(^,\) glutaminolysis\(^3\)\(^,\)\(^,\) and anti-oxidant response\(^4\). In contrast to its role in promoting apoptosis during DNA-damaging stress, p53 can promote cell survival during metabolic stress\(^5\), a function that may contribute not only to tumour suppression but also to non-cancer-associated functions of p53\(^6\). Here we show that human cancer cells rapidly use exogenous serine and that serine deprivation triggered activation of the serine synthesis pathway and rapidly suppressed aerobic glycolysis, resulting in an increased flux to the tricarboxylic acid cycle. Transient p53-p21 (also known as CDKN1A) activation and cell-cycle arrest promoted cell survival by efficiently channeling depleted serine stores to glutathione synthesis, thus preserving cellular anti-oxidant capacity. Cells lacking p53 failed to complete the response to serine depletion, resulting in oxidative stress, reduced viability and severely impaired proliferation. The role of p53 in supporting cancer cell proliferation under serine starvation was translated to an in vivo model, indicating that serine depletion has a potential role in the treatment of p53-deficient tumours.

As p53 contributes to the survival of cells deprived of glucose\(^2\), we investigated whether removal of other nutrients found in normal medium induced a differential response in p53\(^+/+\) and p53\(^{-/-}\) HCT116 cells. Whereas removal of the non-essential amino acids serine and glycine impaired proliferation of p53\(^+/+\) cells, p53\(^{-/-}\) cells showed a more marked loss of proliferation (Fig. 1a) and substantial loss of viability (Fig. 1b, c). The contribution of p53 to growth and survival during serine and glycine depletion was also seen in RKO cells (Supplementary Fig. 2a–c) and primary mouse embryonic fibroblasts (MEFs; Supplementary Fig. 2d). By removing serine or glycine individually, we established that serine depletion was the major contributor to the starvation phenotype (Fig. 1a–c), as removal of glycine alone had no detrimental effect. Whereas serine and glycine may be inter-converted by serine hydroxymethyltransferase (SHMT), serine to glycine conversion supports proliferation via methyl-tetrahydrofolate (THF) production (Supplementary Fig. 1). The reverse reaction (glycine to serine) depletes methyl-THF, which is presumably why excess glycine has been shown to inhibit proliferation\(^7\). As expected, removal of lysine (an essential amino acid) did not cause a differential response, being equally incompatible with proliferation in p53\(^+/+\) and p53\(^{-/-}\) cells (Supplementary Fig. 2e).

Analysis of cell culture medium by liquid chromatography–mass spectrometry (LC–MS) revealed rapid serine consumption by p53\(^+/+\) and p53\(^{-/-}\) cells (Fig. 1d), whereas glycine uptake was low (Supplementary Fig. 2f). A recent screen of NCI-60 cancer cell lines showed that elevated SHMT expression and glycine uptake were correlated with rapid proliferation\(^8\). Notably, all 60 lines consumed more serine than glycine, including seven lines with the shortest doubling times (<22 h), which on average consumed 7.7-fold more serine than glycine\(^9\). Furthermore, cells with the shortest doubling times and highest glycine uptake also consumed most of the available serine\(^10\), raising the possibility that these highly proliferative cells switch to glycine consumption because they have exhausted the available serine. Overall this demonstrates that cancer cells avidly consume serine, which may, through high SHMT expression, be used to generate glycine and methyl-THF.

Unlike essential amino acids, the chronic depletion of non-essential amino acids can be tolerated in vivo. Mice tolerated diets lacking serine and glycine well (Supplementary Fig. 3a), and LC–MS confirmed a significant drop in serum levels of serine and glycine, but not other amino acids (Supplementary Fig. 3b). HCT116 cells rapidly formed tumours in animals fed control diet, without a significant difference in volume between p53\(^+/+\) and p53\(^{-/-}\) tumours (Fig. 1e). However, animals fed matched diet lacking serine and glycine displayed significant reduction in the volume of tumours of both genotypes, and survived significantly longer before tumour size or ulceration endpoints (Fig. 1e, f). As with our in vitro studies, serine and glycine starvation had a more dramatic effect on p53\(^+/+\) xenografts, which had significantly reduced volume compared to p53\(^{-/-}\) tumours in serine- and glycine-deprived animals (Fig. 1e).

