

ROS-mediated amplification of AKT/mTOR signalling pathway leads to myeloproliferative syndrome in Foxo3^{-/-} mice

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Reactive oxygen species (ROS) participate in normal intracellular signalling and in many diseases including cancer and aging, although the associated mechanisms are not fully understood. Forkhead Box O (FoxO) 3 transcription factor regulates levels of ROS concentrations, and is essential for maintenance of hematopoietic stem cells. Here, we show that loss of Foxo3 causes a myeloproliferative syndrome with splenomegaly and increased hematopoietic progenitors (HPs) that are hypersensitive to cytokines. These mutant HPs contain increased ROS, overactive intracellular signalling through the AKT/mammalian target of rapamycin signalling pathway and relative deficiency of Lnk, a negative regulator of cytokine receptor signalling. *In vivo* treatment with ROS scavenger N-acetyl-cysteine corrects these biochemical abnormalities and relieves the myeloproliferation. Moreover, enforced expression of Lnk by retroviral transfer corrects the abnormal expansion of Foxo3^{-/-} HPs *in vivo*. Our combined results show that loss of Foxo3 causes increased ROS accumulation in HPs. In turn, this inhibits Lnk expression that contributes to exaggerated cytokine responses that lead to myeloproliferation. Our findings could explain the mechanisms by which mutations that alter Foxo3 function induce malignancy. More generally, the work illustrates how deregulated ROS may contribute to malignant progression.

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Introduction

Oxidative stress, broadly defined as an imbalance between generation and detoxification of reactive oxygen species (ROS), is deleterious to cells and implicated in a number of degenerative diseases and malignancies (reviewed in Beckman and Ames, 1998). In addition, excess accumulation of ROS impacts cellular aging, whereas the ability to resist oxidative stress is associated with evolutionary conserved enhanced longevity (Beckman and Ames, 1998). Although ROS are considered to be toxic byproducts of cellular metabolism, increasing evidence support the notion that ROS have a critical role in normal cellular signalling. In particular, ROS are generated by cytokine signalling and impact the function of a rapidly expanding list of numerous effectors (reviewed in Thannickal and Fanburg, 2000). How these activities affect normal and pathological physiology is not fully understood.

ROS are particularly deleterious to hematopoietic stem cells, specifically as they age (Ito *et al*, 2004; Miyamoto *et al*, 2007; Tothova *et al*, 2007; Yalcin *et al*, 2008; and reviewed in Ghaffari, 2008). A tightly controlled balance between hematopoietic stem and progenitor cell compartments maintains normal blood cell homeostasis throughout life. Alterations of this balance result in various disorders including leukemias or bone marrow failure. For instance, myeloproliferative disorders are a group of hematopoietic malignancies whose incidence increase with age, exhibit enhanced proliferation and survival of one or more myeloid lineage cells that arises from an unbalanced expansion of hematopoietic myeloid progenitor cells (Tefferi and Gilliland, 2007).

The Forkhead FoxO family of transcription factors are critical regulators of oxidative stress and exert this function at least partly by upregulating the expression of several antioxidant enzymes (Kops *et al*, 2002; Nemoto and Finkel, 2002; Murphy *et al*, 2003; Marinkovic *et al*, 2007; Tothova *et al*, 2007; Yalcin *et al*, 2008). FoxO1, FoxO3 and FoxO4 are widely expressed while FoxO6 is predominantly expressed in neuronal tissues. Loss of a single FoxO leads to distinct phenotypes in mice, underscoring their diverse non-redundant functions *in vivo* (Castrillon *et al*, 2003; Hosaka *et al*, 2004). In particular, female Foxo3-deficient mice exhibit a premature infertility associated with ovarian follicle depletion early on in life (Castrillon *et al*, 2003; Hosaka *et al*, 2004). In addition, both Foxo3^{-/-} hematopoietic stem and erythroid cell compartments exhibit enhanced susceptibility to oxidative stress (Marinkovic *et al*, 2007; Miyamoto *et al*, 2007; Yalcin *et al*, 2008).

FoxOs also regulate cellular responses to genotoxic stress, consistent with a tumour suppressor function (Paik *et al*, 2007). In response to stress such as DNA damage or oxidative stress, FoxOs induce cell cycle arrest, repair damaged DNA or initiate apoptosis by modulating genes that control these processes (Brunet *et al*, 1999; Dijkers *et al*, 2000; Medema

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et al, 2000; Nakamura *et al*, 2000; Tran *et al*, 2002; Alvarez *et al*, 2003; Ghaffari *et al*, 2003; Marinkovic *et al*, 2007; Yalcin *et al*, 2008). FoxO genes are also found at chromosomal breakpoints in certain cancers, including acute myeloid leukaemias (FoxO3 and FoxO4) (Borkhardt *et al*, 1997; Hillion *et al*, 1997). Moreover, FoxO3 regulates the expression and activity of ataxia telangiectasia-mutated protein kinase, suggesting an important role in the maintenance of genomic stability (Tsai *et al*, 2008; Yalcin *et al*, 2008).

Function of FoxO is restrained primarily by the phosphoinositide-3-kinase (PI3-kinase)/AKT signalling pathway (Biggs *et al*, 1999; Brunet *et al*, 1999; Dijkers *et al*, 2000; Kashii *et al*, 2000; Nakae *et al*, 1999; Rena *et al*, 1999; Tang *et al*, 1999; and reviewed in Greer and Brunet, 2008). The AKT serine threonine protein kinase regulates a wide range of metabolic processes through phosphorylation of numerous effectors, including FoxO and mammalian target of rapamycin (mTOR) (Gingras *et al*, 1998; Brunet *et al*, 1999; Inoki *et al*, 2002; Manning *et al*, 2002) a kinase that stimulates cell growth and proliferation through multiple effectors including ribosomal S6 kinase (S6K1) and the eukaryotic initiation factor 4E-binding protein.

In response to cytokines, growth factors or oncoproteins, activated AKT kinase directly phosphorylates FoxO on three conserved residues, resulting in their nuclear exclusion and subsequent degradation (Biggs *et al*, 1999; Brunet *et al*, 1999; Matsuzaki *et al*, 2003; Plas and Thompson, 2003; Hu *et al*, 2004). In contrast, stress stimuli, or inhibition of PI3-kinase/AKT signalling pathway by growth factor/cytokine withdrawal, induce FoxO's nuclear localization, thereby enhancing their transcriptional activity (Essers *et al*, 2004; Lehtinen *et al*, 2006; van der Horst *et al*, 2006). The PI3-kinase/AKT signalling pathway is activated in numerous human and animal malignancies, although how this contributes to the pathogenesis of these diseases is not entirely clear (Ugo *et al*, 2004; Bellacosa *et al*, 2005; Dai *et al*, 2005; Yilmaz *et al*, 2006; Zhang *et al*, 2006). In addition to AKT, a number of kinases regulate the activity of FoxO both positively and negatively. In addition to phosphorylation, FoxO proteins are subject of several other post-translational modifications such as acetylation, methylation and ubiquitination whose combined integrated signals determine the activity of FoxOs.

