FoxO3 coordinates metabolic pathways to maintain redox balance in neural stem cells

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Forkhead Box O (FoxO) transcription factors act in adult stem cells to preserve their regenerative potential. Previously, we reported that FoxO maintains the long-term proliferative capacity of neural stem/progenitor cells (NPCs), and that this occurs, in part, through the maintenance of redox homeostasis. Herein, we demonstrate that among the FoxO3-regulated genes in NPCs are a host of enzymes in central carbon metabolism that act to combat reactive oxygen species (ROS) by directing the flow of glucose and glutamine carbon into defined metabolic pathways. Characterization of the metabolic circuit observed upon loss of FoxO3 revealed a drop in glutaminolysis and filling of the tricarboxylic acid (TCA) cycle. Additionally, we found that glucose uptake, glucose metabolism and oxidative pentose phosphate pathway activity were similarly repressed in the absence of FoxO3. Finally, we demonstrate that impaired glucose and glutamine metabolism compromises the proliferative potential of NPCs and that this is exacerbated following FoxO3 loss. Collectively, our findings show that a FoxO3-dependent metabolic programme supports redox balance and the neurogenic potential of NPCs.

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Introduction

Stem cells maintain tissue homeostasis by replacing damaged or worn-out cells and the deterioration of stem-cell functions, including self-renewal capacity, is one of the key components of organismal ageing (Janzen *et al*, 2006; Molofsky *et al*, 2006; Rossi *et al*, 2007, 2008). Distinct metabolic programmes in stem cells are necessary to protect genomic stability and to

generate precursors for macromolecular synthesis to facilitate continued self-renewal. Reactive oxygen species (ROS) may contribute to the functional decline in stem cells by inflicting chronic damage to cellular macromolecules, including genomic DNA, which accumulates during cellular division, ultimately leading to cytostasis or cytotoxicity (Rossi *et al*, 2008). In addition, excessive ROS drives stem cells out of quiescence and eventually lead to depletion of stem-cell reserves (Rossi *et al*, 2008). Animal models of precocious stem-cell depletion or dysfunction consistently emphasize the role of key molecules involved in oxidative defense in maintaining stem-cell reserves: *Atm* (Ito *et al*, 2004), *Tsc1* (Chen *et al*, 2008), *Prdm16* (Chaikov *et al*, 2010), and *FoxO3* (Miyamoto *et al*, 2007; Yalcin *et al*, 2008; Paik *et al*, 2009; Renault *et al*, 2009). Furthermore, stem cells have intrinsic antioxidant and stress-resistance systems that maintain low levels of ROS (Ivanova *et al*, 2002; Ramalho-Santos *et al*, 2002).

As a metabolic by-product, endogenous ROS is intimately tied to cellular metabolic activity. Mitochondria are the primary source for ROS production through oxidative phosphorylation. While glucose is generally regarded as a major substrate of aerobic oxidation, recent studies indicate that other nutrients, including glutamine (Gln), are metabolized into intermediates of the tricarboxylic acid (TCA) cycle and therefore may drive mitochondrial oxidative phosphorylation and ROS production (DeBerardinis *et al*, 2007). On the other hand, metabolic programmes also tightly regulate cellular defense against oxidative stress. One such metabolism-dependent antioxidant defense is glutathione (GSH) production. While the availability of amino acids such as Gln, glutamate (Glu), and cysteine regulates the biosynthesis of cellular GSH, intracellular NADP(+)/NADPH level controls the oxidative state of GSH (Beatty and Reed, 1980; Whillier *et al*, 2011). Under physiological conditions, the oxidative pentose phosphate pathway (PPP) generates reducing potential in the form of NADPH using the glucose metabolite glucose-6-phosphate (G6P). As such, the shunting of glucose carbon into the PPP plays an important role in the maintenance of redox homeostasis (Pandolfi *et al*, 1995). In fact, metabolic anti-oxidant defense programmes respond to and are activated by cellular ROS levels. For example, a recent study demonstrated that ROS disrupts the active tetrameric state of pyruvate kinase M2 (PKM2), a rate-limiting glycolytic enzyme that catalyses the reaction generating pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP, through the direct oxidation of Cys358. The inactivation of PKM2 creates a bottleneck at the end of glycolysis thereby redirecting glycolytic metabolites into the PPP and forming a feedback redox balancing mechanism that generates reducing potential in the form of NADPH (Anastasiou *et al*, 2011).

Among the many molecular determinants of ageing and oxidative stress responses, the PI3K-AKT-FoxO signalling pathway plays a central role. To date, studies from experimental model organisms have demonstrated primary roles of FoxO in dietary restriction-induced longevity and suppression

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of ROS (Kops *et al*, 2002; Nemoto and Finkel, 2002; Greer *et al*, 2007). The latter serves to maintain the homeostasis of adult tissue stem cells and partly explains the core mechanism of lifespan extension by activated FoxO (Miyamoto *et al*, 2007; Tothova *et al*, 2007). For example, haematopoietic stem cells (HSCs) deficient for multiple FoxO isoforms showed a decrease in the expression of ROS-detoxifying enzymes, such as catalase and MnSOD (Tothova *et al*, 2007). Furthermore, we demonstrated that loss of FoxO function led to a transient increase in proliferation followed by progressive loss of self-renewal in neural stem/progenitor cells (NPCs), a phenotype tightly associated with excessive ROS (Paik *et al*, 2009). However, the mechanisms through which FoxO controls metabolic programmes that maintain redox potential, and therefore sustains stem-cell reserves, remain to be determined.

To understand FoxO-mediated metabolic regulation of redox homeostasis, we set out to dissect the metabolic alterations induced by the loss of FoxO3, the predominant FoxO isoform expressed in NPCs. By combining global analysis of metabolites with tracing experiments, we identified glycolysis and Gln metabolism as two major metabolic modules affected by FoxO3 deficiency. Impaired utilization of the Gln carbon skeleton contributes to oxidative stress that in turn downregulates PKM2 activity. At the same time, FoxO3 deficiency leads to downregulation of glucose uptake and depression of oxidative PPP activity. Collectively, these metabolic alterations contribute to a more oxidative cellular environment that may lead to the progressive accumulation of oxidative damage. In addition to previously characterized MnSOD or Catalase-dependent protective functions of FoxO, our study demonstrates an unexpected role of FoxO3 in the maintenance of metabolic homeostasis in NPCs that counteracts oxidative stress and preserves their long-term proliferative potential.

