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Mitochondrial metabolism in hematopoietic stem cells requires functional FOXO3

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Abstract

Hematopoietic stem cells (HSC) are primarily dormant but have the potential to become highly active on demand to reconstitute blood. This requires a swift metabolic switch from glycolysis to mitochondrial oxidative phosphorylation. Maintenance of low levels of reactive oxygen species (ROS), a by-product of mitochondrial metabolism, is also necessary for sustaining HSC dormancy. Little is known about mechanisms that integrate energy metabolism with hematopoietic stem cell homeostasis. Here, we identify the transcription factor FOXO3 as a new regulator of metabolic adaptation of HSC. ROS are elevated in *Foxo3*$^{-/-}$ HSC that are defective in their activity. We show that *Foxo3*$^{-/-}$ HSC are impaired in mitochondrial metabolism independent of ROS levels. These defects are associated with altered expression of mitochondrial/metabolic genes in *Foxo3*$^{-/-}$ hematopoietic stem and progenitor cells (HSPC). We further show that defects of *Foxo3*$^{-/-}$ HSC long-term repopulation activity are independent of ROS or mTOR signaling. Our results point to FOXO3 as a potential node that couples mitochondrial metabolism with HSC homeostasis. These findings have critical implications for mechanisms that promote malignant transformation and aging of blood stem and progenitor cells.

Keywords FOXO3; HSC; metabolism; mitochondria; ROS
Subject Categories Metabolism; Stem Cells
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Introduction

Like all stem cells, hematopoietic stem cells (HSC) are characterized primarily by self-renewal and multipotency [1]. Blood stem cells in adults are largely dormant in the bone marrow (BM) hypoxic niches and divide extremely rarely [2–4]. Despite quiescence, however, a single HSC has the potential to reconstitute the entire hematopoiesis in response to damage or loss. This implies that blood stem cells have the ability to be efficiently activated and rapidly divide to regenerate hematopoietic tissue in a relatively short period of time. This intrinsic potential of HSC to reconstitute all blood cells in a mouse in which bone marrow is ablated is an exquisite measure of their activity [5]. The quiescence of blood stem cells and their self-renewal are highly coupled [1], making the tight balance between quiescence and proliferation of HSC key to the maintenance of the HSC pool throughout adult life. HSC must therefore be poised with plasticity to adapt metabolically to either quiescence or the highly active state.

HSC dormancy is maintained by low metabolic activity supplied by glycolytic metabolites in hypoxic niches [6,7]. In agreement with this, dormant HSC exhibit very low levels of reactive oxygen species (ROS) that are intimately tied to cellular metabolic activity [7–9]. Although oxygen radicals are mostly known for their deleterious properties, they also serve as signaling messengers that variably influence cell fate [10,11]. Dormant HSC are acutely sensitive to oxidative stress, a cellular state instigated by an imbalance between the generation and the detoxification of ROS [12–17]. In many cases, unbalanced accumulation of ROS mediates deficiencies of HSC function [12,18,19]; however, the impact of ROS on HSC activity when mitochondrial function is defective is less clear [20–26].

Mitochondria are the major site of ATP production through oxidative phosphorylation and constitute the metabolic center of the cell. During the process of oxidative phosphorylation, ROS are

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produced as the by-product of mitochondrial respiration [11]. HSC contain relatively few and inactive mitochondria [7,27] consistent with their low levels of ROS. Recent evidence suggests that mitochondria have a key function in the maintenance of HSC quiescence and their potency to rapidly switch from dormancy to a metabolically active state [20,22–24,27–31] (and reviewed in [32]). mTOR signaling in particular has been implicated in HSC mitochondrial biogenesis [20]. Despite this importance, relatively little is known about the mechanisms that control mitochondria or the metabolic adaptation in HSC.

Among potential candidates that may regulate stem cell metabolism are homeostatic FOXO proteins [33]. Transcription factors FOXO (FOXO1, FOXO3, FOXO4, FOXO6 in mammals) are critical regulators of oxidative stress [34–39]. In addition, FOXOs are key regulators of some fundamental biological processes including cell cycle, apoptosis, and metabolism that by integrating various signals insure tissue homeostasis [30,33]. Notably, FOXOs are among the very few transcription factors that are essential for the maintenance of pluripotency/multipotency in several types of stem cells including adult hematopoietic and neural, and embryonic pluripotent stem cells [14–16,40–44] as well as both mouse and human leukemic stem cells [45–47]. FOXO3 is the principal FOXO required to maintain normal adult hematopoietic and leukemic stem cells [15,16,45–48]. In addition, genetic variation within *FOXO3* gene is associated with human longevity [49]. These functions combined with other FOXO3 attributes including its key role in communicating mitochondrial–nuclear signals [50,51] and its potential function in HSC aging [15,17,48] make FOXO3 a suitable candidate for regulating HSC metabolism. Consistent with a potential metabolic function in HSC, FOXO3 is critical for the regulation of oxidative stress in HSC and hematopoietic progenitors; loss of FOXO3 results in elevated ROS associated with defective HSC activity [15–17], as well as ROS-mediated myeloproliferation in mice [41]. Whether FOXO3 is implicated in the mitochondrial regulation of HSC remains unexplored.

Here, we show that FOXO3 is critical for the regulation of mitochondrial respiration in HSC. We further show that the deficiency of *Foxo3*$^{-/-}$ HSC activity as measured by long-term competitive repopulation is not predominantly mediated by the enhanced levels of ROS or mTOR activation. In addition, we provide evidence that activation of mTOR signaling pathway mediates the abnormal mitochondrial function in the less primitive subset of *Foxo3* mutant HSPC. Our combined results suggest that elevation of ROS is not solely due to the reduced expression of antioxidant enzymes [34] in *Foxo3*$^{-/-}$ HSC *in vivo* [14–16], rather elevated ROS is associated with, and may indicate, an underlying unhealthy mitochondrial state [52] in *Foxo3*$^{-/-}$ HSC. These findings are likely to have important implications for mechanisms that control hematopoietic stem cell homeostasis and aging as well as leukemic stem cell activity.

Results

Loss of FOXO3 represses mitochondrial metabolism in HSC

To address whether FOXO3 regulation of HSC metabolism is restricted to controlling ROS levels or is also implicated in a more global control of energy homeostasis, we investigated the status of mitochondrial function. ROS including mitochondrial superoxide are increased in *Foxo3* mutant Lin$^-$Sca-1$^+$cKit$^+$ (LSK) cells, a population enriched for hematopoietic stem and progenitor cells (HSPC) that comprise < 0.05% of bone marrow (Fig EV1A and B) [15,16]. To further address mitochondrial function, we measured the levels of ATP (adenosine triphosphate) that is generated mainly through glycolysis and oxidative phosphorylation in hematopoietic stem cells [7,32]. Blood stem cells are accessed and isolated by flow cytometry using a combination of cell surface markers to deplete mature cells (Lin$^-$, lineage negative), and enrich for a highly pure population of primitive cells. In our studies, we have used long-term HSC (LT-HSC) (CD34$^-$Flk2$^-$LSK or CD150$^+$CD48$^-$LSK) that are highly quiescent, constitute < 0.01% of total BM, and have the ability to reconstitute blood in a lethally irradiated mouse for at least 4 months [53]. With lineage specification, HSC generate progenitors with more restricted activity and lineage potential. Short-term HSC (ST-HSC) with more limited reconstitution capacity which does not surpass 2 months generate multipotent primitive hematopoietic progenitors (MPP) isolated in Lin$^-$cKit$^+$Sca1$^-$ (c-Kit$^+$) cells. These progenitor cells have also been included in our experiments.