Recent findings have established a critical function for FoxO family members in the regulation of normal and malignant hematopoietic stem cell activity (Miyamoto *et al*, 2007; Tothova *et al*, 2007; Yalcin *et al*, 2008; Naka *et al*, 2010). In particular, FoxO3's suppression of ROS is essential for the maintenance of hematopoietic stem cell quiescence and homeostasis (Miyamoto *et al*, 2007; Yalcin *et al*, 2008). In addition, abnormal repression of FoxO3 has been implicated in the pathogenesis of myeloproliferative disorders and other haematological malignancies (Ghaffari *et al*, 2003; Komatsu *et al*, 2003; Fernandez de Mattos *et al*, 2004; Essafi *et al*, 2005). Despite these findings, of the entire scope of FoxO3 functions, regulation of hematopoietic progenitors (HPs) is not fully defined (Miyamoto *et al*, 2007; Yalcin *et al*, 2008).

Here, we show that loss of Foxo3 results in a myeloproliferative syndrome in mice. We further demonstrate that increased ROS accumulation in Foxo3^{-/-} primitive myeloid progenitors activates the cytokine-induced AKT/mTOR signalling pathway and expands Foxo3-deficient primitive myeloid progenitors. Accordingly, this myeloproliferative

syndrome is ameliorated by systemic administration of ROS scavengers. Moreover, Lnk (SH2B3), a negative regulator of cytokine signalling, is directly implicated in this process. Our combined data indicate that Foxo3 modulates HP homeostasis by controlling cytokine-dependent production of, and response to, ROS. These cumulative findings illustrate new mechanisms through which deregulated ROS could contribute to the development of malignancies.

Results

Foxo3^{-/-} mice exhibit a myeloproliferative syndrome

Foxo3^{-/-} mice display increased white blood cell counts, with an increased circulating neutrophils ($P < 0.03$) and monocytes ($P < 0.01$), and a concomitant reduction of circulating lymphocytes ($P < 0.005$) and red blood cells (Table I; Marinkovic *et al*, 2007). These anomalies of the peripheral blood are associated with myeloproliferative syndrome (Figure 1). Foxo3-deficient mice exhibit an enlarged spleen (Figure 1A), increased number of splenocytes (Figure 1B) and extramedullary hematopoiesis (Figure 1C, D and E, Supplementary Figure 1A), with increased frequency of erythrocytic and granulocytic lineages (Supplementary Figure 1). Concomitantly, the bone marrow is hypocellular (Figure 1B) with decreased production of mature B and erythroid cells (Figure 1D; Supplementary Figure 1; Marinkovic *et al*, 2007). Histopathology analysis corroborated these findings, showing increased extramedullary hematopoiesis containing erythroid and myeloid cells in the spleen and liver of Foxo3^{-/-} mice (Figure 1A and Supplementary Figure 2).

In agreement with a myeloproliferative syndrome, the myeloid progenitor compartment is significantly enhanced in the bone marrow, spleen and peripheral blood of Foxo3-deficient mice (Figure 2A and Supplementary Figure 3). In particular, myeloid colony-forming unit-granulocyte-macrophage-derived colonies are increased in numbers and size (Supplementary Figure 3 and data not shown).

Similarly, the size of primitive myeloid progenitor pool was enhanced in Foxo3^{-/-} bone marrow (Figure 2B). The compartment of colony-forming-spleen day 12 (CFU-S_{day12})-derived colonies was also increased ($P < 0.002$; Figure 2C), further supporting an expansion of the early myeloid progenitors. In addition, Foxo3^{-/-} HP cells were overly sensitive to cytokines (Figure 2D) and generated significantly larger-size colonies *in vitro* (Supplementary Figure 3 and data not shown), which are the hallmarks of myeloproliferative disorders (Ghaffari *et al*, 1999; Levine and Gilliland, 2008).

Table I Comparison of blood parameters of Foxo3^{+/+} and Foxo3^{-/-} mice

Parameters	Foxo3 ^{+/+}	Foxo3 ^{-/-}	P-value
WBC ($\times 1000/\mu\text{l}$)	9.45 \pm 1.13	13.48 \pm 1.16	0.01
Neutrophils (%)	8.98 \pm 1.66	13.80 \pm 1.76	0.03
Lymphocytes (%)	83.69 \pm 1.98	75.55 \pm 2.09	0.005
Monocytes (%)	2.98 \pm 0.58	6.87 \pm 1.59	0.01
Eosinophils (%)	2.70 \pm 0.25	2.33 \pm 0.39	0.21
Basophils (%)	1.34 \pm 0.32	0.95 \pm 0.09	0.14

Results for wild type ($n = 10$) and Foxo3^{-/-} ($n = 21$) blood are shown as mean \pm s.e.m. The analyses are from at least three independent experiments.