Results

Glutamine metabolism, glucose metabolism, and FoxO3 suppress ROS in NPCs

We previously reported that FoxO-null NPCs (derived from FoxO1/3/4 combined KO, *hGFAP-Cre* + : *FoxO1/3/4^{L/L}* mice) show an increase in intracellular ROS and a decrease in self-renewal potential relative to wild-type (WT) controls (Paik *et al*, 2009). In order to gain mechanistic insight, we examined associated changes in cellular functions and pathways. First, genes differentially regulated in FoxO-null NPCs were analysed using gene set enrichment analysis (GSEA). Metabolic gene sets for KEGG pathways (i.e., arginine and proline metabolism, glycolysis/gluconeogenesis, and pentose phosphate pathways) were significantly enriched as functional

categories (Supplementary Figure S1A–C). Independent Ingenuity Pathway Analysis identified glutamate and pyruvate metabolism among the most significantly affected pathways in FoxO-null NPCs, adding additional pathways to the list of FoxO-dependent metabolic alterations (Supplementary Figure S1D). On the basis of these results, we pursued the function of FoxO in NPC metabolism and focussed on the role of FoxO3, the most predominantly expressed FoxO isoform in NPCs (Paik *et al*, 2009). Consistent with previous reports FoxO3 KO NPCs exhibited increased mitochondrial abundance and respiration, presumably leading to the observed accumulation of mitochondrial superoxide (Supplementary Figure S2A–C) (Jensen *et al*, 2011; Ferber *et al*, 2012). Notably, the expression of MnSOD did not change and only a few ROS-detoxifying enzymes downregulated in FoxO3 KO NPCs (Supplementary Figure S2D). ROS accumulation is closely associated with increased production by mitochondria as well as with the rate of clearance that are mediated by metabolic and/or transcriptional programmes. As transcriptional MnSOD regulation was not altered despite the elevated mitochondrial superoxide level, we questioned whether metabolic ROS clearance is compromised in FoxO3 KO NPCs. First, we determined the contribution of glucose and Gln metabolism to ROS production at different time points after lowering glucose (25 mM to 1 mM) and/or Gln (2 mM to 0.2 mM). Depletion of Gln profoundly increased ROS production in both WT and FoxO3 KO NPCs. Additionally, both lowering glucose concentration and FoxO3 deficiency elevated ROS levels under all conditions (Figure 1A). These data suggest that Gln and glucose metabolism as well as FoxO3 expression is important for suppression of ROS. Of note, the cells used for *in vitro* analyses are referred to as NPCs, based on the heterogeneity resulting from 3D culture conditions (Reynolds and Rietze, 2005).

Decreased glutaminolysis in FoxO3 KO NPC

Gln metabolism can control the redox balance through a number of mechanisms; among the most well characterized are its contribution as Glu to GSH biosynthesis and/or the generation of reducing potential in the form of NADPH from cytosolic isocitrate dehydrogenase 1 (IDH1) or malic enzyme 1 (ME1) activity (Ashcroft and Randle, 1970; MacDonald and Marshall, 2001; Son *et al*, 2013). Given the increase in oxidative stress observed in FoxO3 KO NPCs, which is exacerbated following Gln withdrawal, we investigated FoxO3-mediated changes in Gln metabolism. To trace Gln metabolism, we grew FoxO3 KO NPCs in growth media containing uniformly ¹³C-labelled Gln [¹³C₅]-Gln and analysed the Gln metabolome by metabolomic profiling after steady-state labelling. Importantly, we used WT and FoxO3 KO NPCs with comparable growth kinetics (4–7 times passaged)

Figure 1 Decreased glutaminolysis in FoxO3 KO NPCs. WT and FoxO3 KO NPCs were cultured with high (25 mM) or low (1 mM) glucose and high (2 mM) or low (0.2 mM) Gln containing media for the indicated times. (A) Intracellular ROS level was measured by DCF-DA staining and (J) the NADP/NADPH ratio was measured. Error bars represent $\pm$ s.d. values of the mean. ¹³C-Gln-derived metabolite pools (B) and total metabolite pools (C) in FoxO3 KO NPCs were measured using LC-MS/MS ($n = 3$). Mean $\pm$ s.d. values are shown. (D) GLS and (E) GLUD activity is assayed in WT and FoxO3 KO NPCs. Mean $\pm$ s.d. values are shown. Gln-derived Glu tracing into GSH (¹³C-GSH) and GSSG (¹³C-GSSG) was analysed by targeted LC-MS/MS (F) as well as the level of GSH and the GSH/GSSG ratio (G) were measured in WT and FoxO3 KO NPCs. Mean $\pm$ s.e. values are shown. (H) Intracellular ROS level was measured by DCF-DA staining in WT and FoxO3 KO NPCs cultured with Gln-free media and treated with Gln metabolites (2 mM Gln, 4 mM Glu, or 2 mM GSH ethyl ester) for 16 h. Mean $\pm$ s.e. values are shown. (I) Intracellular ROS level was measured by DCF-DA staining in FoxO3 KO NPCs overexpressing GLS1 and GLUD1 or knocking down for GLS1. The expression of GLS1 and GLUD was confirmed by V5 immunoblotting and RT-qPCR, respectively. Error bars represent $\pm$ s.d. values of the mean, and comparison was made with one-way ANOVA. * $P < 0.05$, ** $P < 0.01$

and that retained >99% nestin expression to avoid an indirect consequence of compromised proliferation and aberrant differentiation, which can influence anaplerosis of Gln (DeBerardinis *et al*, 2007). Interestingly, FoxO3 KO NPCs exhibited decreased glutaminolysis as evidenced by decreased abundance of Gln-derived metabolic intermediates

(Figure 1B). Consistently, the total metabolite pools for many of the TCA cycle intermediates were universally decreased (Figure 1C). This observation suggests that FoxO3 KO cells utilize less Gln for anaplerotic filling of the TCA cycle. Collectively, our results demonstrate that loss of FoxO3 impairs metabolism of imported Gln in NPCs.