Wild-type and *Foxo3*$^{-/-}$ LT-HSC were freshly isolated from the bone marrow and subjected to ATP bioluminescence assay [7]. To our surprise, ATP was depleted by almost 50% in *Foxo3* mutant LT-HSC as compared to controls (Fig 1A). Oxygen consumption that is a major indicator of oxidative phosphorylation was also markedly reduced (almost by 50%) in *Foxo3* mutant HSC as analyzed by an Oxygen Biosensor (Fig 1B). Lower rates of mitochondrial respiration may reflect lower energy requirements. That is unlikely since *Foxo3* mutant HSC in contrast to their wild-type counterparts have exited the quiescence state and are likely subject to higher energy demand [15,16]. Alternatively, lower respiration rates may indicate that despite loss of quiescence, *Foxo3* mutant HSC increase glycolysis for energy production instead of increasing oxidative phosphorylation. In agreement with this, using gas chromatography–mass spectrometry we found increased ^{13}C lactate production in the *Foxo3* mutant HSC, suggesting the glycolytic flux was enhanced in these cells (Fig 1C). Collectively, these results indicated (Fig 1A–C) a shift in the ATP production from oxidative phosphorylation in mitochondria to glycolysis in the cytosol of *Foxo3* mutant HSC. Glycolysis is a relatively inefficient means for generating ATP [54]. Nonetheless, the increased glycolysis associated with ATP depletion by half and impaired mitochondrial respiration in *Foxo3* mutant HSC suggests that oxidative phosphorylation is compromised. These results were highly unexpected as HSC use glycolysis as their main source of energy [7,9,28,55]. Mutations that cause HSC loss of quiescence associated with increased ROS as observed in *Foxo3*$^{-/-}$ HSC are often associated with decreased glycolysis and increased oxidative phosphorylation that is the major alternative source of energy to glycolysis in HSC [18–20].

Mitochondrial mass was increased in primitive hematopoietic stem cell compartment of *Foxo3*$^{-/-}$ mice including *Foxo3*$^{-/-}$ LSK cells and LT-HSC (although in these latter cells the increase did not reach significance in the limit of the number of replicates used) as compared to controls according to the MitoTracker Green probe that measures mitochondrial mass independently of membrane potential (Fig 1D). Notably however, the mitochondrial mass was not significantly modulated in *Foxo3*$^{-/-}$ c-Kit$^+$ cells enriched for hematopoietic progenitors. As the mitochondrial proton gradient

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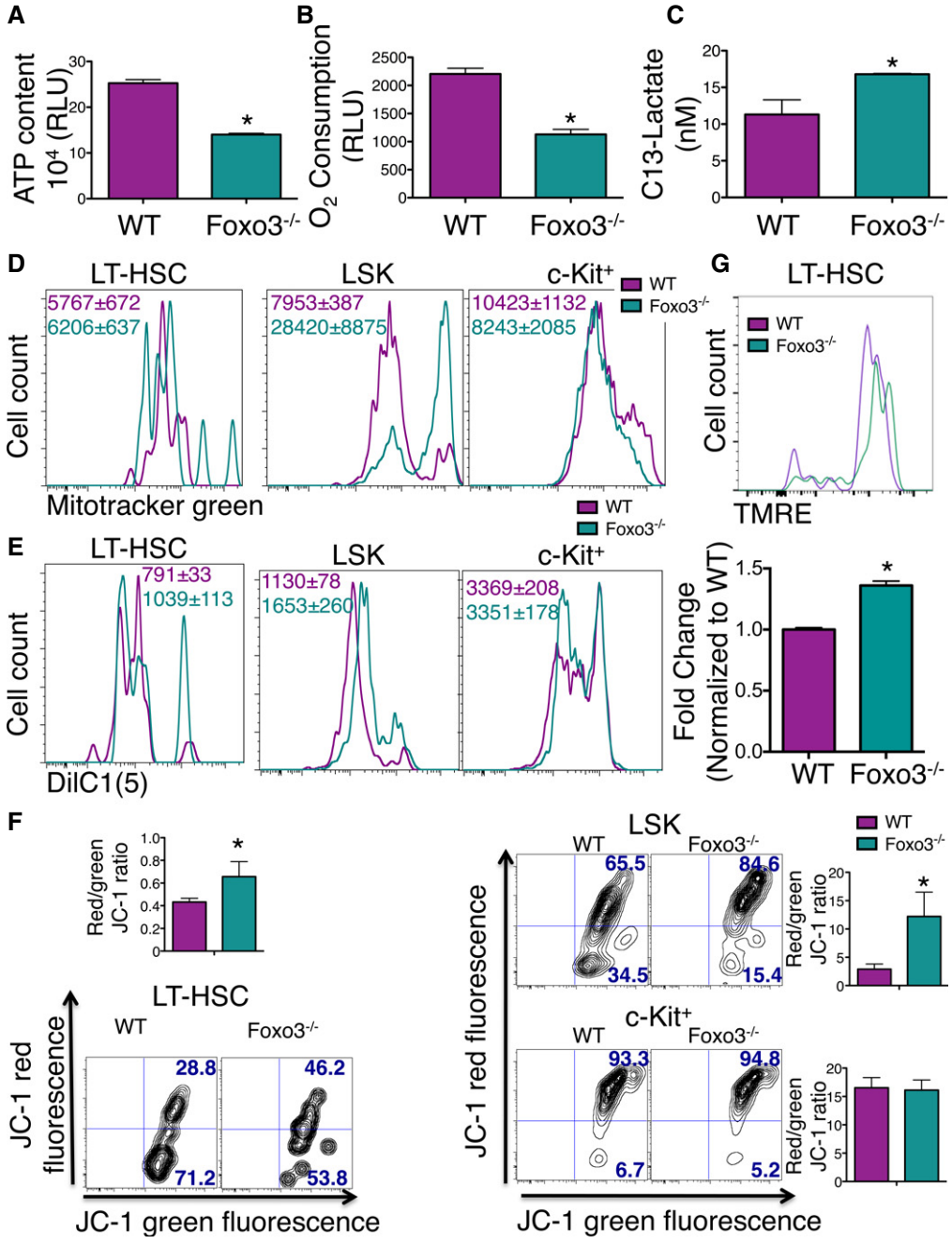

Figure 1. Mitochondrial dysfunction in *Foxo3*$^{-/-}$ HSC.

A–C Mitochondrial parameters as ATP level (A), oxygen consumption (B), and lactate production (C) were measured in WT and *Foxo3*$^{-/-}$ LT-HSC (LSKCD34^{-}Flk2^{-}) (n = 10 mice in each group), and experiments were performed in triplicate.

D–F Mitochondrial mass (D) and membrane potential (E, F) were measured in freshly isolated primitive hematopoietic stem and progenitor cells. (D) One representative FACS plot of the mitochondrial mass measured by the geometric mean fluorescence intensity of MitoTracker Green of 2 (LT-HSC, LSKCD48^{+}CD150^{-}) or 3 (LSK and c-Kit^{+}) independent experiments (n = 3 mice per genotype) is shown: LT-HSC, P = 0.324; LSK, P = 0.021; and c-Kit^{+}, P = 0.092. (E) One representative FACS plot of the mitochondria membrane potential measured by the geometric mean fluorescence intensity of 1,1′,3,3,3′,3′-hexamethylindodicarbo-cyanine iodide [DiIC1(5)] of 2 (LT-HSC, LSKCD48^{+}CD150^{-}) or 3 (LSK and c-Kit^{+}) independent experiments (n = 3 mice per genotype) is shown: LT-HSC, P = 0.046; LSK, P = 0.042; and c-Kit^{+}, P = 0.478. (F) Mitochondria membrane potential was also measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) probe by flow cytometry. Monomeric JC-1 has a green fluorescent emission spectrum while its aggregated form has a red fluorescent emission spectrum. As JC-1 probe accumulates, its aggregates and shifts fluorescent color. Representative FACS plots of JC-1 red and green fluorescence and relative $\Delta\Psi_m$ measured by the red/green fluorescence ratio are shown for each population (n = 3 mice per genotype for LT-HSC and n = 6 mice per genotype for LSK and c-Kit^{+} cells).