Mammalian cells synthesize serine de novo by channelling the glycolytic intermediate 3-phosphoglycerate into the ‘phosphorylated pathway’ of serine synthesis\(^11\) (Supplementary Fig. 1). Flux through this biosynthetic pathway is controlled primarily by the demand for serine, rather than the availability of substrate 3-phosphoglycerate\(^12\). The serine synthesis pathway (SSP) supports anabolism by providing precursors for biosynthesis of proteins, nucleotides, creatine, porphyrins, phospholipids and glutathione, and SSP upregulation occurs in some breast cancers\(^13\)–\(^16\). A recent study demonstrated that serine starvation activates the SSP\(^17\); we found that serine starvation induced strong p53-independent upregulation of PHGDH and PSAT1, with a modest increase in PSPH (Fig. 1g and Supplementary Fig. 4a, b). The failure of p53\(^{-/-}\) cells to proliferate under serine starvation could therefore not be attributed to a deficiency in SSP enzyme expression. p53 has been shown to downregulate PGAM\(^18\)—potentially allowing 3-phosphoglycerate to be channeled to the SSP. However, PGAM expression did not vary greatly during serine starvation (Supplementary Fig. 4a, b). Consistent with their ability to activate the SSP, both p53\(^+/+\) and p53\(^{-/-}\) cells achieved de novo serine synthesis, as detected using \([U\text{-}^{13}\text{C}]\)glucose labelling (Fig. 1h). However, p53\(^{-/-}\) cells had lower serine levels, indicating some defect in the ability of these cells to adapt to de novo serine synthesis. We therefore sought to explore the mechanisms through which cells adapt to serine starvation.

The mTOR pathway senses amino acid availability, and whereas mTORC1 activity was lowered by serine starvation, it was maintained at very similar levels in p53\(^+/+\) and p53\(^{-/-}\) cells (Supplementary Fig. 5). This demonstrated that the effect of serine starvation on mTORC1 was p53-independent and therefore unlikely to contribute to the enhanced sensitivity of p53\(^{-/-}\) cells. A similar maintenance of mTORC1 activity in serine-starved cells has recently been shown, and is promoted by PKM2 expression\(^19\). Serine activates PKM2\(^20\) and decreased PKM2 activity following serine starvation causes an accumulation of upstream glycolytic intermediates for diversion to the SSP\(^20\). To balance lower

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As expected, p53<sup>−/−</sup> cells showed lower O<sub>2</sub> consumption than p53<sup>+/+</sup> cells under fed conditions. Surprisingly, p53<sup>−/−</sup> cells responded to serine starvation with increased O<sub>2</sub> consumption, whereas p53<sup>+/+</sup> cells showed lower O<sub>2</sub> consumption (Fig. 2c). Closer analysis of metabolic flux into the TCA cycle revealed that whereas both p53<sup>+/+</sup> and p53<sup>−/−</sup> cells showed an increase in glycolytic TCA cycle flux in the immediate response to serine starvation, this response was reversed over time in p53<sup>+/+</sup> cells, but sustained in the p53<sup>−/−</sup> cells (Fig. 2d, e). This correlated with the changes in O<sub>2</sub> consumption observed during serine starvation, indicating that the initial response of p53<sup>+/+</sup> and p53<sup>−/−</sup> to serine depletion is similar, but p53<sup>−/−</sup> cells sustain a metabolic profile indicative of low PKM2 activity, including elevated O<sub>2</sub> consumption.

We predicted that the disruption to glycolysis in the immediate response to serine starvation would impede ATP production, indeed ATP levels dropped in both p53<sup>+/+</sup> and p53<sup>−/−</sup> cells (Fig. 2f). We considered that as glycolytic flux to pyruvate is lowered by serine starvation, increasing pyruvate levels (by adding exogenous pyruvate) would potentially alleviate the ATP shortage by further enhancing flux to the TCA cycle. We observed a significant recovery in proliferation of p53<sup>−/−</sup> cells after adding pyruvate (Fig. 2g).