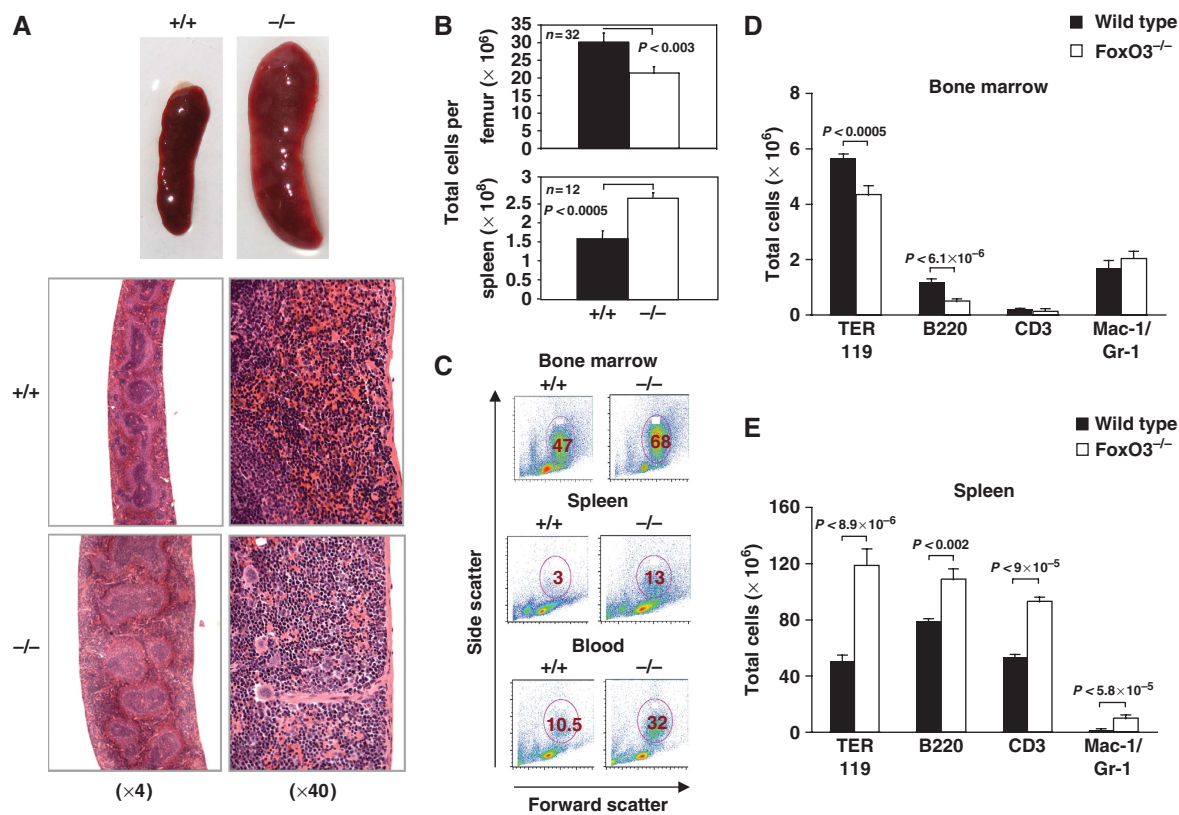


Figure 1 Myeloproliferative-like syndrome in Foxo3^{-/-} mice. (A) Representative whole-mount (upper panels) and histological sections (lower panels) of spleens from 11-week-old wild-type (+/+) and Foxo3^{-/-} (-/-) mice. Increased extramedullary myeloid hematopoiesis in the red pulp and minimal depletion of marginal zone lymphocytes with the retention of the T-cell regions in Foxo3^{-/-} spleen, as compared with the wild-type are shown. (B) Total number of bone marrow ($n = 32$) and spleen cells ($n = 12$) is shown, Student's *t*-test. (C) Representative FACS plots of FSC versus SSC of bone marrow, spleen and blood of wild-type and Foxo3^{-/-} mice are shown. Percentages of FSC^{high}SSC^{high} (granulocytic) cells are marked. (D) Total number of bone marrow cells in each lineage is plotted. Total number of bone marrow erythroid (TER 119, $n = 11$), B (B220, $n = 14$) and T cells (CD3, $n = 11$) and neutrophils (Gr-1/Mac-1, $n = 11$) is shown. (E) Total number of cells in each lineage of the spleen, TER 119 ($n = 8$), B220 ($n = 8$), CD3 ($n = 11$) and Gr-1/Mac-1 ($n = 11$) (Student's *t*-test). The analyses are from at least four independent experiments.

These data suggest that Foxo3 suppresses HP production and proliferation by inhibiting cytokine signalling. These findings were surprising, as Foxo3^{-/-} hematopoietic stem cells are not highly cycling *in vivo* and do not generate excessive number of HPs in culture (Yalcin *et al*, 2008), suggesting that these observations were not simply the result of a highly proliferative hematopoietic stem cell compartment in Foxo3^{-/-} mice.

ROS-mediated amplification of AKT/mTOR signalling pathway enhances primitive HP compartment in Foxo3^{-/-} mice

Foxo3 suppresses ROS in many cell types, including in hematopoietic cells, by regulating a programme of anti-oxidant gene expression (Kops *et al*, 1999; Nemoto and Finkel, 2002; Marinkovic *et al*, 2007; Miyamoto *et al*, 2007; Yalcin *et al*, 2008). Accordingly, Foxo3-mutant lineage-negative bone marrow cells exhibited reduced expression of several anti-oxidant enzyme genes (Supplementary Figure 4). In addition, ROS were significantly overaccumulated in different Foxo3^{-/-} subpopulations of lineage-negative cells enriched for myeloid progenitors (Figure 3 and Supplementary Figure 5). ROS concentrations were highly enhanced (approximately 1.6-fold, $P < 0.02$; Figure 3) in Foxo3^{-/-} Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cells, a population that encompasses all myeloid progenitors (Akashi *et al*, 2000), and increased significantly in freshly isolated Foxo3^{-/-} common myeloid progenitors

(CMP), as compared with their wild-type counterparts (approximately 1.2-fold, $P < 0.03$; Supplementary Figure 5). CMP is a highly pure population of hematopoietic cells giving rise to megakaryocyte/erythrocyte and granulocyte/monocyte progenitors (Akashi *et al*, 2000). Similar results were obtained from analysis of ROS accumulation in total Foxo3-deficient bone marrow depleted from lineage-restricted cells (data not shown). *In vivo* treatment of mice with ROS scavenger *N*-acetyl-cysteine (NAC, 100 mg/kg), normalized the levels of ROS in Foxo3^{-/-} Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cell population (Figure 3), supporting the specificity of ROS measurement. Interestingly, these experiments revealed two distinct populations of ROS-containing cells (ROS-high or ROS-hi and ROS-low) in primitive myeloid progenitors of both wild-type and Foxo3^{-/-} origin. The significant increase of ROS observed in Foxo3^{-/-} Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cell population was entirely in ROS-hi fraction (Figure 3). This ROS-hi subpopulation may be the one that elicits cellular responses, such as increased cell cycle or apoptosis, to oxidative stress in primitive myeloid progenitors.

To investigate the mechanisms of enhanced myeloproliferation caused by loss of Foxo3, we interrogated cytokine-mediated activation of principal signalling pathways in bone-marrow-derived HPs. Freshly isolated bone marrow cells were depleted of mature lineages by immunoselection (Lin⁻ cells), and subjected to cytokine starvation followed by