G Histogram (top) and quantification (bottom) of TMRE fluorescence intensity comparing mitochondrial membrane potential between WT and *Foxo3*$^{-/-}$ LT-HSC. TMRE florescence normalized to WT TMRE levels in LT-HSC (n = 3 mice per group).

Data information: All data are expressed as mean ± SEM (Student's t-test, *$P < 0.05$).

generated by the respiratory chain drives ATP synthesis [56], and given the reduced mitochondrial respiration and ATP levels in *Foxo3* mutant HSC, we suspected the mitochondrial membrane potential would be decreased. Unexpectedly however, the mitochondrial membrane potential was increased in *Foxo3*$^{-/-}$ LT-HSC and LSK according to DilC1(5) (Fig 1E), a probe that is actively transported into mitochondria in a mitochondrial membrane potential ($\Delta\Psi_m$)-dependent manner. To confirm these results, we used JC-1 probe combined with flow cytometry. As anticipated [7], mitochondrial membrane potential was increased with differentiation and maturation of wild-type HSC (Fig 1F, compare LT-HSC, to LSK to c-Kit$^+$ cells). However, the conversion of green to red fluorescence was increased in *Foxo3*$^{-/-}$ LT-HSC (1.5-fold), and LSK (fourfold) as compared to wild-type controls independently of any other mitochondrial parameter indicating enhanced mitochondrial membrane potential (Fig 1F). Increased mitochondrial membrane potential was specific to *Foxo3*$^{-/-}$ primitive hematopoietic stem cell compartment as it was not detected in *Foxo3*$^{-/-}$ c-Kit$^+$ cells (Fig 1F). Treatment with CCCP (carbonyl cyanide 3-chlorophenylhydrazone), an inhibitor of oxidative phosphorylation (Fig EV1C and D), reduced significantly both DiIC1(5) and JC-1 signals confirming their specificity. To further validate these measurements and exclude any potential aberration [57], we also used TMRE (tetramethylrhodamine, ethyl ester) to label active mitochondria. TMRE is a positively charged dye that readily accumulates in active mitochondria in live cells [57]. As anticipated, TMRE loading of mitochondria detected increased and decreased mitochondrial membrane potential after oligomycin inhibition of ATP synthase and CCCP inhibition of oxidative phosphorylation, respectively (Fig EV1E). Using TMRE, we further confirmed that mitochondrial membrane potential is significantly increased in *Foxo3*$^{-/-}$ LT-HSC relative to controls (Fig 1G).

These results were highly unanticipated as increased mitochondrial membrane potential in HSC is often associated with enhanced oxidative phosphorylation [7]. Loss of quiescence [15,16] associated with increased mitochondrial membrane potential, decreased ATP, and reduced mitochondrial respiration (Figs 1 and EV1) despite high levels of ROS underscore an abnormality of *Foxo3*$^{-/-}$ HSC mitochondrial function. Consistent with this contention, mitochondria was hyper-fragmented and mitochondrial morphology compromised in *Foxo3*$^{-/-}$ HSPC, suggesting that mitochondrial dynamics [58] might be altered (Fig EV2). Collectively, these findings (Figs 1A–G, EV1 and EV2) suggest that mitochondrial function is defective in *Foxo3*$^{-/-}$ HSC compartment. Increased glycolysis associated with enhanced mitochondrial membrane potential might indicate compensatory mechanisms responding to ATP depletion in *Foxo3*$^{-/-}$ HSC.

Inhibition of ROS *in vivo* does not rescue *Foxo3*$^{-/-}$ LT-HSC phenotype

Impaired oxidative phosphorylation was unexpected in *Foxo3* mutant HSC [15–17,59] as defective HSC associated with abnormal accumulation of ROS as observed in *Foxo3* mutant HSC often indicates a switch from glycolysis in quiescent HSC to oxidative phosphorylation in activated HSC [12,18,28,29]. In light of these findings, we suspected that accumulated ROS might not cause HSC defects [15–17]. If true, we reasoned that decreasing ROS levels *in vivo* with a glutathione precursor N-acetyl-cysteine (NAC) would not rescue the defects of *Foxo3* mutant HSC. Indeed, loss of FOXO3 [15–17] (or FOXO [59]) is associated with oxidative stress in HSC. Elevated ROS mediate the increased production of myeloid colony-forming unit-spleen (CFU-S) progenitors in triple *Foxo*$^{-/-}$ (*Foxo1*$^{-/-}$ *xFoxo3*$^{-/-}$*xFoxo4*$^{-/-}$) mice as well as the activation of p38 MAPK in *Foxo3*$^{-/-}$ LT-HSC [15] and to variable degrees the defective production of myeloid progenitors in long-term culture of *Foxo3*$^{-/-}$ long-term culture initiating cell (LTC-IC) *in vitro* [16,17], a population that overlaps with, but is not restricted to, long-term competitive multilineage repopulating cells *in vivo* [60]. However, the contribution of elevated ROS to the dysfunction of *Foxo3* (or *Foxo*) mutant long-term competitive repopulation of HSC *in vivo* remained unexplored [15–17,59]. To investigate this, wild-type and *Foxo3*$^{-/-}$ mice were treated *in vivo* with NAC for 2 weeks (Fig 2A). The NAC regimen used was not toxic and did not significantly alter the BM cellularity in wild-type or *Foxo3*$^{-/-}$ mice (Fig 2A). As anticipated, NAC treatment normalized the levels of ROS in *Foxo3*$^{-/-}$ but did not alter ROS in wild-type LSK cells (Fig 2B). However, inhibition of ROS *in vivo* did not modulate significantly the reduced numbers of *Foxo3*$^{-/-}$ LSK cells (Fig 2C). Similarly, this regimen did not impact significantly the *Foxo3*$^{-/-}$ LT-HSC numbers (Fig 2D). We next injected 100 highly purified (LSKCD48$^-$CD150$^+$) *Foxo3*-deficient HSC into lethally irradiated recipient mice along with 200,000 recipient bone marrow cells in an *in vivo* competitive repopulation assay (Fig 2E). As previously reported [16], *Foxo3*-null HSC were highly compromised in their long-term competitive multilineage reconstitution of lethally irradiated recipients. However, prior treatment of *Foxo3*-deficient mice with NAC *in vivo* did not improve the long-term competitive repopulation ability of isolated *Foxo3*$^{-/-}$ HSC as compared to animals treated with vehicle control in transplanted lethally irradiated recipients (Fig 2E). Although ROS levels were reduced in HSPC at the time of transplantation as a result of the NAC regimen (Fig 2B), we wondered whether NAC treatment for a longer period of time would have a positive effect on the *Foxo3*$^{-/-}$ HSC repopulation ability. Competitive repopulation capacity of isolated LT-HSC from mice treated with NAC or vehicle control for 4 weeks was assayed in lethally irradiated mice as in Fig 2A. We confirmed that NAC is effective in reducing protein oxidation in *Foxo3*$^{-/-}$ bone marrow cells by analyzing protein carbonyl derivatives before and after NAC treatment (Fig EV3). The 4-week NAC regimen improved initially the ability of *Foxo3*$^{-/-}$ HSC to repopulate recipient mice at 4 weeks post-transplantation, but this improvement was lost after 8 weeks and not observed at any later time point up to 16 weeks post-transplantation (Fig 2F). This NAC regimen clearly reduces ROS in transplanted LT-HSC (Fig EV4A). The effect of NAC was maintained during the entire transplantation as judged by ROS levels in CD45$^+$ peripheral blood cells 16 weeks post-transplantation (Fig EV4B). Taken together, these results suggest that in contrast to *Atm*$^{-/-}$ HSC where NAC treatment of only *Atm*$^{-/-}$ (donor) mice improved significantly the output of *Atm*$^{-/-}$ HSC function in transplanted irradiated hosts [12], reducing ROS levels of *Foxo3*$^{-/-}$ HSC had minimal effect on their long-term competitive repopulation ability in transplanted animals (Fig 2F). A potential explanation for these results might be that to insure continuous normal ROS levels, NAC treatment of recipient in addition to donor mice may be required during the entire transplantation for full restoration of the competitive repopulation ability of donor mutant cells [12,18]. Restoration of repopulation under such conditions might