**Figure 1** | p53 promotes cell survival and proliferation during serine starvation *in vitro* and *in vivo*. a, HCT116 cells were grown in complete medium (containing serine and glycine) or equivalent medium lacking these amino-acids (averages of triplicate wells). p53<sup>−/−</sup> (1ex), deletion of p53 exon 2; p53<sup>/−/−</sup> (3ex), deletion of p53 exons 2, 3 and 4. b, c, Viability of HCT116 cells was assessed by analysing sub-G1 DNA content (b, n = 3) and propidium iodide (PI) exclusion (c, n = 3). d, LC–MS was used to determine the relative consumption of serine by HCT116 cells fed complete medium (averages of triplicate wells versus fresh medium). e, Nude mice were subcutaneously injected with HCT116 cells (p53<sup>+/−</sup> right flank, p53<sup>−/−</sup> left flank), and fed diet with or without serine and glycine. Tumour volume is plotted until the first animal in each group reached the experimental end-point (*P < 0.05 control diet group versus –Ser & Gly group; **P < 0.05 for p53<sup>+/−</sup> versus p53<sup>−/−</sup> within –Ser & Gly group). f, Kaplan–Meier plot of survival until experimental end-point for diet groups (mean survival; control = 33.3 days (n = 10), –Ser & Gly = 53.2 days (n = 8), log rank P = 0.001, Wilcoxon P = 0.003). g, Expression of glycolytic and SSP genes (averages of triplicate quantitative PCR). h, Intracellular serine levels in HCT116 cells fed medium containing [U-<sup>13</sup>C]glucose were measured by LC–MS (averages of triplicate wells). Labelled serine (M + 3, mass isotopomer with 3<sup>13</sup>C) and unlabelled serine (M + 0, mass isotopomer with 0<sup>13</sup>C incorporated) is shown in cells grown in complete (Com) or serine- and glycine-deficient (–) medium for the indicated times. All error bars are s.e.m.

 glycolysis following PKM2 inhibition, cells increase flux of pyruvate to the tricarboxylic acid (TCA) cycle, requiring cells depleted of PKM to display increased O<sub>2</sub> consumption to support elevated oxidative phosphorylation<sup>20</sup>. Both p53<sup>+/−</sup> and p53<sup>−/−</sup> cells displayed elevated phosphoenolpyruvate (PEP) levels and decreased pyruvate and lactate levels, evidence of low PKM2 activity following serine starvation (Fig. 2a). The importance of oxidative phosphorylation during serine starvation was demonstrated by treatment with the mitochondrial ATP synthase inhibitor oligomycin (Fig. 2b), which completely inhibited the growth of serine-deprived p53<sup>−/−</sup> cells. As p53 supports oxidative phosphorylation<sup>21,22</sup>, we considered the possibility that p53<sup>−/−</sup> cells would be unable to upregulate oxidative phosphorylation in response to serine starvation.

**Figure 2** | Serine starvation differentially changes energy metabolism in p53<sup>+/−</sup> and p53<sup>−/−</sup> cells. a, HCT116 cells were fed complete (Com) or serine- and glycine-deficient (–SG) medium for 24 h, in the presence of [U-<sup>13</sup>C]glucose for the final 2 h. LC–MS was used to determine metabolic flux into the TCA cycle revealed that whereas both p53<sup>+/−</sup> and p53<sup>−/−</sup> cells showed an increase in glycolytic TCA cycle flux in the immediate response to serine starvation, this response was reversed over time in p53<sup>+/−</sup> cells, but sustained in the p53<sup>−/−</sup> cells (Fig. 2d, e). This correlated with the changes in O<sub>2</sub> consumption observed during serine starvation, indicating that the initial response of p53<sup>+/−</sup> and p53<sup>−/−</sup> to serine depletion is similar, but p53<sup>−/−</sup> cells sustain a metabolic profile indicative of low PKM2 activity, including elevated O<sub>2</sub> consumption.