Importantly, we did not observe a difference in Gln uptake, expression levels of the Gln transporters ASCT2 and LAT1, or intracellular Gln abundance between WT and FoxO3 KO NPCs (Figure 1B and C; Supplementary Figure S3A). These data suggest that FoxO3 may function downstream of Gln uptake, where FoxO3 loss impairs glutaminolysis. To test this hypothesis, we examined the expression of glutaminase (GLS), which converts Gln into Glu, and glutamate dehydrogenase 1 (GLUD1), which converts Glu into α -ketoglutarate (α -KG). The expression levels of *Gls1* and *Gls2* were not altered, whereas that of *Glud1* decreased in FoxO3 KO NPCs compared with WT control (Figure 1D and E; Supplementary Figure S3A). These results suggest that the decreased turnover of Gln to Glu may be due to decreased activity, rather than decreased expression, of GLS. In order to determine whether GLS activity is affected by FoxO deficiency, we examined its enzymatic activity in NPCs. Compared with WT NPCs, ablation of FoxO3 suppressed GLS activity (Figure 1D). Furthermore, GLUD1 activity is also downregulated, though this presumably results from decreased expression (Figures 1E and 5B).

Elevated oxidative stress in FoxO3 KO NPCs

Gln is a crucial metabolite in the defense against oxidative stress (Mates *et al*, 2002), as Gln-derived Glu can serve as a precursor for the biosynthesis of GSH (DeBerardinis and Cheng, 2010). Consistent with the decrease in glutaminolysis, we also observed less Gln-derived Glu in GSH in FoxO3 KO NPCs, as evidenced by [U - $^{13}C_5$] Gln tracing (Figure 1F). In steady state, total GSH was reduced whereas GSSG remained similar leading to reduced GSH/GSSG ratios in FoxO3 KO NPCs (Figure 1G). In order to determine whether ROS accumulation is due to decreased GSH level when Gln is depleted, we treated Gln-starved cells with a cell permeable analogue of GSH. This suppressed the Gln-deprivation induced ROS in both WT and FoxO3 KO NPCs, suggesting its major antioxidant role as a downstream metabolite of Gln (Figure 1H). In addition, supplementing the culture with the GSH precursors Gln or Glu suppressed the ROS level in WT NPCs. In FoxO3 KO NPCs, however, the addition of Gln was not as effective as Glu or GSH in suppressing ROS, consistent with suppressed glutaminolysis through decreased GLS activity (Figure 1D and H). In order to determine the importance of glutaminolysis in anti-oxidative metabolism in FoxO3 KO NPCs, we modulated GLS1 and GLUD1 expression. As shown in Figure 1I, further down-regulation of GLS1 increased ROS compared with control FoxO3 KO NPCs, while ectopic expression of GLS1 or GLUD1 attenuated ROS accumulation. Collectively, our results suggest that decreased glutaminolysis contributes to exacerbated oxidative stress in FoxO3 KO NPCs, and that this may be through decreased Glu production and GSH biosynthesis.

FoxO3 regulates glucose metabolism to maintain NADP/NADPH

Next, we examined the ratio of NADP and NADPH, which is the major determinant of reduced GSH level. While Gln deprivation did not have an appreciable effect on the NADP/NADPH ratio, reducing glucose levels significantly increased NADP/NADPH. This effect was more significant in FoxO3 KO than in WT NPCs (Figure 1J), suggesting that

FoxO3-dependent glucose, but not Gln metabolism, is critical for maintaining NADP/NADPH levels. Indeed, one of the central pathways controlling the NADP/NADPH ratio under physiological conditions is the oxidative arm of the PPP. Together, these data suggest that the reduction in the GSH-to-GSSG ratio is due to both a decrease in the Gln-derived GSH and a decrease in glucose-dependent NADPH generation in FoxO3 KO NPCs.

The role of FoxO3 in stem-cell glucose metabolism has not yet been defined. In order to determine how the lack of FoxO3 affects glucose utilization in NPCs, we measured key steps of glycolysis. Surprisingly, FoxO3 KO NPCs showed decreased glucose uptake as confirmed by 2-NBDG uptake analysis (Figure 2A). To understand the basis of glucose metabolic alterations induced by FoxO3 inactivation in NPCs, we performed global metabolite profiling of polar metabolites in FoxO3 WT and KO NPCs (Ying *et al*, 2012; Yuan *et al*, 2012). We observed a decrease in upstream glycolytic intermediates (i.e., G6P and F6P) and accumulation of downstream metabolites (i.e., BPG and 3PG) with a seven-fold accumulation of PEP, indicating inhibition of the rate-limiting glycolytic step (Figure 2B and C).

Next, we set out to determine the molecular basis of decreased glucose uptake. First, neither the expression of glucose transporters nor the rate-limiting enzyme responsible for phosphorylating and trapping glucose in the cell, hexokinase (HK) 1 or 2, was decreased in FoxO3 KO NPCs (Figure 2D; Supplementary Figure S3A). Rather, FoxO3 KO NPCs showed a decreased glucose metabolism resulting from lower HK activity (Figure 2E). Previous studies have shown that activation of the PI3K-AKT pathway promotes the translocation of HK2 to the outer mitochondrial membrane, thereby increasing its activity (Bustamante and Pedersen, 1977; Gottlob *et al*, 2001). Given the significant decrease in pAKT in FoxO3 KO NPCs, we examined the mitochondrial localization of HK2 in WT and FoxO3 KO NPCs by co-staining with Mitotracker. FoxO3 KO NPCs showed decreased HK2 translocation to mitochondria compared with WT NPCs consistent with the attenuation of HK2 activity (Figure 2F).