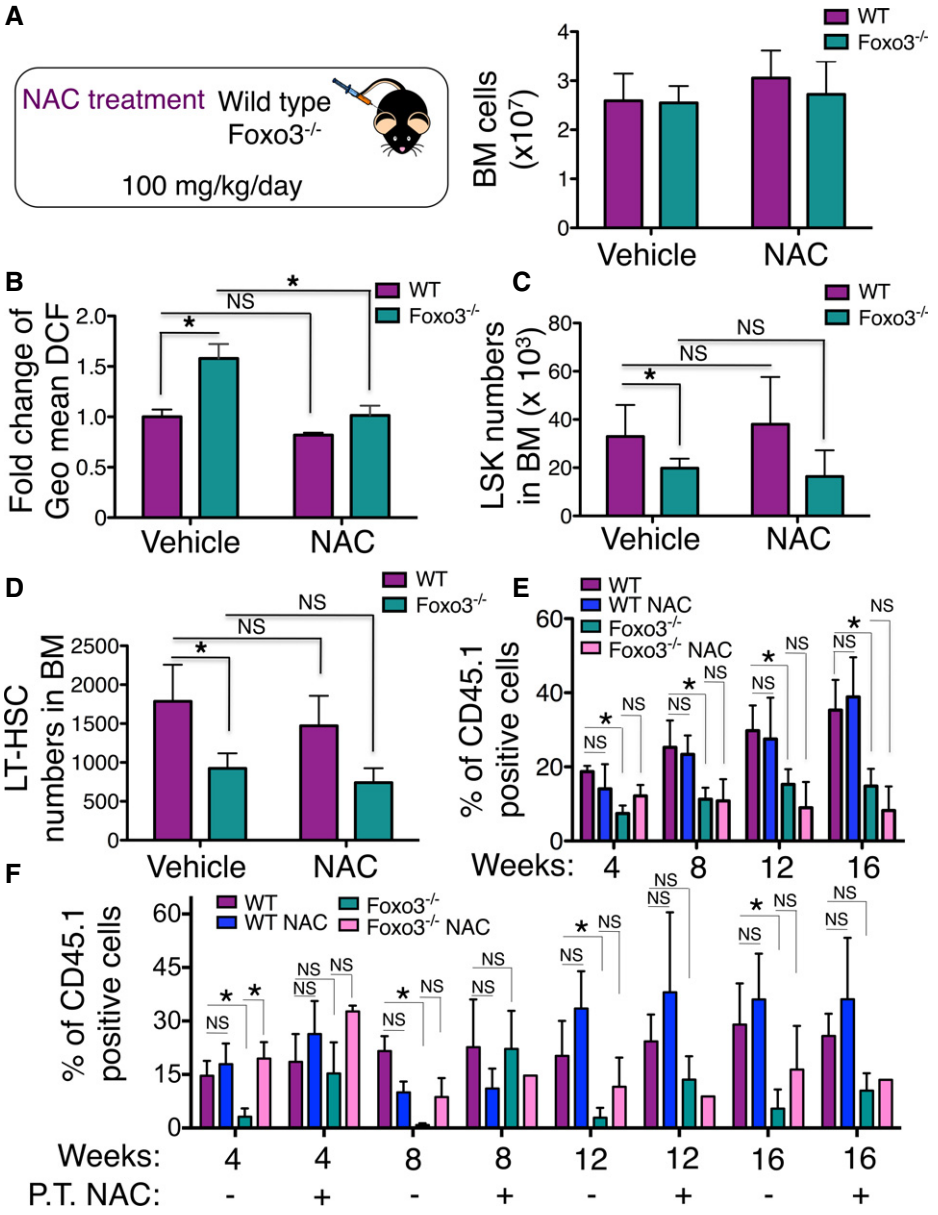

Figure 2. Inhibition of ROS does not rescue *Foxo3*$^{-/-}$ LT-HSC phenotype.

A WT and *Foxo3*$^{-/-}$ mice were injected intraperitoneally daily with 100 mg/kg body weight of N-acetyl-L-cysteine (NAC) or vehicle control for 2 weeks (left panel). Total number of bone marrow (BM) cells isolated from one femur and one tibia (n = 6 mice per genotype) (right panel).

B ROS levels were measured by flow cytometry in WT and *Foxo3*$^{-/-}$ LSK cells isolated after 2 weeks. Fold change of geometric mean normalized with WT of DCF fluorescence (n = 6 mice per genotype).

C Total number of LSK cells in the BM (n = 6 mice per genotype).

D Total number of LSKCD48^{-}CD150^{+} (LT-HSC) cells in the BM (n = 6 mice per genotype).

E Contribution of LT-HSC (CD45.1) from (A) to the peripheral blood (PB) of recipient mice (CD45.2) in a long-term competitive repopulation assay (n = 5 in each group).

F Contribution of LSKCD48^{-}CD150^{+} (LT-HSC) (CD45.1) isolated from NAC- or vehicle control-treated mice for 4 weeks to long-term competitive repopulation of lethally irradiated mice treated with NAC or vehicle control during post-transplantation (P.T.). One of two experiments is shown.

Data information: Data are expressed as mean ± SEM (n = 5 mice transplanted per group); NS, not significant; *P < 0.05, Student's t-test.

indicate potential additional effects of NAC on bone marrow microenvironment. Regardless, NAC treatment of both pre- and post-transplanted *Foxo3*$^{-/-}$ HSC did not improve significantly their repopulation ability beyond 4 weeks post-transplantation (Fig 2F). Similarly, the positive effect of post-transplant NAC treatment of *Foxo3*$^{-/-}$ HSC recipients did not last beyond 8 weeks (Fig 2F). Thus, unlike *Atm*$^{-/-}$ [12] and *Meis1*$^{-/-}$ HSC [18] where reducing elevated ROS rescued their long-term repopulation abnormalities, normalizing ROS levels *in vivo* may not be sufficient to overcome *Foxo3*$^{-/-}$ HSC long-term repopulation defects.

Reducing ROS levels does not improve *Foxo3*$^{-/-}$ HSC mitochondrial dysfunction *in vivo*

As a chronic increase in ROS might impair mitochondrial function [61], we evaluated whether *Foxo3*$^{-/-}$ HSC mitochondrial dysfunction was at least partially due to increased ROS. The *in vivo* NAC treatment did not revert the abnormal increased mitochondrial membrane potential in *Foxo3*$^{-/-}$ LSK cells as measured by both DiIC1(5) (Fig 3A) and JC-1 (Fig 3B) probes. In agreement with these results, TMRE levels of *Foxo3*$^{-/-}$ LT-HSC or LSK cells (Figs 3C and EV4C) despite reduced ROS levels (Fig EV4A and D) were not reduced in response to NAC treatment, suggesting that the increased mitochondrial membrane potential of *Foxo3*$^{-/-}$ HSC was not due to oxidative stress. Altogether, these results indicate that loss of FOXO3 impairs mitochondrial function independent of ROS in HSC. In agreement with this, many genes implicated in the regulation of mitochondrial function, electron transfer chain, and/or metabolism, particularly glycolysis, were significantly deregulated in *Foxo3*$^{-/-}$ HSPC (Fig 4). Among these genes, impaired expression of a master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor-γ coactivator (*Pgc*) 1 was notable. Some of these genes that are potential direct targets of FOXO3 including isocitrate dehydrogenase 1 (*Idh1*) and *Idh2* genes [62,63] were specifically modulated in *Foxo3*$^{-/-}$ HSPC (LSK cells), but not in more committed *Foxo3*$^{-/-}$ c-Kit^{+} hematopoietic progenitor cells. One of the most remarkable impacts among the genes surveyed was on *Atpif1*, the inhibitor of mitochondrial F1F0-ATPase that limits the ATP depletion. *Atpif1* expression was reduced by over 60% (Fig 4A). Reduced expression of ATPif1 maintains mitochondrial membrane potential to protect cells with severe deficiencies in electron transfer chain from apoptosis [64]. It is noteworthy in this context that mitochondrial membrane potential is elevated in *Foxo3*$^{-/-}$ HSPC (Fig 1E–G), and despite high ROS, these cells do not exhibit increased apoptosis [15].