We predicted that the disruption to glycolysis in the immediate response to serine starvation would impede ATP production, indeed ATP levels dropped in both p53<sup>+/−</sup> and p53<sup>−/−</sup> cells (Fig. 2f). We considered that as glycolytic flux to pyruvate is lowered by serine starvation, increasing pyruvate levels (by adding exogenous pyruvate) would potentially alleviate the ATP shortage by further enhancing flux to the TCA cycle. We observed a significant recovery in proliferation of p53<sup>−/−</sup> cells after adding pyruvate (Fig. 2g). LC–MS analysis of

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Serine-starved p53−/− cells fed unlabelled pyruvate (in the presence of [U-13C]glucose) confirmed increased TCA cycle flux (Supplementary Fig. 6). However, this enhanced TCA cycle flux was not sufficient to fully restore proliferation (Fig. 2g). Importantly, although it has recently been shown that serine can be synthesised from pyruvate (reviewed in ref. 30), we detected only labelled serine in the cells fed unlabelled pyruvate (Supplementary Fig. 6), demonstrating that the serine in these cells was derived from glucose.

Serine contributes to the synthesis of purines via glycine, which is incorporated into GMP and AMP via inosine monophosphate (IMP). Serine starvation resulted in lower GMP and AMP levels in p53+/− and p53−/− cells (Fig. 3a). Previous studies show that GMP depletion can activate p53-dependent G1 arrest23,24. Consistently, we found that serine starvation led to a small elevation in p53 expression, accompanied by more marked, but transient, elevation of the p53 target protein p21 (Fig. 3b). Treatment of p53+/− cells with low doses of mycophenolic acid (an inhibitor of GMP synthesis) replicated this subtle p53-p21 response (Supplementary Fig. 7a, b). Recruitment of mycophenolic acid (an inhibitor of GMP synthesis) replicated target protein p21 (Fig. 3b). Treatment of accompanied by more marked, but transient, elevation of the p53 type p53) were grown in medium with or without serine and glycine (averages of triplicate wells). All error bars are s.e.m.

**Figure 3** | Serine starvation causes recruitment of p53 to the p21 promoter and activation of a transient p21-dependent G1 arrest. a. LC–MS was used to quantify total relative amounts of intracellular purine nucleotides GMP and AMP in serine and glycine fed (Com) and starved (– SG) HCT116 cells (averages of triplicate wells). b, p53 and p21 protein expression was quantified in p53+/− and p53−/− HCT116 cells via western blot and detection with infrared-conjugated secondary antibodies (n = 3). CDK4 expression was used as a control for normalisation. c, Chromatin-immunoprecipitation (ChIP) was performed for p53 with quantitative PCR for two p53-response elements (−2350 bp and −1450 bp) and the transcription initiation region (−50 bp) of the p21 promoter (n = 3). HU, hydroxyurea. d, BrdU labelling and PI staining followed by flow cytometry were used to assess cell cycle (n = 3). e, p21 was transiently knocked down in p53+/− HCT116 cells using short interfering RNA (siRNA, averages of triplicate wells). f, p21−/− HCT116 cells (retaining wild-type p53) were grown in medium with or without serine and glycine (averages of triplicate wells). All error bars are s.e.m. confirmed by chromatin-immunoprecipitation (Fig. 3c). As only a modest increase in p53 expression was observed, we speculate that p53 was activated via post-translational modification, rather than MDM2 inhibition. Although our data are consistent with p53 activation in response to nucleotide depletion, activation of AMPK (Supplementary Fig. 7c) due to lower ATP levels could also contribute to the p53 response.

As expected following p53 activation, p53+/− cells starved of serine and glycine initially showed an accumulation of cells in G1 and reduced S-phase, correlating with the increased expression of p21 (Fig. 3d). By 48 h, these cells resumed a normal cell cycle. By comparison, p53−/− cells failed to establish a strong G1 arrest, showing a more gradual decrease in S-phase (corresponding to an increase in sub-G1 cells, Fig. 1b) and did not recover normal cell cycle (Fig. 3d). Because p21 is a major mediator of p53-induced G1 arrest, we examined the effect of p21 loss in p53+/− cells. p21−/− (CDKN1A−/−) cells showed a similar cell-cycle response as p53−/− cells (Fig. 3d), indicating that induction of p21 may be critical for adaptation to serine starvation. Indeed, p53+/− cells depleted of p21 by siRNA were unable to increase in number without serum (similar to p53−/− cells, Fig. 3e), a response even more notable in cells genetically deleted of p21 (Fig. 3f).