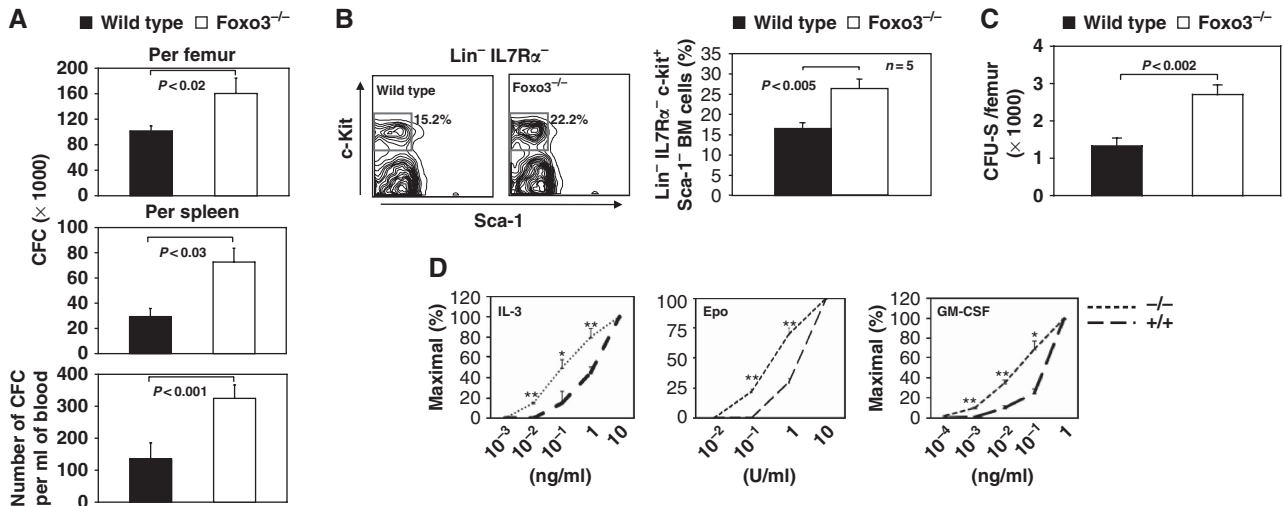


Figure 2 Enhanced hematopoietic progenitor activity in Foxo3^{-/-} mice. (A) Progenitor-derived colonies were measured in the bone marrow, spleen and blood of wild-type and Foxo3^{-/-} mice. The analyses are from four independent experiments in each of which two to three animals were either pooled or analysed independently. (B) Frequency (left panel) of highly enriched myeloid progenitor Lin⁻ IL7R α ⁻ Sca-1⁺ c-Kit⁺ compartment (right panel) in wild-type and Foxo3^{-/-} bone marrow is shown ($n = 5$ in each group, Student's *t*-test). The frequency of Sca-1⁻ c-Kit⁺ cells within Lin⁻ IL7R α ⁻ gated cells is shown (the frequency of Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cells within bone marrow is $1.3 \pm 0.11\%$ for wild type and $2.2 \pm 0.2\%$ for Foxo3^{-/-}). One representative of three independent experiments is shown. (C) CFU-S-derived colonies formed in the spleen were measured 12 days after *in vivo* injection of 10^5 wild-type or Foxo3^{-/-} bone marrow cells into lethally irradiated hosts; representative of two independent experiments, $n = 5$ in each group is shown, Student's *t*-test. (D) Colony-forming cell ability of wild-type and Foxo3^{-/-} progenitors was measured after plating 10^5 cells in semi-solid methylcellulose cultures in three replicates in the presence of limiting doses of the indicated cytokines (colonies of 20 or more cells were counted after 8 days and the numbers of colonies present at each cytokine concentration were calculated as percentages of the number formed in the highest concentration of the indicated cytokine). Mean + s.e.m. of three independent experiments, each pool of two to three mice. Student's *t*-test; * $P < 0.05$, ** $P < 0.01$.

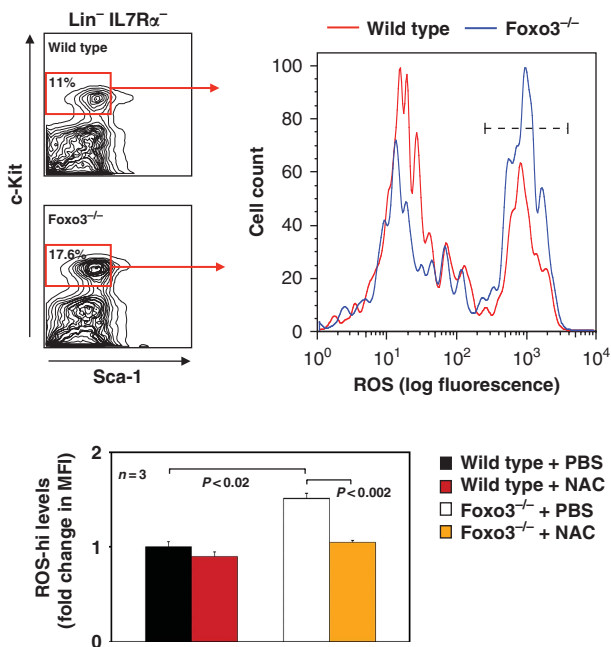


Figure 3 Enhanced ROS accumulation in Foxo3^{-/-} primitive myeloid progenitors. Representative FACS profile of bone marrow Lin⁻ IL7R α ⁻ Sca-1⁺ c-Kit⁺ that contain 98% of all myeloid progenitor cells (Akashi *et al*, 2000) (left panel). Frequency of c-Kit⁺ Sca-1⁺ cells within Lin⁻ IL7R α ⁻ cells is shown. Endogenous ROS concentrations were measured in freshly isolated Lin⁻ IL7R α ⁻ Sca-1⁺ c-Kit⁺ cells (right panel) from wild-type or Foxo3^{-/-} mice treated daily *in vivo* with NAC (100 mg/kg) or PBS for 15 days; fold change in mean fluorescence intensity (MFI) of ROS in gated subpopulations (ROS-hi using ----- gate), as compared with control wild-type cells treated with PBS is shown as mean \pm s.e.m., $n = 3$; Student's *t*-test. One of two independent experiments is shown.

stimulation with interleukin-3 (IL-3). Myeloid progenitors constitute a significant majority of Lin⁻ cells expressing IL-3 receptor at the steady state. To our surprise, IL-3 stimulation of Foxo3^{-/-} Lin⁻ cells led to hyperphosphorylation of AKT, mTOR and mTOR substrate S6K1 (Figure 4A). In contrast, STAT5 proteins, another effector of IL3 signalling, were not affected in these cells (Figure 4A). Similar results showing specific hyperactivation of the AKT/mTOR pathway in Foxo3^{-/-} cells were obtained with other cytokines such as erythropoietin (Epo, data not shown). In agreement with *in vivo* overactivation of AKT/mTOR signalling pathway mediating enhanced generation of early myeloid progenitors in Foxo3^{-/-} mice, *in vivo* administration of the mTOR inhibitor rapamycin resulted in significant reduction of Foxo3^{-/-}-derived CFU-S_{d12}, as compared with controls in lethally irradiated hosts (Figure 4B).