Thus, loss of *Foxo3*$^{-/-}$ HSC long-term competitive repopulation is associated with impaired mitochondrial metabolism, but not mediated by ROS. These combined findings raise the possibility that compromised *Foxo3*$^{-/-}$ HSC mitochondria may be implicated in defects of *Foxo3*$^{-/-}$ LT-HSC activity.

Inhibition of mTOR signaling improves ROS levels but does not ameliorate *Foxo3*$^{-/-}$ LT-HSC function *in vivo*

The mammalian target of rapamycin (mTOR) is critical for the regulation of mitochondrial biogenesis including in HSC [20,65,66].

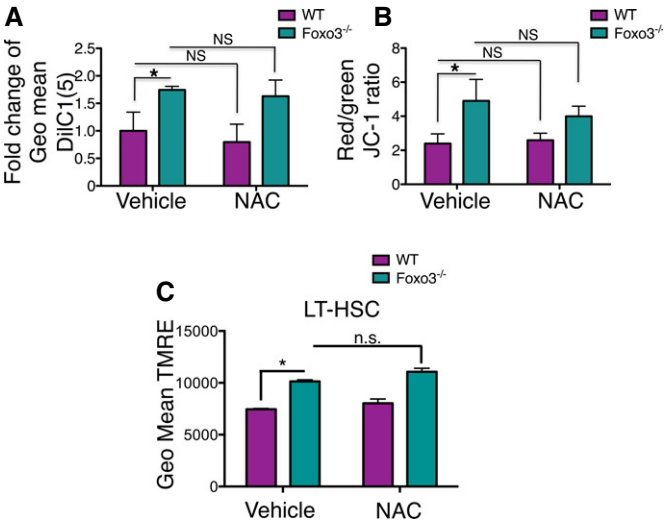

Figure 3. Reducing ROS levels does not improve mitochondrial dysfunction in *Foxo3*$^{-/-}$ HSPC.

A Mice were treated with NAC or vehicle control, and mitochondrial membrane potential was measured in freshly isolated cells after 2 weeks. Fold change of geometric mean of DilC1(5) fluorescence measured in LSK cells (n = 3 mice per genotype) normalized with vehicle-treated (WT).

B Relative $\Delta\Psi_m$ measured in LSK cells by the red/green fluorescence ratio of JC-1 probe (n = 6 mice per genotype).

C TMRE quantification of mitochondrial membrane potential in LT-HSC isolated from mice treated with NAC or vehicle control (n = 3 mice).

Data information: All data are expressed as mean ± SEM; NS, not significant; *P < 0.05, Student's t-test. Controls are the same as in Fig 1G.

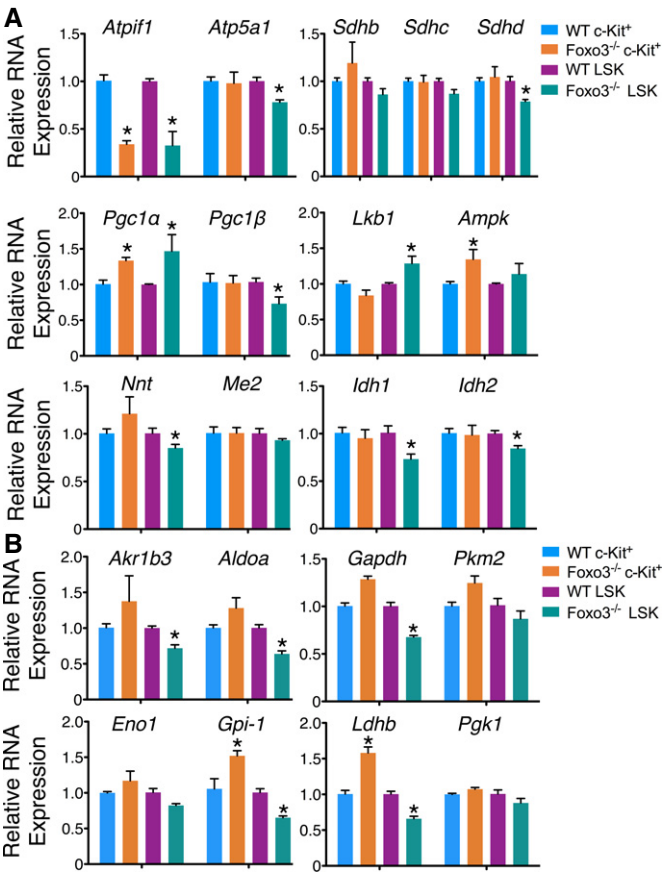

Figure 4. Alteration of mitochondrial and metabolic gene expression in *Foxo3*$^{-/-}$ HSPC.

A qRT–PCR analysis of genes whose products are implicated in mitochondrial metabolism. Results are relative to WT set to one in each population.

B qRT–PCR analysis of glycolytic genes. Results are relative to WT set to one in each population.

Data information: Results are from three independent experiments each based on three replicates of one cDNA generated from a pool of cells isolated from three mice. All data are expressed as mean ± SEM (Student's t-test, *P < 0.05).

mTOR signaling senses nutrients availability and is a key regulator of cell growth. mTOR protein kinase exists in two distinct core complexes, mTOR complex I and II (mTORC1 and mTORC2, respectively) which differ in their regulation and functions as well as in their sensitivity to rapamycin [67]. As an interplay between mTORC1 and FOXO3 is implicated in the myeloid progenitor homeostasis [41,62] and the glycolytic enzyme regulation [62,68], we investigated whether mTORC1 signaling was involved in the defective mitochondrial function in *Foxo3*$^{-/-}$ HSC. Interestingly, loss of FOXO3 increased the fraction of HSPC expressing the phosphorylated form of S6 that is a reliable indicator of mTORC1 activity [67] as measured by flow cytometry (Fig 5A). These results suggest that as in myeloid progenitors [41,62], FOXO3 might inhibit mTOR signaling in primitive HSPC. To investigate this, we evaluated the impact of pharmacological inhibition of mTORC1 signaling on the phenotype of *Foxo3*$^{-/-}$ HSC. Wild-type and *Foxo3*$^{-/-}$ mice were treated *in vivo* for 2 weeks with rapamycin [67] that is a specific inhibitor of mTORC1 (Fig 5A). As anticipated, this treatment normalized the levels of S6K1 target ribosomal protein S6 phosphorylation (Fig 5A, upper panel) and reduced significantly the frequency of *Foxo3*$^{-/-}$ LSK cells positive for pS6 to levels similar to wild-type control cells (Fig 5A, lower panel), without any significant impact on wild-type control cells. Treatment with rapamycin *in vivo* did not modulate the mitochondrial membrane potential as measured by either DiIC1(5) (Fig 5B) or JC-1 (Fig 5C) probes in WT LSK cells (Fig 5B and C). Notably however, this treatment mitigated the increased $\Delta\Psi_m$ levels in *Foxo3*$^{-/-}$ LSK cells (Fig 5B and C). In addition, the *in vivo* treatment with rapamycin normalized ROS levels in freshly isolated *Foxo3*$^{-/-}$ LSK (Fig 5D) cells as has been previously observed in *Foxo3*$^{-/-}$ committed myeloid progenitors [41]. These results suggest that activation of mTOR signaling mediates the abnormalities of mitochondrial membrane potential in *Foxo3*$^{-/-}$ HSPC *in vivo*.