We next considered whether p53+/− and p53−/− cells use the low levels of intracellular serine available under conditions of starvation differently. Specifically, we analysed the balance between purine-nucleotide and glutathione synthesis, both of which require serine/glycine. LC–MS showed that after 24 h, p53−/− cells retained flux of de novo serine/glycine into IMP (M+7), but this was inhibited in p53+/− cells (Fig. 4a). Re-feeding serine-starved cells with [U,13C]N]-serine confirmed sustained serine flux into IMP, GMP and AMP in p53−/− cells (Supplementary Fig. 8a). However, in serine-starved p53+/− cells, flux of the replenished labelled serine to nucleotides was blocked, despite plentiful intracellular serine. This demonstrated that a feature of p53-p21-induced cell-cycle arrest in response to serine starvation is inhibition of nucleotide synthesis, a known function of p2126,27. Reduced glutathione (GSH) is the principal cellular anti-oxidant, and we found that levels of GSH dropped significantly in p53+/− and p53−/− serine-starved cells (Fig. 4b). However, in contrast to nucleotides, p53+/− cells maintained and, over time, enhanced flux to GSH synthesis during starvation. Notably, this maintenance of flux to glutathione was not seen in p53−/− and p21−/− cells (Fig. 4b and Supplementary Fig. 8b). Consequently, total GSH levels showed recovery in p53+/− but not p53−/− or p21−/− cells (Fig. 4c and Supplementary Fig. 8c).

The failure of p53−/− cells to recover GSH levels combined with their elevated oxygen consumption suggested that these cells would accumulate increased intracellular reactive oxygen species (ROS) levels, p53 has well-established anti-oxidant functions4,8, but hydrogen peroxide treatment demonstrated that p53−/− cells were only slightly more susceptible than p53+/− to oxidative stress under normal conditions (Fig. 4d). Although serine and glycine starvation increased the sensitivity of both genotypes to peroxide treatment, this effect was more marked in p53−/− cells (Fig. 4d), and rescued by adding GSH (Fig. 4e). Staining with an oxidation-activated fluorescent dye confirmed increased intracellular ROS in p53−/− cells during serine starvation (Fig. 4f and Supplementary Fig. 9a). To assess the importance of ROS in limiting proliferation, we tested the effect exogenous GSH or ROS-limiting treatments almost completely rescued the proliferation of serine-starved p53−/− cells in combination with pyruvate (Fig. 4g). We confirmed that the exogenous GSH did not lead to accumulation of intracellular glycine or serine, demonstrating that rescue was achieved by increasing the intracellular GSH pool (Supplementary Fig. 9b). Adding GSH to serine-starved p53+/− cells did not greatly enhance proliferation, supporting the theory that p53+/− cells are able to maintain GSH pools independently (Supplementary Fig. 9c).
Figure 4 | p53-p21 activation allows serine-deprived cells to synthesize GSH in preference to nucleotides. a–c, LC-MS was used to detect relative intracellular quantities of IMP (a) and GSH (b) in HCT116 cells fed complete medium (Com) or medium lacking serine and glycine (−SG) for 24 h, in the presence of [U-13C]glucose for the final 2 h, and in HCT116 cells fed −SG medium for the indicated times in the presence of [U-13C]glucose for the final hour (averages of triplicate wells). d, e, HCT116 cells were grown with or without serine and glycine (d) or medium lacking serine and glycine with 5 mM glutathione (GSH) (e) for 48 h in the presence of hydrogen peroxide (H2O2) for the final 24 h. f, HCT116 cells were treated with an oxidation-activated fluorescent dye and analysed by flow cytometry. g, HCT116 p53+/− (1ex) cells were grown with or without serine, glycine, pyruvate 5 mM (Pyr) and/or GSH 5 mM, or N-acetyl cysteine 0.2 mM (NAC) (averages of triplicate wells). All error bars are s.e.m.