Normal cytokine signalling, including signalling by IL-3 (Sattler *et al*, 1999), is mediated in part by ROS in *in vitro* cultured cells (Thannickal and Fanburg, 2000; Finkel, 2003). Thus, we investigated whether abnormal increase of ROS contributes to the hyperactivation of AKT/mTOR signalling pathway caused by loss of Foxo3 in primary cells *in vivo*. We treated mice with the ROS scavenger NAC (100 mg/kg), and tested IL-3 signalling responses by examining phosphorylation of downstream targets AKT, mTOR and S6K1 (Figure 4A). *In vivo* treatment with NAC specifically reduced the intensity of IL-3-mediated phosphorylation of AKT and mTOR in Foxo3-mutant cells enriched for HPs. Surprisingly, reduction in phospho-mTOR in response to NAC did not impact phosphorylation of the mTOR target S6K1 in Foxo3 mutants, as compared with normal hematopoietic cells (Figure 4A). Although the mechanism of lack of pS6K1

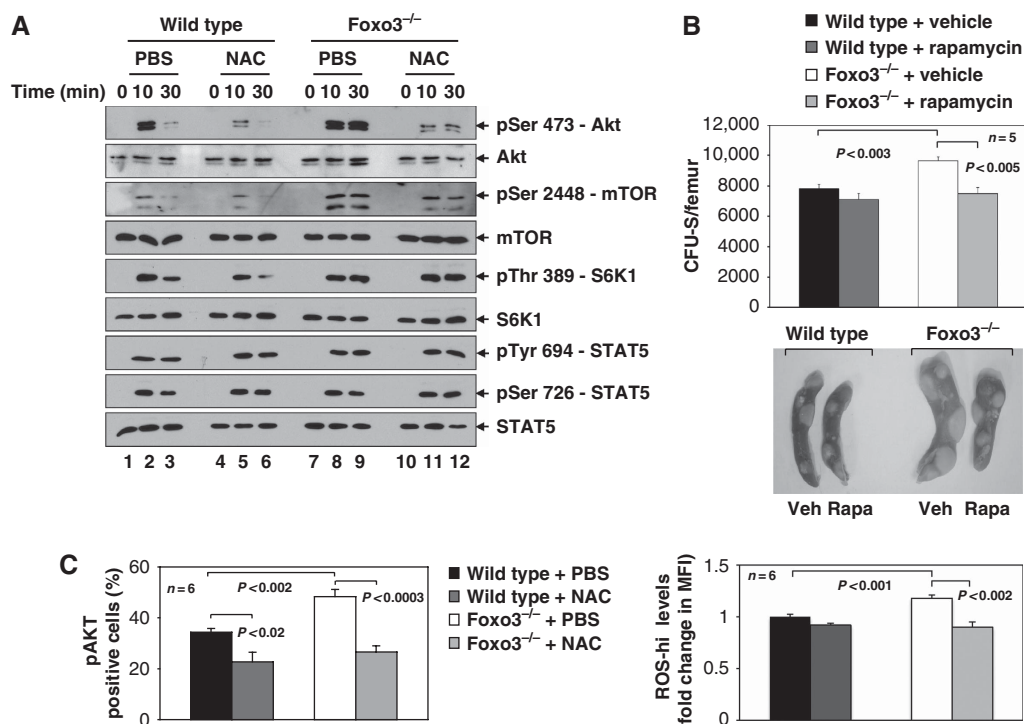


Figure 4 mTOR mediates the enhancement of Foxo3^{-/-} hematopoietic progenitor cell compartment. (A) Western blot analysis of phosphorylation of signalling proteins in lineage-negative bone marrow cells isolated from wild-type and Foxo3^{-/-} mice ($n = 4$). Mice were administered daily with NAC (100 mg/kg) or PBS *in vivo* for 3 days, after which lineage-negative cells were isolated, serum- and cytokine starved for 2 h and stimulated with IL-3 (20 ng/ml) for the indicated time points (0, 10 and 30 min) *in vitro* in the absence or presence of NAC (100 μ M) before preparing the whole cell extract; representative immunoblot of three independent experiments is shown. (B) Number of CFU-S_{d12}-derived colonies formed in the spleens of lethally irradiated mice reconstituted with 10⁵ wild-type or Foxo3^{-/-} bone marrow cells detected after 12 days during which mice were administered either rapamycin (Rapa; 4 mg/kg) or vehicle (Veh) intraperitoneally for 5 days a week. Results shown are mean \pm s.e.m. ($n = 5$ in each group, Student's *t*-test). One representative of three independent experiments is shown. Representative spleen from each group is shown in the lower panel. (C) ROS levels (right panel) and phosphorylated AKT protein kinase (left panel) were measured by FACS in Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ bone marrow cells of wild-type and Foxo3^{-/-} mice treated daily with NAC or PBS for 2 weeks *in vivo*, after which isolated cells were serum- and cytokine starved for 2 h and stimulated with IL-3 (20 ng/ml) *in vitro* for 10 min. Results are mean \pm s.e.m. of percentage of Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ bone marrow cells that express phosphoAKT (pAKT), as detected by FACS ($n = 6$ in each group, Student's *t*-test). Endogenous ROS-hi levels were measured in Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ bone marrow cells of the same mice as in the left panel, at the end of the 2 week *in vivo* treatment (right panel), and shown as fold change in MFI of experimental as compared with control wild-type cells ($n = 6$ in each group, Student's *t*-test). Animals were analysed individually. One of two independent experiments is shown.

response to NAC in Foxo3-mutant cells is not clear, it is possible that the residual pmTOR kinase activity, despite the presence of NAC (Figure 4A, lanes 11 and 12), is sufficient for phosphorylation of S6K1 to the same extent as controls, especially if the activity of a phospho-S6K1-specific phosphatase is reduced in Foxo3^{-/-} cells. NAC treatment did not alter STAT5 activity, as determined by phosphorylation on tyrosine 694 or serine 726 (Figure 4A).

Interestingly, NAC treatment specifically attenuated IL-3-mediated phosphorylation of AKT and S6K1 in wild-type HPs (Figure 4A), suggesting that ROS participate in specific cytokine signalling pathways in primary bone marrow cells *in vivo*. Next, we asked whether this data remains valid in populations of lineage-negative bone marrow cells that contain all myeloid but not lymphoid progenitors. We found that AKT was hyperphosphorylated in response to IL-3 in Foxo3^{-/-} myeloid (Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺) progenitors, as measured by flow cytometric analysis of intracellular pAKT (Figure 4C). NAC treatment reduced pAKT significantly in this highly enriched population of myeloid progenitors, in both wild-type and Foxo3^{-/-} mice (Figure 4C,

Supplementary Figure 6). As anticipated, *in vivo* treatment with NAC reduced ROS concentrations significantly in Foxo3^{-/-} Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ bone marrow cells (Figure 4C, right panel).

Importantly, and in agreement with the results above, *in vivo* administration of NAC normalized the number and the size of multipotential Foxo3^{-/-}-derived CFU-S_{d12} in lethally irradiated hosts without any significant impact on wild-type CFU-S_{d12} ($P < 0.0003$, $n = 5$; Figure 5). Taken together, these results indicate that ROS specifically amplify cytokine-mediated AKT/mTOR signalling pathway to stimulate the expansion of HPs in Foxo3^{-/-} mice.