We next investigated whether activation of mTOR signaling contributes to *Foxo3*$^{-/-}$ HSC activity defects. As anticipated, the 2-week *in vivo* treatment with rapamycin did not impact the BM cellularity (Fig 6A). Interestingly, while rapamycin treatment did not modulate significantly the HSC pool in wild-type mice (Fig 6B–D), this regimen rescued the total numbers (Fig 6B) and the frequency of *Foxo3*$^{-/-}$ LSK cells (Fig 6C). In contrast to these effects, the rapamycin regimen did not alter the numbers (Fig 6D) or the frequency (not shown) of *Foxo3*$^{-/-}$ LT-HSC in the bone marrow (Fig 6D). This regimen was sufficient to inhibit mTOR signaling (Fig 5A); furthermore, similar rapamycin regimen had positive effects on *Lkb1*$^{-/-}$ LT-HSC numbers [22–24], together raising the possibility that mTOR activation impacts specifically *Foxo3*$^{-/-}$ HSPC subset that excludes cells with long-term competitive repopulation competence. In agreement with this interpretation, rapamycin-treated *Foxo3*$^{-/-}$ HSC repopulated recipients only up to 8 weeks post-transplantation (Fig 6E). However, rapamycin- and vehicle control-treated *Foxo3*$^{-/-}$ HSC were similarly defective in their ability to competitively reconstitute hematopoiesis at 12 and 16 weeks post-transplantation in irradiated hosts (Fig 6E), suggesting that rapamycin did not have a major impact on *Foxo3*$^{-/-}$ LT-HSC competitive repopulation ability. Relative to their wild-type counterparts, a significant fraction of *Foxo3*-defective HSPC exit quiescence (G0) [15,16] (Fig 6F). In agreement with the lack of effect on *Foxo3*$^{-/-}$ HSC long-term repopulation, the rapamycin treatment modulated only marginally the *Foxo3*$^{-/-}$ HSPC cycling status or the quiescent (G0) fraction of *Foxo3*$^{-/-}$ HSPC as measured by Ki-67^{+} proliferating cells and DAPI staining (Fig 6F). Unexpectedly however, we observed that rapamycin-treated wild-type HSPC exited quiescence state (Fig 6F).

Altogether, these results suggested that short-term but not long-term *Foxo3*$^{-/-}$ HSC defects were mediated by the activation of mTOR signaling.

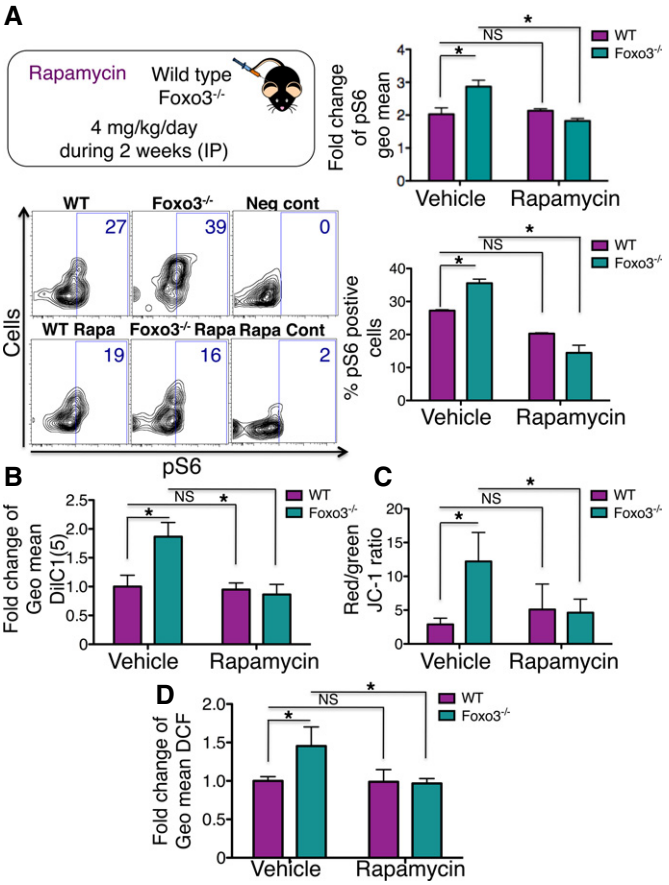

Figure 5. Inhibition of mTOR signaling normalizes ROS levels and mitochondrial parameters in *Foxo3*$^{-/-}$ HSPC.

A WT and *Foxo3*$^{-/-}$ mice were injected intraperitoneally with 4 mg/kg body weight of rapamycin on 5 consecutive days/week daily for 2 weeks (left upper panel). Analysis of phosphorylated form of S6K1 target ribosomal protein S6 (pS6) by flow cytometry in LSK cells isolated from WT and *Foxo3*$^{-/-}$ mice treated with rapamycin or vehicle control by flow cytometry measuring fold change of geometric mean of pS6 compared to negative control (Neg cont: anti-rabbit IgG-PE antibody only; right upper panel) and the percentage of pS6-positive cells (right lower panel). Representative FACS plots of pS6 expression (left lower panel). The anti-rabbit IgG-PE antibody only (Neg cont) and rapamycin treatment (3 μM) for 1 h *in vitro* before the LSK staining were used as negative controls of pS6 antibody. One representative of three independent experiments (n = 3 mice per group) is shown.

B Fold change of geometric mean normalized with WT of DiIC1(5) fluorescence measured in LSK cells isolated in (A) (right panel) (n = 6 mice per genotype).

C Relative $\Delta\Psi_m$ measured in LSK cells isolated in (A) by the red/green fluorescence ratio of JC-1 probe (n = 6 mice per genotype).

D Fold change of geometric mean normalized with WT of DCF fluorescence (n = 6 mice per genotype).

Data information: All data are expressed as mean ± SEM; NS, not significant; *P < 0.05, Student's t-test.

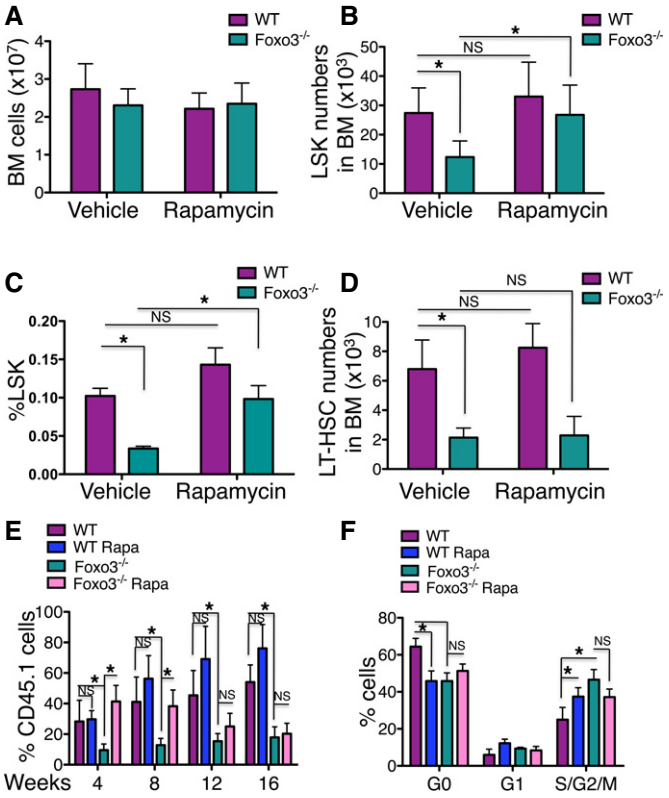

Figure 6. Inhibition of mTOR does not rescue the *Foxo3*$^{-/-}$ HSC long-term repopulation activity.