Our data therefore show that the sensitivity of p53−/− cells to serine depletion is due to a combination of impaired glycolysis and elevated ROS. Whereas the initial response of cells with or without p53 to serine depletion was similar, p53+/− cells underwent p21-dependent G1 arrest, allowing the limited levels of de novo serine to be channelled to GSH production to counter oxidative stress. Depletion of p21 (while retaining p53) caused severe sensitivity to serine depletion (Fig. 3e, f), indicating that activation of other arms of the p53 response may contribute to the death of these cells. Our data also demonstrate that the ability of p53-deficient cells to engage higher rates of oxidative phosphorylation is not entirely defective, but that oxidative phosphorylation in these cells is probably limited by a requirement to prevent ROS generation. This observation ties in with the suggestion that cancer cells adopt aerobic glycolysis (the Warburg effect) to avoid generation of metabolic ROS28. It has recently been shown that increased ROS levels inhibit PKM229, providing an explanation for why p53−/− cells show evidence of sustained PKM2 inhibition during serine starvation.

In conclusion, our study underlines the importance of p53 in coordinating metabolic remodelling in response to metabolic stress. We demonstrate that serine uptake supports the Warburg effect, indicating that many cancer cells may show some sensitivity to serine depletion, particularly those lacking p53. However, it is likely that other genetic alterations (such as PHGDH amplification14–16) may circumvent serine-dependence in other cancer cells. Taken together, our work suggests the therapeutic utility of serine depletion—either by removal from the diet, enzymatic depletion in vivo, or other means—is worthy of further investigation.

METHODS SUMMARY
HCT116 p53+/−/p21−/− (parental), p53−/− (1ex) (deletion of p53 exon 2), p53−/− (3ex) (deletion of p53 exons 2, 3 and 4), p21−/− and RKO p53−/− and p53+/− cells were a gift of B. Vogelstein. Litter-matched p53+/+ and p53−/− MEFs were prepared from embryonic day 14.5 (E14.5) embryos derived from mating p53−/− mice. For starvation, cells were initially grown in DMEM (which contains serine 0.4 mM and glycine 0.4 mM); complete medium (Com) was formulated by supplementing MEM with additional nutrients to closely match the nutrient content of DMEM. Serine- and glycine-deficient medium (−SG) was formulated in the same way as complete medium, without addition of serine and glycine. Bilateral subcutaneous injections of HCT116 cells were carried out on CD-1-Foxn1nu female mice (Charles River); p53+/− on right flank and p53−/− (1ex) on the left. Following injection, mice were placed either on control diet (containing serine and glycine as part of the amino-acid mix) or diet deficient in serine and glycine (TestDiet, International Product Supplies). The diets had equal caloric value and equal total amino acid content. Animals were housed in sterile IVC cages, monitored thrice weekly and killed humanely when tumours reached clinical endpoint of predetermined size (volume = (length × width2)/2) or ulceration. All animal work was undertaken in line with the UK Animals (Scientific Procedures) Act of 1986 and the EU directive 2010. Cell-cycle analysis was performed by labelling with 5-bromo-2′-deoxyuridine (BrDU) and propidium iodide (PI) staining followed by flow cytometry. Oxygen consumption rates were measured with an XF-24 extracellular flux analyser (Seahorse Bioscience). ROS levels were detected with CellROX deep red reagent (Invitrogen). ATP levels were assayed with a luciferase-based kit. The relative quantities of intracellular metabolites after labelling with [U-13C]glucose or [U-13C,15N]L-serine were analysed by LC-MS and plotted as the peak area for each metabolite.

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

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Author Contributions K.H.V. and O.D.K.M. conceived the project and wrote the manuscript with C.R.B.’s help. C.R.B. and L.Z. performed and optimized LC–MS, C.R.B. and O.D.K.M. analysed LC–MS raw data. E.G. contributed to the design and interpretation of LC–MS experiments. K.B. and S.M.M. carried out the xenograft experiment, from which K.B. and O.D.K.M. analysed the data. O.D.K.M. performed all other experiments and data analysis. All the authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.H.V. (k.vousden@beatson.gla.ac.uk).
Cell culture. Unless otherwise stated, chemicals were obtained from Sigma-Aldrich, and cell culture reagents from Gibco (Invitrogen). HCT116 p53−/−/p21−/− (parental), p53−/− (1ex) (deletion of p53 exon 2), p53−/− (3ex) (deletion of p53 exons 2,3 and 4), p21−/− and RKO p53−/− and p53+/− cells were a gift of B. VogelsteinL. Litter-matched wild-type and p53−/− MEFs (passage <4) were prepared from E14.5 embryos derived from mating p53−/− mice and p53−/− mice (C57Bl/6J background, >20 gen). Cells were kept at 37 °C in humidified 5% CO2 in air, stock HCT116 and RKO cells were maintained in McCoy’s 5A medium (26600) containing 2 mM l-glutamine and 10% FBS (PAA Laboratories). For experiments cells were seeded in DMEM (21969) with 10% FBS and 2 mM l-glutamine. Complete (Com) medium was formulated to closely match the nutrient composition of DMEM (which contains 0.4 mM serine and 0.4 mM glycine); complete medium consisted of DMEM supplemented with the additional L-1X MEM vitamins (11120), 10% dialysed FBS (Hyclone, Thermo Scientific), l-glutamine 2 mM, additional d-glucose (to 25 mM), serine 0.4 mM and glycine 0.4 mM. For starvation experiments cells were fed the same medium formulation without serine and glycine (–SG medium).