To understand the mechanism how the loss of FoxO3 decreases AKT phosphorylation, we tested the role of *Pik3ca* (p110 α) and *Rictor*, upstream activators of AKT. Expression of both *Pik3ca* and *Rictor* was decreased in FoxO3 KO NPCs (Figure 2G). Consistently, the expression of a constitutively active form of FoxO3 (ca-FoxO3) in FoxO3 KO NPCs robustly induced phosphorylation of AKT, which was accompanied by strong upregulation of both *Pik3ca* and *Rictor* expressions (Figure 2G and H). In order to determine the contribution of these signal transducers to AKT activation, we treated cells with PI3K inhibitors BYL719 (p110 α specific) or BKM120 (pan) and inhibited mTORC2 with rapamycin (Sarbasov *et al*, 2006; Young *et al*, 2013). Inhibition of PI3K by BYL719 and BKM120 attenuated the increased pAKT observed in ca-FoxO3 expressing FoxO3 KO cells, whereas rapamycin did not (Figure 2H). Our results suggest that FoxO3 activates PI3K-AKT signalling as a feedback regulatory response that is mediated through PIK3CA. Next, NPCs expressing ca-FoxO3, and therefore activated AKT, showed partially restored HK activity compared with FoxO3 KO NPCs (Figure 2I). In agreement, dominant-negative AKT (DN-AKT) expression caused a decline in glucose uptake in WT NPCs

Figure 2 Decreased glucose metabolism in FoxO3 KO NPCs. (A) Uptake of 2-NBDG for 2 h analysed by flow cytometry in WT and FoxO3 KO NPCs. Values are mean $\pm$ s.d. (B) Schematic summary of changes observed in glucose metabolism in FoxO3 KO NPCs and (C) the corresponding data were measured by LC-MS/MS. Error bars represent $\pm$ s.d. values of the mean. (D) The indicated protein expression in WT and FoxO3 KO NPCs was analysed by immunoblotting. Ratios of pAKT band intensities from a representative experiment are presented. (E, I) HK activity was assayed in WT and FoxO3 KO NPCs (E) and ca-FoxO3 adenovirus-infected FoxO3 KO NPCs (I). The values are mean $\pm$ s.d. (F) Mitochondrial translocation of HK2 (green) stained with Mitotracker (red) was determined as percent co-localization (doubly green/red pixels) in WT and FoxO3 KO NPCs. Clotrimazole (CTZ) treatment was used to disrupt HK2 localization from the mitochondria. Error bars represent $\pm$ s.d. values of the mean. (G) mRNA expression of *Pik3ca* and *Rictor* was measured by RT-qPCR analysis in WT and FoxO3 KO NPCs and GFP or ca-FoxO3 adenovirus-infected FoxO3 KO NPCs. The values are mean $\pm$ s.e. (H) GFP or ca-FoxO3 adenovirus-infected FoxO3 KO NPCs were treated with 10 μ M BYL719 (BYL) and 3 μ M BKM120 (BKM) for 3 h or with 100 nM Rapamycin (Rapa) for 24 h, and the expression of the indicated proteins was analysed by immunoblotting. (J, K) The uptake of 2-NBDG and HK activity was measured in either control GFP (Ad-GFP) or NPCs expressing dominant-negative AKT1 (DN-AKT) after adenoviral infection. Values are mean $\pm$ s.d. of fold change. DN-AKT1 expression was confirmed by immunoblotting of HA epitope. * P < 0.05, ** P < 0.01. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose biphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; B(1,3)PG, 1,3-bisphosphoglycerate; B(2,3)PG, 2,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate. Source data for this figure is available on the online supplementary information page.

(Figure 2J), and significantly reduced HK activity (Figure 2K). These data suggest that the decreased AKT and HK activity is at least partially responsible for the decreased glucose uptake in FoxO3 KO NPCs.

ROS inhibits PKM2 activity in FoxO3 KO NPCs

Notably, the level of PEP in FoxO3 KO NPCs was increased seven-fold, suggesting that the rate-limiting enzyme PK, which converts PEP into pyruvate, was inhibited. Indeed,

PK activity was reduced to 70% in FoxO3 KO NPCs compared with WT controls, clearly suggesting the inhibition of this rate-limiting step of glycolysis (Figure 3A). Furthermore, expression of ca-FoxO3 partially restored the PK activity in FoxO3 KO NPCs, suggesting that FoxO3-dependent cellular changes are necessary to maintain PK activity in NPCs (Figure 3B).

PK exists as four isozymes (PK-L, PK-R, PK-M1, and PK-M2) encoded by two genes, *PKLR* and *PKM*. Among these, PKM2 is predominantly expressed in murine NPCs (Figure 3C). In particular, PKM2 is sensitive to oxidizing agents and prone to oxidative modification preventing the formation of active tetramers (Anastasiou *et al*, 2011). In agreement, pretreatment with diamide (thiol oxidizing) or glutathione-depleting buthionine sulfoximine (BSO) oxidants suppressed PKM2 activity, confirming that PKM2 is inhibited by an oxidizing environment in NPCs (Figure 3D). Importantly, PKM2 activity was reduced in FoxO3 KO NPCs, while its protein and mRNA expression were maintained (Figure 3A and C). Thus, we examined PKM2 multimer formation in FoxO3 knock-down (KD) 293T cells. Importantly, FoxO3 KO in this system manifests increased ROS accumulation (Supplementary Figure S2B). Our results showed decreased interaction between endogenous PKM2

and Flag-tagged PKM2 subunits in FoxO3 KD (Figure 3E). These results form the basis for our hypothesis that accumulated ROS inhibits PKM2 multimerization and activity in FoxO3 KO NPCs.

Given that our data indicate that deficiency in Gln metabolism leads to increased ROS, we queried the role of glutaminolysis-dependent anti-oxidant capacity in maintaining PK activity in NPCs. In order to examine the contribution of GLS1 activity in sustaining PKM2 activity, we evaluated the effect of 968 and BPTES, potent and selective allosteric GLS1 inhibitors (Robinson *et al*, 2007; Wang *et al*, 2010). Inhibition of GLS1 with 2.5 μ M 968 as well as 0.1 and 1 μ M BPTES increased ROS (Figure 3F). In these cells, PK activity was significantly inhibited, suggesting that glutaminolysis-dependent maintenance of redox potential is necessary to maintain PKM2 activity (Figure 3G). Taken together, these results strongly illustrate that the suppression of glutaminolysis in FoxO3 KO NPCs leads to increased ROS that impairs PKM2 activity.