A Total number of bone marrow (BM) cells isolated from one femur and one tibia from WT and *Foxo3*$^{-/-}$ mice treated with rapamycin or vehicle control (n = 10 mice per group).

B Total number of LSK cells in the BM of mice from (A) (n = 10 mice per genotype).

C Frequency of Lin$^-$Sca-1$^+$c-Kit$^+$ cells (n = 10 mice per genotype).

D Total number of LSKCD48$^-$CD150$^+$ (LT-HSC) cells in the BM of mice from (A) (n = 6 mice per genotype).

E The contribution of transplanted LT-HSC (CD45.1) cells isolated from (A) to the PB of recipient mice (CD45.2) in a long-term competitive repopulation assay (n = 8 in each group).

F Mean values of cell cycle distribution measured by Ki-67/DAPI staining of LSK cells isolated from (A) (n = 6 mice per genotype).

Data information: All data are expressed as mean ± SEM; NS, not significant; *P < 0.05, Student's t-test.

Discussion

FOXO3 is critical for mitochondrial metabolism in HSC

Here, we showed that FOXO3 is critical for mitochondrial respiration in LT-HSC independently of its regulation of mTOR activity or ROS levels. Although mitochondria are relatively inactive in HSC, mitochondrial function is required for the proper maintenance of blood stem cell activity [21–24]. Elevation of ROS in defective mutant HSC associated with loss of quiescence often signals a switch to mitochondrial oxidative phosphorylation [18–20]. *Foxo3*$^{-/-}$ HSC loss of quiescence [15–17], however, was paradoxically associated with increased glycolysis, elevated ROS and significant reduction of oxidative phosphorylation, abnormalities of mitochondrial membrane potential and mass (Figs 1 and 2) as well as aberrant expression of critical genes implicated in mitochondrial functions (Fig 4). In agreement with previous findings [7,27], our data support the notion that the regulation of mitochondria is distinct between hematopoietic stem and progenitor cells; furthermore, that FOXO3 is required for mitochondrial metabolism in LT-HSC (Figs 1–4). Given the expression and subcellular localization of FOXOs in *Foxo3*$^{-/-}$ HSPC [16] (R. Liang and S. Ghaffari, unpublished findings), it is unlikely that these observations are mediated through compensatory mechanisms by other FOXOs. These results raise the possibility that impaired mitochondrial metabolism may be implicated in abnormalities of *Foxo3*$^{-/-}$ HSC long-term repopulation.

These findings are in agreement with compromised *Foxo3*$^{-/-}$ HSPC function in generating colony-forming cells *in vitro* and with their delayed transition through the G2/M cell cycle phase [16,17]. They are also consistent with the lack of enhanced apoptosis in *Foxo3*$^{-/-}$ HSC despite elevated ROS [17] (C.L. Bigarella and S. Ghaffari, unpublished findings). These findings may also provide some explanation as to why FOXO3 is required for leukemic stem cell maintenance [45–47]. It will be interesting to see how mechanistically FOXO3 regulates mitochondrial metabolism and how this is integrated with the regulation of ROS and HSC activity.

The defective *Foxo3*$^{-/-}$ HSC activity is not mediated by elevated ROS *in vivo*

FOXO3 regulation of ROS in stem cells [15–17,41,42] is mediated in part by transcriptional regulation of several antioxidant enzymes [34] (and reviewed in [33,69]). In addition, FOXO3 regulates NADPH generation and glutathione biosynthesis [63,68]. Here, we found that in addition to the control of oxidative stress, FOXO3 regulates HSC mitochondrial metabolism (Figs 1–4). Our findings suggest that while increased ROS are implicated in enhanced generation of *Foxo3*- [41] or *Foxo*-deficient [59] multipotent colony-forming unit-spleen (CFU-S), and cell cycle-related abnormalities of HSPC [14,16,59], elevated ROS do not mediate the functional defects of *Foxo3*$^{-/-}$ long-term HSC but rather might reflect an abnormal mitochondrial function. These results are consistent with the notion that NAC treatment only partially rescued the p38 MAPK activation in *Foxo3*$^{-/-}$ HSPC [15]. These findings raise the interesting possibility that underlying mechanisms that uncouple ROS from mitochondrial potential might disengage HSC cycling from HSC repopulation potential.

The outcome of FOXO3 regulation of mitochondria may be context dependent [70–72]. Furthermore, FOXO3 appears to have relatively distinct metabolic functions in neural [68] versus hematopoietic stem cells (current study). Given that loss of quiescence in *Foxo3*$^{-/-}$ HSC is paradoxically associated with increased glycolysis, these results raise questions regarding FOXO3 regulation of additional metabolic pathways including the pentose phosphate pathway in HSC. These findings together with the regulation of autophagy in aged and stressed HSC [73] underline critical and diverse FOXO3 metabolic functions in stem and progenitor cells.

Inhibition of mTOR signaling rescues the phenotype of the less primitive subset of *Foxo3*$^{-/-}$ HSPC

mTOR signaling is one the major signaling pathways implicated in the regulation of mitochondrial metabolism and oxygen

consumption in somatic and embryonic stem cells [20,65,74,75]. However, the activation of mTOR signaling in *Foxo3*$^{-/-}$ HSPC seems to mediate the defects of *Foxo3*$^{-/-}$ ST-HSC, but not LT-HSC. Rapamycin treatment rescued the numbers of *Foxo3*$^{-/-}$ LSK cells and normalized the mitochondrial membrane potential and ROS in *Foxo3*$^{-/-}$ LSK cells (Fig 5). In addition, rapamycin rescued the competitive repopulation ability of *Foxo3*$^{-/-}$ HSC up to 8 weeks, suggesting that mTOR mediates the *Foxo3*$^{-/-}$ ST-HSC defects (Fig 6). In contrast, while this 2-week period of treatment was clearly sufficient to reduce pS6 downstream of mTOR signaling and rescue *Tsc1* and *Lkb1* mutant LT-HSC numbers [20,22–24], rapamycin did not rescue the number of *Foxo3*$^{-/-}$ LT-HSC in treated mice or their competitive repopulating potential in lethally irradiated transplanted hosts (Figs 5 and 6). These findings combined with our previous results [41] indicate that mTOR activation mediates defects of *Foxo3*$^{-/-}$ myeloid and multipotential progenitors, but not *Foxo3*$^{-/-}$ LT-HSC abnormalities. These results were not too surprising after all as the phenotype of *Foxo3*$^{-/-}$ and *Tsc1*$^{-/-}$ LT-HSC in which mTOR is constitutively activated is quite distinct [20]. Consistent with PTEN-independent regulation of FOXO3 in HSC [48,76], these results (Figs 5 and 6) further delineate differences of mTOR activation in HSC on a background of PTEN versus FOXO3 loss of function [76]. In addition, they suggest that mTOR activation in *Foxo3*$^{-/-}$ ST-HSC might result in their defective function leading to proliferation of downstream hematopoietic progenitors [41]. As both rapamycin and NAC treatments normalized ROS but not the competitive repopulation of *Foxo3*$^{-/-}$ LT-HSC, our results support the notion that elevated ROS do not mediate the defective competitive repopulation of *Foxo3*$^{-/-}$ LT-HSC. However, despite similarities of *Foxo*$^{-/-}$ and *Foxo3*$^{-/-}$ HSC [15–17,41,59], our results do not rule out the potential oxidative stress mediation of *in vivo Foxo*$^{-/-}$ HSC competitive repopulation defects [59].

Overall, our results identify FOXO3 as essential for HSC mitochondrial metabolism. They also support a model in which mitochondria is key to the maintenance of LT-HSC (Fig 7). In addition, these findings provide a platform for elucidating FOXO3 metabolic contributions to HSC aging and leukemic stem cell maintenance [45,47].