Proliferation assays. HCT116 and RKO cells were seeded in 24-well plates (8 × 104 cells per well), MEFs in 12-well plates (p53−/− 2 × 106 cells per well, p53−/− 1 × 106 cells per well) in DMEM and allowed to grow for 16–24 h. Cells were then washed with PBS and received complete or –SG medium supplemented with the stated nutrients and/or drugs. To maintain constant nutrient levels and remove nutrients liberated from dead cells, medium was replaced every 24 h. Cell counts were performed with a Casy TT cell counter (Innovatis, Roche Applied Science).

RNA for p21 (sc-29427, Santa Cruz Biotechnology, UGCAGAACGGGCUGGGGA and UCCCGAGGCGGUGUUGACCA) or non-targeting control (si-ctr, ON-TARGETplus, Dharmacon D-001801-20-20, UGGUUAACAGU CGACUAA, UGGUUAACAGGUUGUG, UGGUUAACAGUCAUUGU GA and UGGUUAACAGUAAUCCUA) was transfected with Metafectene SI (Bionon) in DMEM for 24 h before washing and adding complete or –SG medium.

Xenografts. Bilateral subcutaneous injections of 3 × 106 HCT116 cells were carried out on 8 week CD-1 Foxn1nu female mice (Charles River); ried out on 8 week CD-1-Foxn1nu female mice (Charles River); unless otherwise stated, chemicals were obtained from Sigma-Aldrich. All experiments were undertaken in line with the UK Animals (Scientific Procedures) Act of 1986 (PPL 60/4181) and the EU directive 2010/63/EU (University of Glasgow) and received the appropriate authority or ethical approval.

METHODS

Oxygen consumption rates (OCR). An XF24 Extracellular Flux Analyzer (SeaHorse Bioscience) recorded OCR. HCT116 cells were grown in XF-plates for 48 h in complete or –SG medium (90–100% confluent at measurement). OCR was recorded after equilibration and after CCCP (0.5 μM) and antimycin (1.5 μM) treatments, these values were used to calculate basal and maximal OCR. All OCR measurements were normalized with well-by-well haemocytometer cell counts.

Peroxide sensitivity assay. HCT116 cells were seeded in 24-well plates in DMEM. After 16–20 h cells were washed with PBS and received complete or serine- and glycine-deficient medium. After 24 h the medium was replaced with matching medium containing the stated concentrations of hydrogen peroxide, after a further 24 h cells were washed and images captured using a low microscope. We noted that cell number had a large impact on peroxide sensitivity; therefore cell seeding was carefully titrated so that cell number was equal across the different experimental conditions at the time of peroxide addition.

Reactive oxygen species detection. CellROX deep red reagent (Invitrogen) was added to cell culture medium for 30 min. For flow cytometry, cells were detached by washing with PBS-EDTA followed by treatment with PBS-EDTA with trypsin (0.025%) and analysed on a FACSCalibur cytometer (BD Bioscience). For confocal microscopy, live cells were imaged using an inverted confocal fluorescence microscope (Fluoview FV1000, Olympus).