The oxidative arm of the PPP is impaired in FoxO3 KO NPCs

Inhibition of PKM2 has been shown to promote the redirection of glucose into the oxidative arm of PPP in a feedback

Figure 3 Increased ROS inhibits PK activity in FoxO3 KO NPCs. PK activity was assayed in WT and FoxO3 KO NPCs (A) and FoxO3 KO NPCs expressing ca-FoxO3. (B) Values are shown as mean $\pm$ s.d. (C) mRNA expression of PKM1 and PKM2 was measured by RT-qPCR analysis, and protein levels of PKM2, total PKM, and PKLR were examined by immunoblotting. (D) PK enzyme activity was determined after treating cells with 250 μ M diamide or 1 μ M BSO (G) and 0.1 or 1 μ M BPTES or 2.5 μ M 968 in WT NPCs. Values represent mean $\pm$ s.d. and statistical significance was determined by ANOVA. (E) PKM2-Flag expressing WT and FoxO3 KD 293T cell lysates were used for immunoprecipitation with Flag antibody. The interaction of endogenous PKM2 with Flag-tagged exogenous PKM2 was determined by immunoblotting with the PKM2 antibody. Ratio indicates density of endogenous PKM2 co-immunoprecipitated over PKM2 in input. Increased ROS (F) and decreased PK activity (G) in WT NPCs treated with GLS inhibitors. * P < 0.05, ** P < 0.01. Source data for this figure is available on the online supplementary information page.

regulatory loop that suppresses ROS by generating reducing power in the form of NADPH (Anastasiou *et al*, 2011; Gruning *et al*, 2011). Despite having higher ROS, the abundance of PPP metabolites was surprisingly lower in FoxO3 KO NPCs than in control NPCs. These data suggest that ROS-mediated activation of the PPP is impaired in this context (Figure 4A and B). To measure the activity of the oxidative arm of the PPP, we monitored the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ -glucose. The carbon at the 1-position of glucose is selectively lost during metabolism in the oxidative arm of the PPP. As previously reported (Anastasiou *et al*, 2011), diamide treatment increased $^{14}\text{CO}_2$ production (Figure 4C). To determine whether ROS induced by FoxO3 deficiency or Gln depletion enhances glucose flux into the oxidative arm of the PPP, both WT and FoxO3 KO NPCs were grown in Gln-free media. Consistent with metabolite profiling results, FoxO3 KO NPCs did not increase PPP-specific $^{14}\text{CO}_2$ production in the presence of Gln. Gln deprivation increased the oxidative PPP activity of FoxO3 KO NPCs to a lesser degree than that observed in WT cells, suggesting that ROS-dependent glucose flux into PPP is impaired in FoxO3 KO NPCs (Figure 4C). Therefore, we examined expression of enzymes in the oxidative arm of PPP. The levels of phosphogluconate dehydrogenase (*Pgd*) mRNA were reproducibly decreased in FoxO3 KO NPCs, whereas the rest of the enzymes remained unchanged (Figure 4D). PGD mediates the reaction that generates NADPH and its decreased expression likely serves as the bottleneck impairing both NADPH generation from, and total

flux through the oxidative PPP in FoxO3-deficient NPCs. These results suggest that ROS-mediated redirecting of glucose into PPP is less effective in FoxO3 KO NPCs.

FoxO3 transcriptionally regulates a host of metabolic enzymes

In order to understand how FoxO3 depletion impinges on metabolic enzymes, we examined the expression of putative transcriptional targets. We chose significantly affected metabolic pathways based on the GSEA of differentially expressed genes in FoxO3 KO NPCs. The KEGG 'arginine-proline metabolism' pathway, that is downstream of glutaminolysis, was a top-ranked gene set downregulated in FoxO3 KO NPCs. In addition, gene sets for glucose metabolism (KEGG_glycolysis and gluconeogenesis, KEGG_pentose phosphate pathway) were enriched (Supplementary Figure S1A–C). Interestingly, only a small number of ROS-detoxifying enzymes (i.e., Cat and Sesn3) were downregulated and did not serve as a signature for global gene expression changes in FoxO3 KO NPCs (Supplementary Figure S2D). We selected candidate transcriptional targets based on the metabolic changes that are mediated by specific enzymes significantly changed in these gene lists. First, we determined whether FoxO3 occupies promoter regions of these targets by chromatin immunoprecipitation (ChIP) assay (Figure 5A). In doing so, we identified several enzymes (e.g., *Glud1* and *Pgd*) of which upstream sequences are enriched by FoxO3 ChIP (Figure 5A). The gene expression changes were confirmed by qRT-PCR in

Figure 4 Impaired oxidative PPP activity in FoxO3 KO NPCs. (A) The metabolic alterations of the PPP in FoxO3 KO NPCs were measured by LC-MS/MS compared with WT NPCs in triplicate. Relative metabolite abundances were normalized by protein amount with values from WT set as 1. Error bars represent $\pm$ s.d. values of the mean. (B) The schematic of glucose flux to PPP. (C) WT and FoxO3 KO NPCs were cultured in 2 mM Gln-supplemented or Gln-free media and treated with $[1-^{14}\text{C}]$ glucose or $[6-^{14}\text{C}]$ glucose. Diamide (100 μM) was included as a positive control. Released $^{14}\text{CO}_2$ was measured after 3 h, and the rate of $^{14}\text{CO}_2$ production from glucose via the PPP ($1-^{14}\text{C}$ -CO₂) was normalized to TCA cycle-derived $^{14}\text{CO}_2$ ($6-^{14}\text{C}$ -CO₂). Data show mean $\pm$ s.d. ($n = 5$). (D) mRNA expression of oxidative PPP enzymes was measured by RT-qPCR analysis, and the values are mean $\pm$ s.e. * $P < 0.05$, ** $P < 0.005$. GdL6P, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; G3P, glyceraldehyde-3-phosphate; S7P, sedoheptulose-7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; E4P, erythrose-4-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; PRPP, 5-phospho-d-ribosyl α -1-pyrophosphate; G6pd, glucose-6-phosphate-dehydrogenase; PglS, gluconolactonase; Pgd, 6-phosphogluconate dehydrogenase; Rpe, ribulose-5-phosphate 3-epimerase; Rpia, ribulose-5-phosphate isomerase; Tkt, transketolase; Taldo, transaldolase.

Figure 5 FoxO3 transcriptionally regulates metabolic enzymes. (A) ChIP-qPCR analysis of NPCs using normal rabbit-IgG or FoxO3 antibodies; (B) qRT-PCR was performed to confirm FoxO3-dependent target gene expressions. Data are shown as mean $\pm$ s.e. values of relative enrichment based on the chromatin inputs (A) and of relative expressions (B). PDK4, pyruvate dehydrogenase kinase-4; ACSS, acetyl CoA synthetase. * $P < 0.05$, ** $P < 0.005$.

WT and FoxO3 KO NPCs, with an exception of the post-transcriptionally regulated GLS1 (Figure 5B). Our results support that FoxO3 regulates a host of metabolic enzymes for both glucose and Gln metabolism at the transcriptional level.