To investigate whether accumulation of ROS in myeloid progenitors contributes to the pathogenesis of the myeloproliferative syndrome, we subjected wild-type and Foxo3-deficient mice to a short, 15-day treatment with NAC. Interestingly, *in vivo* NAC administration treated some of the myeloproliferative symptoms in Foxo3-deficient mice (Figure 6, Supplementary Figure 6). The *in vivo* treatment with NAC normalized the total number of bone marrow and spleen cells in Foxo3^{-/-} mice (Figure 6A). This treatment

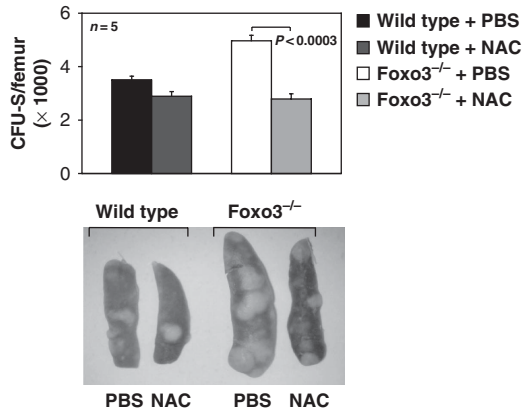


Figure 5 NAC treatment corrects the expansion of Foxo3^{-/-} primitive multipotential hematopoietic progenitor compartment *in vivo*. CFU-S-derived colonies were measured 12 days after injection of 10⁵ wild-type or Foxo3^{-/-} bone marrow cells into lethally irradiated hosts and treated daily with NAC (100 mg/kg) or PBS. One representative of three independent experiments is shown (*n* = 5 in each group, Student's *t*-test). Representative spleen of each group is shown in the bottom panel.

also had a specific and significant effect on the frequency and total number of immature myeloid (Mac-1/Gr-1 positive) cells in the bone marrow without any impact on B cells (Figure 6B and Supplementary Figure 6).

To identify the mechanism by which ROS regulate the myeloproliferative syndrome, we asked whether ROS impact the proliferation or apoptosis rate of Foxo3^{-/-} myeloid progenitor cells. Thus, mice were treated *in vivo* with NAC and the rate of 5-bromo-2-deoxyuridine (BrdU) incorporation in Lin⁻ IL7Rα⁻ Sca-1⁻ c-Kit⁺ cells was measured. We found that Foxo3^{-/-} myeloid progenitors exhibit a highly and significantly increased proliferation, as compared with their wild-type counterparts (Figure 6C, top panel). This was not accompanied by any modification of their apoptotic rate (Figure 6C, bottom panel). NAC treatment significantly reduced the percentage of cycling Foxo3-deficient myeloid progenitor cells (Figure 6C, top panel), without having any effect on their apoptotic rate (Figure 6C, bottom panel). ROS regulation of proliferation of Foxo3^{-/-} primitive myeloid progenitor cells is in accord with our earlier findings of ROS-induced amplification of AKT/mTOR signalling pathway

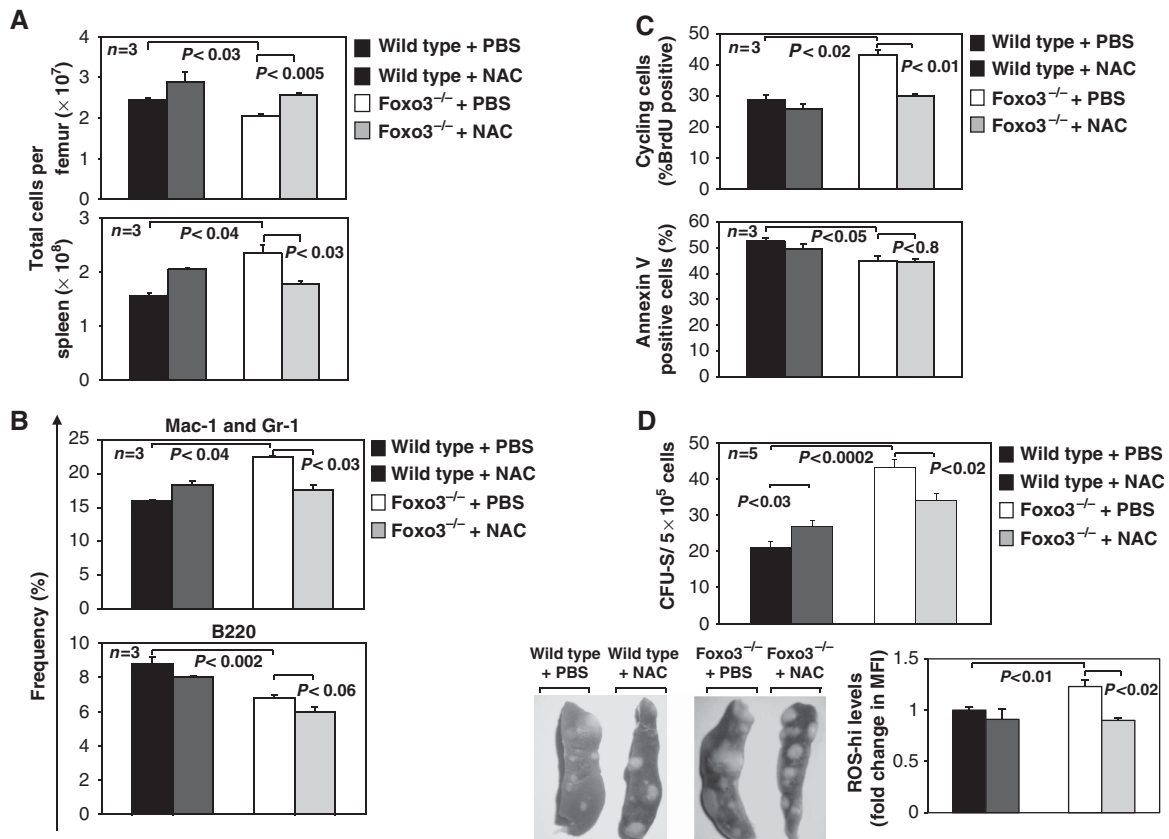


Figure 6 NAC treatment ameliorates myeloproliferative syndrome in Foxo3^{-/-} mice *in vivo*. (A) Mice were treated daily with NAC (100 mg/kg) or PBS and their total number in the bone marrow and spleen was measured after 15 days (*n* = 3 in each group). (B) Frequency of myeloid (Mac-1 and Gr-1 positive) and B (B220 positive) cells in the bone marrow of mice from A (*n* = 3 in each group). (C) Percentage of BrdU- (upper) and annexin-V-binding positive (lower) cells in wild-type and Foxo3^{-/-} Lin⁻ IL7Rα⁻ Sca-1⁻ c-Kit⁺ cells was analysed by flow cytometry after 15 days *in vivo* of NAC (100 mg/kg) or PBS treatment of mice from A (*n* = 3 in each group). (D) Wild-type or Foxo3^{-/-} mice were treated daily with NAC (100 mg/kg) or PBS for 2 weeks after which bone marrow cells were isolated and injected (5 × 10⁵ cells) into lethally irradiated hosts in the absence of any further treatment. CFU-S-derived colonies were counted in the hosts 12 days after cell injection, *n* = 5 in each group; Student's *t*-test. Representative spleen from each group is shown in the lower left panel. ROS-hi concentrations were measured in an aliquot of Lin⁻ IL7Rα⁻ Sca-1⁻ c-Kit⁺ cells isolated from each mouse 15 days after *in vivo* NAC or PBS treatment. ROS levels are shown as fold change in MFI as compared to control wild type cells (bottom right panel). One representative of two independent experiments is shown.