Liquid chromatography–mass spectrometry (LC–MS). HCT116 cells (0.5–1.5 × 106) were seeded in triplicate wells of 6-well plates in DMEM, duplicate plates were seeded for cell counts. After 16–24 h cells were washed with PBS and received complete or –SG medium for the stated times. For glucose flux experiments, medium was replaced with HEPES-buffered Krebs–Ringer solution with 25 mM U-13C-glucose (Cambridge Isotopes), 10% dialysed FBS, 1X MEM amino acids (11130), 2X MEM vitamins and 2 mM l-glutamine. For serine flux experiments, medium was replaced with complete medium, with serine substituted for 0.4 mM U-13C-glucose (Cambridge Isotopes). Cells were washed with PBS and metabolites extracted using methanol:acetonitrile (5:32) and received complete medium for 10–20 h. Samples were filtered, thawed at 4 °C, spun at 16 000g for 15 min and supernatants collected and filtered through 0.45 μm PTFE membranes (Millipore). Serum samples were collected at time of killing. 20 μl of serum was added to 980 μl of extraction buffer and prepared as above. LC–MS analyses were performed on an Orbitrap Exactive (Thermo Scientific) in line with an Acella autosampler and an Acella 600 pump (Thermo Scientific). The Exactive operated in the polarity-switching mode with positive voltage 4.5 kV and negative voltage 3.5 kV. Column hardware consisted of a Sequant ZIC-phILIC column (2.1 × 150 mm, 5 μm) coupled to a Sequant ZIC-phILIC guard column (2.1 × 20 mm, 5 μm) (Merck). Flow rate was 100 μl/min, buffers consisted of acetonitrile (ACN) for A, and 20 mM (NH4)2CO3, 0.1% NH4OH in H2O for B. Gradient ran from 80% to 40% ACN in 20 min, followed by a wash at 20% ACN and re-equilibration at 80% ACN. Metabolites were identified and quantified using LCquan software (Thermo Scientific). Metabolites were positively identified on the basis of exact mass within 5 p.p.m., further validated by concordance with standard retention times and plotted as the peak area for each metabolite.

Quantitative PCR. Primers: PKG1 forward: CTGTTTGCTTCTGGCATACCT, PKG1 reverse: CGAAGTGACACCGTCTACGATA; PHGDH forward: ATTCCTCA CGGGGTTTGTGG, PHGDH reverse: AGGCTGGCATGACTGTCG, PSAT1 forward: CCGTCCTGGAATACAAAGTGTG, PSAT1 reverse: ACAAAGGCGGCTTGTA, PHGDH forward: GAGCCGACTCCCTTATTAAOG, PHGP1 reverse: AGGCTGCAATGCACACT, PHGDH forward: GGGCTGCACTTTCACGGG, PHGDH reverse: AATTGCCGAGTTTGGATACGTA, actin forward: ACTCTCTCGGAGAGGAGG, actin reverse: ATTCCTTTTCTGTGAGTACT.

Chromatin-immunoprecipitation. Assays were performed as described previously. Cells (6–8 × 106) were seeded in 6-well plates in DMEM, allowed to grow for ~40 h, then washed with PBS and received complete or –SG medium for 15 h, hydroxyurea (HU, 0.4 mM) was added as a positive control.

Cell-cycle analysis. For sub-G1 analysis, cells were grown and detached from plates as described above, then fixed and stained with propidium iodide (PI, 50 μg/ml) for 30 min. For cell-cycle analysis BrDU (Invitrogen) was added to live cells for 100 min and detected as described previously. Flow cytometry was performed on a FACSCalibur cytometer.

Pl exclusion. PI was added to cell culture medium (1 μg/ml) for 5 min. Non-adherent and adherent cells were collected and analysed by Flow cytometry on a FACSCalibur cytometer.
ATP assay. Aliquots of cell suspension were added to TE buffer (pH 7.75) heated to 99 °C and assayed with an ATP determination kit (Invitrogen) on a Veritas Microplate luminometer (Turner Biosystems). Cell suspensions were counted to normalize for cell number.

Statistics. Survival was assessed by non-parametric distribution analysis (right censoring), using log-rank and Wilcoxon tests calculated on Minitab 16 (Minitab Ltd). The following t-test comparisons were performed with Microsoft Excel (v12.3.4): tumour volume between diet groups; unpaired, one tail. Tumour volume within diet groups; paired, one tail. Serum amino acids between diet groups; unpaired, one tail. Oxygen consumption rate within genotype; paired, one tail. Oxygen consumption rate between genotypes; unpaired, one tail.

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