Attenuated mTOR activation in FoxO3 KO NPCs

Impaired glucose and Gln metabolism, due to both transcriptional and post transcriptional changes, was accompanied by a significant AMPK activation and low intracellular ATP in FoxO3 KO NPCs (Figure 6A and B). In fact, AMPK activation could be suppressed by expressing ca-FoxO3, suggesting that FoxO3-dependent metabolism is necessary to maintain energy homeostasis in NPCs (Figure 6A). mTOR intimately interacts with AMPK as a major switch for cellular growth and proliferation (Inoki *et al*, 2012). In addition, recent studies have reported reciprocal interaction between glutaminolysis and mTOR activation (Duran *et al*, 2012; Csibi *et al*, 2013). In order to determine whether impaired glutaminolysis and activated AMPK correlate with mTOR pathway activity, we surveyed downstream effectors of mTOR. Decreased phosphorylations of the downstream effectors 4EBP1 and S6 were observed in FoxO3 KO NPCs, consistent with the activation of AMPK antagonizing mTOR activity (Figure 6C). Notably, there was marked elevation of phosphorylated p42/44 MAPK and p70S6K, in accordance

with the known role of FoxO in suppressing the Ras-MAPK pathway (Paik *et al*, 2007) (Figure 6C). Activation of p42/44 MAPK was sensitive to an MEK inhibitor, PD98059, suggesting that hyperactivation of p70S6K is largely mediated by MEK rather than by mTOR activation in FoxO3 KO NPCs (Figure 6D). Accordingly, p70S6K-dependent phosphorylation of mTOR (S2448) was increased (Figure 6E) (Chiang and Abraham, 2005; Holz and Blenis, 2005).

Consistent with other cell types, phosphorylation of S6 was dependent in part on the availability of Gln in NPCs. Attenuated levels of pS6 were consistent with suppressed glutaminolysis, a finding that agrees with a previous report showing no increase of cell size in FoxO3 KO NPCs (Figure 6F) (Paik *et al*, 2009). Next, we examined the lysosomal localization of mTOR as a readout of glutaminolysis-dependent activation (Duran *et al*, 2012; Kim *et al*, 2013). FoxO3 KO NPCs exhibited reduced localization of mTOR to LAMP1-positive lysosomes, indicating decreased activation (Figure 6G). Together, the decreased PI3K-AKT activity, AMPK activation, and glutaminolysis agree with attenuated mTOR activation. Additionally, activation of MAPK-p70S6K may further suppress IRS1-dependent PI3K-AKT activity in FoxO3 KO NPCs (Figure 6H).

A recent study demonstrated that the activation of FoxO3 upregulated the expression and enzyme activity of glutamine synthetase (GS), leading to an induction of autophagy by

Figure 6 PI3K-AKT-mTOR signalling is attenuated in FoxO3 KO NPCs. (A) The levels of AMPK activation were measured by immunoblotting in GFP or ca-FoxO3 adenovirus-infected FoxO3 KO NPCs (left two lanes) and WT and FoxO3 KO primary NPCs derived from three mice (right three lanes). Representative blots are shown. (B) ATP was determined by mass spectrometry in WT and FoxO3 KO NPCs. Mean $\pm$ s.d. of fold change values are shown. $**P < 0.01$. (C) Immunoblot of WT and FoxO3 KO NPC lysates for indicated proteins. All the panels were probed on the same membrane. (D) Activation of p42/44 MAPK is sensitive to 10 μ M PD98059 in FoxO3 KO NPCs. (E) The components of mTOR pathway were analysed by immunoblotting in WT and FoxO3 KO NPCs. (F) Phosphorylation of S6 is responsive to the availability of Gln. NPCs (left) and HeLa cells (right) grown in the presence and absence of 2 mM Gln for 24 h. Representative blots from multiple experiments are presented. (G) Lysosomal localization of mTOR in WT and FoxO3 KO NPCs. Percent of mTOR overlapping with LAMP1 was increased in FoxO3 KO NPCs. Representative images are shown. Insets are higher magnification images of boxed regions. Bar = 5 μ m. (H) Schematic summary of changes in PI3K-AKT-mTOR signalling in FoxO3 KO NPCs. Molecules shown to be activated (red) or suppressed (blue) in this study are depicted. Spry, Sprouty. Source data for this figure is available on the online supplementary information page.

inhibiting the mTOR signalling pathway (van der Vos *et al*, 2012). Similarly, we observed $\sim 40\%$ decrease in GS expression in FoxO3 KO NPCs (Supplementary Figure S3A). However, neither activation of the mTOR pathway nor inhibition of downstream autophagy transducer ULK1 was observed in FoxO3 KO NPCs, suggesting a cellular context-dependent regulation of mTOR by GS (Figure 6E).

Altered metabolism contributes to the decreased proliferative potential of FoxO3 KO NPCs

In order to determine the contribution of dysfunctional metabolic programmes to the self-renewal capacity of FoxO3 KO NPCs, we tested the role of FoxO-regulated metabolic pathways in the proliferative potential of NPCs by measuring neurosphere formation. As previously demon-

strated, FoxO3 KO NPCs showed decreased neurosphere forming capacity during the extended culture period (Figure 7A). Next, we determined the proliferative potential of WT and FoxO3 KO NPCs grown in high or low Gln- or glucose-containing media. Similarly to the increase in ROS levels, the number of neurospheres was decreased more significantly by lowering Gln than glucose. Notably, this effect was more profound in FoxO3 KO NPCs and inversely correlated with intracellular ROS levels (Figures 1A and 7B). These results suggest that impaired Gln and glucose metabolism suppress the proliferative potential of FoxO KO NPCs under growth conditions elevating ROS.