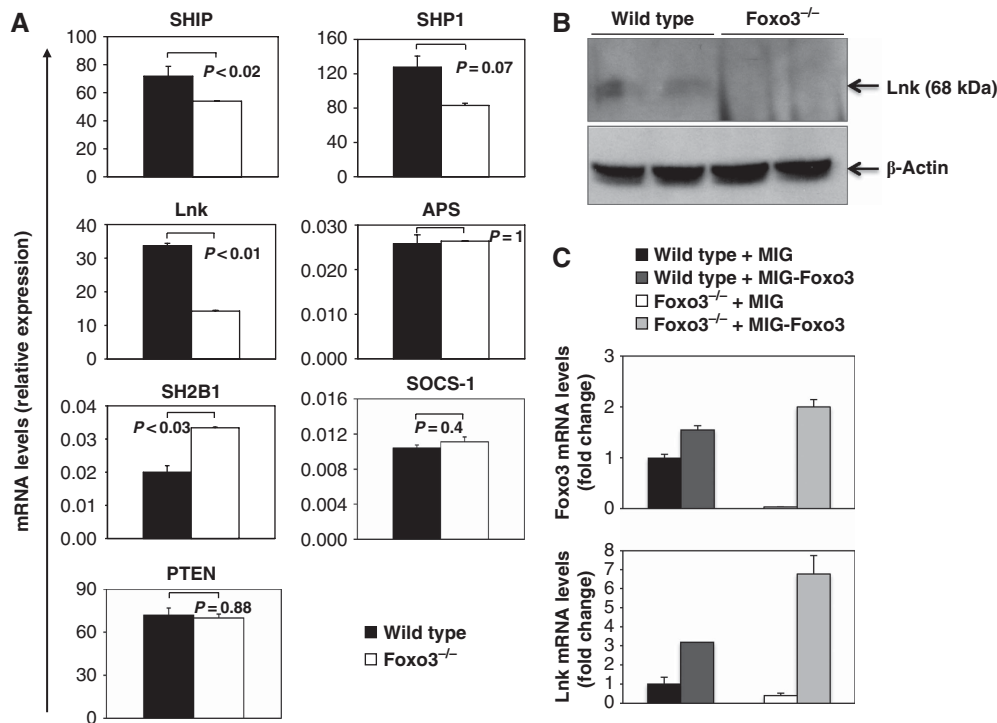


Figure 7 Deregulated expression of modulators of cytokine signalling in Foxo3^{-/-} hematopoietic progenitor cells. (A) QRT-PCR expression analysis of cytokine receptor signalling regulatory genes in lineage-negative wild-type and Foxo3^{-/-} cells. Quantification of target genes is relative to β-actin. Results are mean ± s.e.m. of duplicate analysis of at least three cDNAs, each generated from a pool of two to three wild-type or Foxo3^{-/-} mice; Student's *t*-test. (B) Western blot analysis of endogenous Lnk protein in total bone marrow cells isolated from two wild-type and two Foxo3^{-/-} mice; representative immunoblot of three independent experiments is shown. (C) QRT-PCR analysis of Foxo3 (upper panel) and Lnk (lower panel) expression in mononuclear cells derived from 5-FU-treated wild-type and Foxo3^{-/-} mice, transduced with MSCV-IRES-GFP (MIG) vector control or MIG-Foxo3; results are mean ± s.e.m. of three independent experiments.

in these cells (Figure 4). We further asked whether pretreatment with NAC is sufficient to normalize the activity of Foxo3^{-/-} primitive HPs in transplanted hosts *in vivo*. As shown in Figure 6D, pretreatment of Foxo3^{-/-} mice with NAC significantly decreased the number of multipotential CFU-S_{d12}-derived colonies formed in the spleen of lethally irradiated hosts (upper panel and lower left panel), likely through reduction of the levels of ROS in primitive myeloid progenitors (Figure 6D, bottom right panel). These results strongly suggest that the myeloproliferation in Foxo3^{-/-} mice is mediated by ROS and is sensitive to NAC. Altogether, these results indicate that the treatment with ROS scavenger NAC improves, at least partially, the myeloproliferative phenotype of Foxo3^{-/-} mice.

Lnk, a negative regulator of cytokine signalling, is directly implicated in enhanced Foxo3^{-/-} primitive HP cell activity

To further examine how Foxo3 regulates HP expansion, we used real-time QRT-PCR to compare the expression of numerous regulators of cytokine signalling in lineage-depleted (Lin⁻) cells from WT and Foxo3^{-/-} bone marrow. The function of some of negative regulators is known to be modulated by ROS (Thannickal and Fanburg, 2000); thus, we initially focused on negative regulators. The expression of most negative regulators of cytokine signalling was not significantly affected by loss of Foxo3. These include protein tyrosine phosphatase (SHP1, PTPN6) a negative regulator of JAK2 signalling, suppressor of cytokine signalling 1, which negatively regulates STATs, and PTEN, a negative regulator

of PI3-kinase/AKT signalling (Figure 7A). However, the expression of SH2-containing inositol-5-phosphatase (SHIP), another negative regulator of PI3-kinase signalling, was significantly reduced. In addition, the expression of the adaptor protein Lnk (SH2B3), a negative regulator of cytokine signalling (Takaki *et al*, 2002; Velazquez *et al*, 2002; Tong and Lodish, 2004; Tong *et al*, 2005), was significantly down-regulated in Foxo3^{-/-} lineage-negative progenitors, as determined by QRT-PCR (Figure 7A) and confirmed by western blot analysis (Figure 7B). Altered Lnk expression is due to loss of Foxo3, as overexpression of Foxo3 in primitive bone marrow cells significantly enhanced Lnk expression (Figure 7C). Lnk (SH2B3) belongs to a family of SH2-containing adaptor proteins, which also includes SH2-B1 and APS (SH2B2) (Rudd, 2001). Expression of SH2-B1, which is a positive regulator of cytokine signalling, was significantly increased in Foxo3-mutant cells (Figure 7A). Thus, deregulation of Lnk and SH2-B1 could contribute to the enhanced cytokine responses observed in Foxo3^{-/-} progenitors.