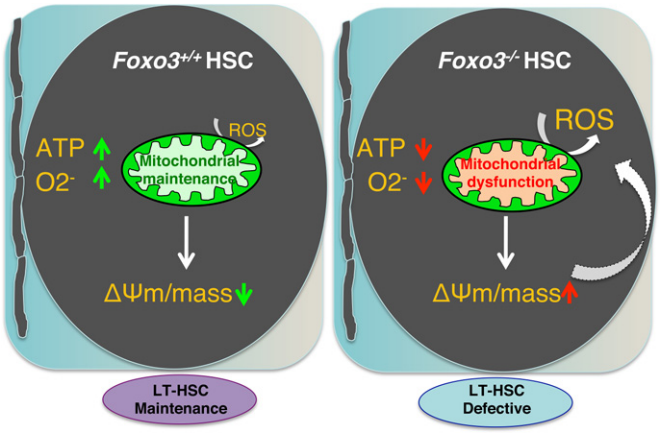

Figure 7. Model of FOXO3 regulation of mitochondrial function in HSC.
Loss of FOXO3 leads to mitochondrial defects that might be implicated in the loss of *Foxo3*-deficient HSC long-term competitive repopulation ability.

Materials and Methods

Mice

Foxo3$^{-/-}$ mice were backcrossed 10 generations onto C57BL6 (CD45.1) background [16]. Mice were used according to the protocols approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Flow cytometry

Antibody staining and flow cytometry analysis were performed as previously described [16,41]. For Lin$^-$Sca-1$^-$c-Kit$^+$ (c-Kit$^+$) and Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells, freshly isolated bone marrow cells were pre-incubated with 5% rat serum and biotinylated hematopoietic multilineage monoclonal antibody cocktail (StemCell Technologies), containing CD5 (lymphocytes), CD11b (leukocytes), CD19 (B cells), CD45R (lymphocytes), 7-4 (neutrophils), Ly-6G-Gr-1 (granulocytes), TER119 (erythroid cells) antibodies to remove mature cells, stained with PE-Sca-1 and APC-c-Kit antibodies (BD Biosciences) prior to two rounds of wash followed by incubation with Pacific Blue–streptavidin. In addition to LSK staining, total bone marrow cells were stained with FITC-CD48 (eBioscience) and PECy7-CD150 (BioLegend) antibodies to isolate the long-term HSC (LSKCD48$^-$CD150$^+$). The MitoTracker and JC-1 probes were used in combination with the lineages antibody cocktail—Pacific Blue–streptavidin, APC-c-Kit, V500-Sca-1, APCCy7-CD48 (BD Biosciences) and PECy7-CD150 antibodies. The DilC1(5) probe was used in combination with the lineages antibody cocktail—Pacific Blue–streptavidin, PE-c-Kit (eBioscience), V500-Sca-1, FITC-CD48, and PECy7-CD150 antibodies.

To sub-fractionate the long-term HSC (LSKCD34$^-$Flk2$^-$), bone marrow cells were stained as described [7]. The frequencies and numbers of HSC in the BM are measured per femur and per tibia. Freshly isolated bone marrow cells stained with LSK were fixed with fix/permeabilization buffer (BD Biosciences) and incubated with 1:100 dilution of anti-pSer235/236 S6 antibody (Cell Signaling Technology) followed by incubation with 1:800 dilution of PE-conjugated secondary antibody (BD Biosciences) to measure intracellular phosphorylated S6 (pS6). Samples were washed, and protein phosphorylation was analyzed by flow cytometry.

Long-term repopulation assay

Lethally irradiated (12 Gy as a split dose, 6.5 and 5.5 Gy, 4–5 h apart) [16] congenic C57BL6-CD45.1 mice (NCI) were reconstituted with intravenous injections of 100 donor LSKCD48$^-$CD150$^+$ cells from C57BL6 mice (CD45.1) along with 2×10^5 competitor bone marrow cells (CD45.2). Reconstitution of donor-derived cells was distinguished from host cells by the expression of CD45.1 versus CD45.2 antigens (BD Biosciences) in the peripheral blood.

Cell cycle analysis

To measure the fraction of quiescent cells, bone marrow cells were stained for LSK, fixed, permeabilized, and incubated with Ki-67 antibody (BD Pharmingen) and DAPI to determine the G0 state (1 μg/ml). Samples were immediately analyzed by flow cytometry.

NAC treatment

WT and *Foxo3*$^{-/-}$ mice were injected intraperitoneally with 100 mg/kg body weight of N-acetyl-L-cysteine (NAC; Sigma, MO) in phosphate-buffered saline (PBS) solution (pH 7.4) daily, as previously described [16,41]. For long-term treatment, NAC was alternatively injected every day and added to the water every other week.

Rapamycin treatment

WT and *Foxo3*$^{-/-}$ mice were injected intraperitoneally with 4 mg/kg body weight of rapamycin (Enzo Life Sciences, NY) in PBS with 5% Tween-80, 5% PEG400 and 4% ethanol during five consecutive days/week for 2 weeks, as previously described [41].

Measurement of intracellular ROS

ROS was measured using CM-H_2DCFDA (Molecular Probes) as previously described [16,41]. To measure the concentration of superoxide anion, 10^6 cells stained first for LSK were resuspended in PBS 2% FBS, loaded with 5 μM mitoSOX Red (M36008; Molecular Probes) and incubated in the dark for 30 min at 37°C. The fluorescent product was measured immediately by flow cytometry.

Measurement of mitochondrial mass and membrane potential

To measure the mitochondrial mass, 3×10^6 cells stained first to gate c-Kit$^+$, LSK, LT-HSC were resuspended in PBS 2% FBS and loaded with 20 nM MitoTracker Green (M7514; Molecular Probes), in the dark for 30 min at 37°C. To measure the mitochondrial membrane potential, cells were loaded with 2 μM JC-1 probe (M34152; Molecular Probes) or 25 nM DilC1(5) (M34151; Molecular Probes) in accordance with the manufacturer's recommendations and incubated in the dark for 20 min or 25 min, respectively, at 37°C. To test the specificity, cells were incubated with 50 mM CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a $\Delta\Psi_m$ inhibitor for 20 min at 37°C. For each probe, the fluorescent product was measured immediately by flow cytometry.

Metabolic assays

Oxygen consumption was measured with the BD Oxygen Biosensor System in accordance with the manufacturer's recommendations, as previously described [7]. ATP levels were quantified with ATP Bioluminescence Assay Kit HS II (Roche) in accordance with the manufacturer's recommendations, as previously described [7]. Lactate production was measured with gas chromatography–mass spectrometry, as previously described [7].

Protein oxidation detection

Cell lysate proteins were treated as previously described [39], reacted with 2,4-dinitrophenylhydrazine to derivatize carbonyl groups (OxyBlot Protein Oxidation Detection kit; Chemicon International) to dinitrophenylhydrazone (DNP) and resolved by 12% SDS–PAGE and detected by rabbit anti-DNP antibody.

Real-time quantitative RT–PCR

Total RNA was isolated using RNeasy MicroPlus kit (Qiagen). First-strand cDNA was synthesized using SuperScriptII (Invitrogen). cDNA obtained from 300 cells was used per well for RT–PCR performed using SYBR Green JumpStart Taq ReadyMix (Takara) in triplicates, using the primers indicated in the figures and ABI Prism 7900 HT Cycler (Applied Biosystems, see Primer sequences in Appendix Table S1). All the results were normalized to β-actin RNA levels.

Statistical analysis

Unpaired two-tailed Student's *t*-test was used for all experiments. *P*-value < 0.05 was considered significant.

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Author contributions
PR, RL, CLB, FK and JX designed experiments, performed experiments, and analyzed data. PR and RL wrote the paper; HS and CCZ designed experiments and analyzed data; MNS and JC provided valuable tools; JC edited the paper and SG conceived the project, designed experiments, analyzed data, and wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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