Next, we determined the dose-dependent effect of the GLS1 inhibitors BPTES and 968 on the neurosphere formation and GLS activity in WT and FoxO3 KO NPCs. BPTES and 968

Figure 7 Defective glucose and Gln metabolism decreases proliferative potential of FoxO3 KO NPC. (A) Neurosphere formation was assayed in (A) WT and FoxO3 KO NPCs (B) cultured under high (25 mM) or low (1 mM) glucose and high (2 mM) or low (0.2 mM) Gln conditions. Values represent mean $\pm$ s.d. WT and FoxO3 KO NPCs were treated with indicated concentrations of BPTES (C, D) or 968 (E, F) and the number of neurospheres was determined after 14 days of culture (C, E) and GLS activity was assayed (D, F). Values represent $\pm$ s.d. values of the mean. (G) Mock, ca-FoxO3, WT-AKT, or PGD-V5 viruses-infected FoxO3 KO NPCs were cultured and the number of neurospheres was determined as above. The expression of virus-encoded proteins was confirmed by immunoblotting. Neurospheres larger than 130 μ m in diameter were scored. * P < 0.05, ** P < 0.005.

suppressed neurosphere formation in both WT and FoxO3 KO NPCs. Notably, FoxO3 KO NPCs exhibited higher sensitivity towards GLS inhibitors. This effect was consistent with heightened inhibition of GLS activity, confirming that self-renewal of NPCs requires glutaminolysis (Figure 7C–F). Finally, we enforced expression of ca-FoxO3, AKT, and PGD to overcome the metabolic deficiency in FoxO3 KO NPCs. Such restoration increased neurosphere formation (Figure 7G), providing further evidence in support of the notion that the PI3K-AKT-FoxO3-dependent metabolic programme is necessary for the proliferative potential of FoxO3 KO NPCs.

In addition, we attempted to rescue previously characterized defects of FoxO-null NPCs *in vivo* based on the

forementioned mechanistic findings. We administered N-acetyl cysteine (NAC) to the brain-specific FoxO-null mice (Paik *et al*, 2009) to suppress the chronic effect of ROS in NPCs (Supplementary Figure S4A). FoxO-null mice brains showed a decreased GSH/GSSG ratio that was restored to the level of WT upon feeding with NAC (Supplementary Figure S4B). Moreover, nitro-tyrosine-positive spots, which are indicative of ROS-modified protein adducts, were more prevalent in FoxO-null brains, while NAC-fed animals showed reduced staining (Supplementary Figure S4C). Congruent with above-measured oxidative stress indices and our neurosphere analysis, long-term NAC-fed FoxO-null mice showed increased numbers of NPC and doublecortin

(DCX)-positive neuroblasts in the subventricular zone, comparable to age-matched WT littermate control animals (Supplementary Figure S4D–F). Collectively, our *in vitro* and *in vivo* results support the notion that metabolic abnormalities and elevated oxidative stress in FoxO-null NPCs may serve as causes of undue loss of NPCs and accompanying neurogenesis.

Discussion

The role of FoxO in organismal metabolism has long been appreciated. However, the mechanisms by which FoxO regulated metabolism in adult stem cells, and especially those that counteract ROS accumulation, remained to be established. In this study, we determined that FoxO3 deficiency caused an unexpected suppression of Gln and glucose metabolism, providing insights into the long-term basis for oxidative stress-mediated dysfunction in FoxO3 KO NPCs (Figure 8). Our global metabolic profiling and mechanistic studies further clarified FoxO3-dependent transcriptional regulation as an essential metabolic rheostat that supports the oxidative PPP and Gln utilization in NPCs. The complex interaction of these programmes together contributes to energy homeostasis and long-term proliferative potential of adult stem cells.

Recent studies support an indispensable role of FoxO3 for metabolic adaptation under stress conditions. Another study demonstrated that FoxO3 activation blocks hypoxia-induced elevation of ROS and consequential HIF-1 α stabilization, an effect independent of MnSOD, but mediated through inhibiting c-Myc function (Jensen *et al*, 2011; Ferber *et al*, 2012). We propose that FoxO3, as a critical factor in stem-cell homeostasis and longevity, coordinates metabolic activity that confers resistance towards both intrinsic and extrinsic oxidative challenges. Indeed, our findings extend the previous reports pinpointing chronically elevated ROS as the cause of the aberrant biphasic outcome of proliferation followed by precocious loss of self-renewal potential in FoxO-deficient stem cells (Tothova *et al*, 2007; Paik *et al*, 2009) by defining several mechanisms through which this process is regulated. For example, the rise in ROS may account for the re-directing of glucose into macromolecule (e.g., RNA, protein, and lipid) biosynthetic pathways. This would be

initially compatible with decreased cellular quiescence and enhanced proliferation. However, the increased energy stress and attenuated growth signalling eventually suppress the long-term proliferative potential of FoxO3 KO NPCs. This may be an underlying need for FoxO3 to balance anabolic glutaminolysis and catabolism (e.g., autophagy) in NPCs.

Previously, several Gln-dependent mechanisms were shown to activate mTORC1. For example, the counter transport of Gln with essential amino acids activates mTORC1 and blocks autophagy (Nicklin *et al*, 2009). α -KG produced by glutaminolysis stimulated lysosomal translocation and activation of mTORC1 (Duran *et al*, 2012) and, conversely, glutamine biosynthesis through GS inhibits mTOR and promotes autophagy (van der Vos *et al*, 2012). In our study, there were no gross changes in uptake or steady-state intracellular levels of Gln in FoxO3 KO NPCs (Figure 1B and C). Instead, decreased glutaminolysis and α -KG were observed, which may account for the attenuated mTOR signalling.

FoxO has been implicated in the regulation of the mTOR signalling pathway under various cellular contexts. For example, transcriptional targets of FoxO, sestrin3 and Rictor, antagonize mTORC1 activity (Chen *et al*, 2010). In haematopoietic progenitors, loss of FoxO3 causes ROS accumulation which in turn inhibits Lnk, a negative regulator of cytokine receptor signalling and thus activates AKT/mTOR signalling (Yalcin *et al*, 2010). Unexpectedly, our study in NPCs revealed multiple points where FoxO3 is necessary to maintain mTOR-dependent growth signalling: upregulation of *Pik3ca* and glutaminolysis supports PI3K-AKT-mTOR signalling. The differences between these studies and the results presented herein may stem from varying cellular contexts, which require different pathways for homeostasis.

The aberrant activation of p42/44 MAPK observed in FoxO3 KO NPCs might be due to decreased expression of negative regulators of Ras-MAPK signalling (e.g., sprouty) (Paik *et al*, 2007). This may prevent hyperactivation of PI3K-AKT-mTOR pathway by way of p70S6K-mediated inhibitory phosphorylation of IRS1 (Takano *et al*, 2001). Interestingly, dampened phosphorylation of S6 did not agree with hyperactivation of p70S6K, suggesting that other kinases for S6 (i.e., S6K2) may predominantly phosphorylate S6 in NPCs. Future work will be required to precisely define the signalling programmes and mechanisms of regulation controlled by FoxO3 that maintain homeostasis in NPCs.

In proliferating cells, the Gln carbon skeleton sustains anaplerotic TCA cycle activity as a major source of α -KG. Additionally, Gln-derived Glu can be used in GSH biosynthesis (DeBerardinis and Cheng, 2010; Levine and Puzio-Kuter, 2010; Wise and Thompson, 2010). Interestingly, we observed a decline in Glu and α -KG levels despite similar uptake of Gln. These data suggested that the steps mediated by GLS and GLUD1 are suppressed in FoxO3 KO NPCs. GLS1 converts Gln into Glu and can be induced by Myc (Yuneva *et al*, 2012). The GLS2 isoform, on the other hand, can be upregulated in response to DNA damage or oxidative stress in a p53-dependent manner where it acts to facilitate the suppression of intracellular ROS (Hu *et al*, 2010; Suzuki *et al*, 2010). In our study, suppressing FoxO3 did not affect expression of GLS1, but various factors, including forced FoxO3 activation or AKT activation, altered its activity (Figure 1D; Supplementary Figure S5A and B). Defining the

Figure 8 Schematic summary of FoxO3 action in NPC metabolism. FoxO3 deficiency suppresses glutaminolysis, glucose uptake and metabolism and oxidative PPP activity. Less GSH derived from Gln together with reduced NADPH generation from PPP lowers GSH/GSSG and cellular redox potential. Increased ROS inhibits PKM2 without effectively redirecting glucose into the PPP, further exacerbating oxidative stress in FoxO3 KO NPCs.

conditions and mechanisms of GLS1 activity modulation warrants further investigations. In summary, our findings that FoxO3 controls central carbon metabolism by direct transcriptional activation of a subset of metabolic enzymes substantiate its role as a guardian of stem cells against oxidative stress.

Materials and methods

Primary cultures

Primary NPCs were isolated from the forebrain SVZ of neonatal FoxO3^{L/L} mice (Paik *et al*, 2007). Isolated NPCs were maintained in NPC culture medium supplemented with 20 ng/ml EGF and bFGF. To induce the recombination and deletion of FoxO, NPCs were infected with Adeno-CMV-Cre or empty virus (Vector Biolab).

Viral production and infection

To enforce the expression of exogenous WT-AKT, DN-AKT, and constitutively active FoxO3-AAA mutants (ca-FoxO3, three known phosphorylation sites were substituted into alanine) (Brunet *et al*, 1999), NPCs were infected with adenovirus containing control GFP (Ad-GFP), Adeno-CMV-WT-AKT1-HA, Adeno-CMV-DN-AKT1-HA, or ca-FoxO3 viruses for 12 h (Vector Biolabs). To generate retroviruses, 293T cells were transiently transfected with 10 µg of the replication-incompetent helper vector pCL and the target retroviral vectors, such as pMSCV-GLS1-V5, pMSCV-GLUD1-V5, and pMSCV-PGD-V5. Retroviral supernatant was collected at 48–72 h and precipitated with PEG10000. NPCs were infected with retroviral particles with 8 µg/ml of polybrene. GLS1 lentiviral shRNA was purchased from Sigma (clone ID, TRCN0000253163). Lentivirus was generated by co-transfecting lentiviral vector, pCMV R8.91, and pMDG packaging vectors in 10:9:1 ratio in 293T cells. At least two shRNAs for each target gene were tested.

Glucose uptake analysis

NPCs were kept in glucose-free media for 2 h, and 1 mM 2-NBDG was added to the media for 2 h at 37°C. The amount of 2-NBDG uptake was analysed by flow cytometry.

Immunoprecipitation

pLNCX-PKM2-Flag plasmid (Anastasiou *et al*, 2011) was transfected into 293T control and FoxO3 KD cells. After 72 h, cells were lysed with de-gassed RIPA buffer containing complete protease and phosphatase inhibitors. Lysates were treated with 250 µM diamide for 15 min and 80 µg of lysates was incubated with 0.5 µg of Flag-M2 antibody (Sigma) for 12 h and then incubated with GammaBind sepharose protein G beads for 2 h at 4°C. Then, the beads were washed, heated in Laemmli's sample buffer and the supernatant was used for immunoblot analysis. The densitometry was performed using ImageJ.

Intracellular ROS measurement

Intracellular ROS was detected using an intracellular ROS dye, dichlorodihydrofluorescein (DCF-DA). NPCs were incubated with 10 µM DCF-DA for 30 min at 37°C. The level of fluorescent adduct

was determined by flow cytometry with excitation at 488 nm. To assess the effect of Gln metabolites on intracellular ROS accumulation, NPCs were cultured in both high (25 mM), low (1 mM) glucose and with or without 2 mM Gln. To determine the effect of Gln metabolites on ROS, Gln-starved NPCs were treated with 4 mM Glu or 2 mM cell permeable GSH for 16 h prior to analysis. The same conditions were used to measure intracellular NADP/NADPH using an assay kit (Abcam).

Neurosphere formation

NPCs were dissociated and seeded at 2 cells/µl density in multi-well plates. Cells were cultured for 14 days with treatment of 968 at concentrations of 0.5, 1.25, and 2.5 µM and BPTES at concentrations of 0.1, 1, and 10 µM. Neurospheres that appeared greater than 130 µm in diameter were scored under bright-field microscopy.

Statistical analysis

The unpaired two-tail Student's *t*-test was used for experiments comparing two sets of data unless otherwise noted. Otherwise, one-way analysis of variance (ANOVA) was conducted with Tukey HSD as a post test for significant differences (**P* < 0.05 or ***P* < 0.01) as noted.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: HYeo, CAL, and YZ participated in experiments. HYeo coordinated the mouse work and HYeo, CAL, and JMA analysed the data. LCC provided new reagents. HYeo, CAL, HYing, and J-HP designed research, organized the study, and wrote the manuscript.

Conflict of interest

LCC owns equity in, receives compensation from, and serves on the Board of Directors and Scientific Advisory Board of Agios Pharmaceuticals. Agios Pharmaceuticals is identifying metabolic pathways of cancer cells and developing drugs to inhibit such enzymes in order to disrupt tumour cell growth and survival.

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