Lnk-deficient mice exhibit a myeloproliferative-like disorder similar to what we observe here with loss of Foxo3 (Takaki *et al*, 2002; Velazquez *et al*, 2002). Thus, we asked whether decreased expression of Lnk contributes to the myeloproliferation observed in Foxo3-mutant mice. To address this, mice were treated with high-dose 5-fluorouracil (5-FU), which ablates proliferating hematopoietic cells (Suda *et al*, 1983; Lemieux *et al*, 1995), and populations of bone marrow mononuclear cells highly enriched for primitive hematopoietic stem and progenitors were isolated and transduced with the bicistronic retroviral vector MSCV-IRES-GFP

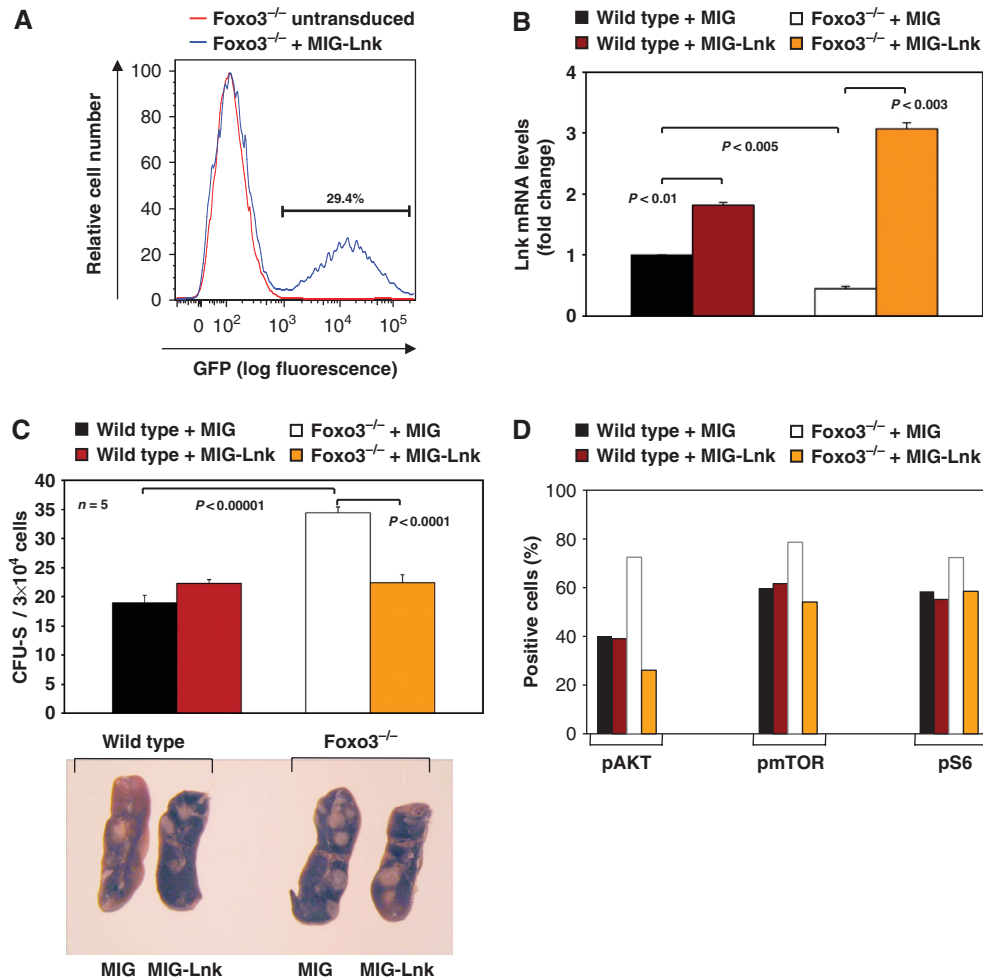


Figure 8 Decreased expression of Lnk is critical for increased activity of primitive hematopoietic progenitor cell compartment in Foxo3-deficient mice. **(A)** Flow cytometry profile of GFP expression in wild-type or Foxo3^{-/-} bone marrow lineage-negative cells derived from 5-FU-treated mice and transduced with MIG encoding for Lnk (MIG-Lnk; FACS profile was almost identical with MIG vector control). **(B)** QRT-PCR analysis of Lnk expression in FACS-sorted retrovirally transduced GFP⁺ gated cells from **A**; results are mean \pm s.e.m. of three independent experiments; Student's *t*-test. **(C)** Number of CFU-S-derived colonies formed in the spleens of lethally irradiated hosts ($n = 5$ in each group) reconstituted with 5-FU-treated bone marrow mononuclear cells transduced with retroviral vector MIG or MIG-Lnk. FACS-sorted GFP-positive (3×10^4)-transduced primitive hematopoietic wild-type or Foxo3^{-/-} cells (from **A**, **B**) were injected into lethally irradiated hosts and spleen colonies were counted 12 days later. Results shown as mean \pm s.e.m. ($n = 5$ mice); Student's *t*-test. Representative spleens are shown in the lower panel. One representative of three independent experiments is shown. **(D)** Quantification of phosphorylated AKT, mTOR and S6 proteins in mononuclear cells derived from 5-FU-treated wild-type or Foxo3^{-/-} mice transduced with MIG vector control or MIG-Lnk in response to IL-3. The percentage of cells with phosphorylated AKT, mTOR or S6 proteins was measured in GFP⁺ gated cells. Results shown are mean of two independent experiments.

(MIG) alone or with MIG encoding for Lnk (MIG-Lnk). Forced expression of Lnk (Figure 8A) was confirmed in an aliquot of transduced GFP-positive wild-type or Foxo3^{-/-} primitive hematopoietic cells (Figure 8B). Retrovirally transduced cells from Figure 8A and B were injected into lethally irradiated hosts and CFU-S_{d12} were measured after 12 days. Ectopic expression of Lnk in contrast to vector control normalized the number of CFU-S_{d12} derived from Foxo3-mutant mice detected in lethally irradiated hosts (Figure 8C), indicating that the relative loss of Lnk contributes to the overactivation of the CFU-S_{d12} compartment in Foxo3^{-/-} mice. This is likely by constraining cytokine-mediated hyperactivation of AKT/mTOR signalling pathway, as ectopic expression of Lnk, but not of vector control, in primitive Foxo3^{-/-} hematopoietic cells reduced the phosphorylation of AKT, mTOR and the S6K1-substrate S6 ribosomal protein in these cells in response to IL-3 (Figure 8D, Supplementary Figure 7).

Foxo3 could regulate Lnk expression through a number of different mechanisms. We could not detect any consensus FoxO binding site in regulatory regions of mouse *Lnk* gene that is conserved among mammals, suggesting the absence of direct transcriptional effects. Therefore, we investigated whether elevated ROS concentrations inhibit *Lnk* gene expression. Thus, mice were treated with NAC (100 mg/kg) or PBS for 3 days *in vivo*, and bone marrow myeloid progenitors (Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺) were analysed for Lnk expression. NAC treatment significantly increased the expression of *Lnk* mRNA in Foxo3^{-/-} myeloid progenitors, without having any impact on the expression levels of SHIP or SHP1 (Figure 9A). In addition treatment with NAC did not affect the expression of *Lnk* in wild-type cells. This was confirmed by western blot analysis of Lnk (Figure 9B). Consistent with these results, *in vitro* treatment of bone marrow cells with hydrogen peroxide reduced Lnk

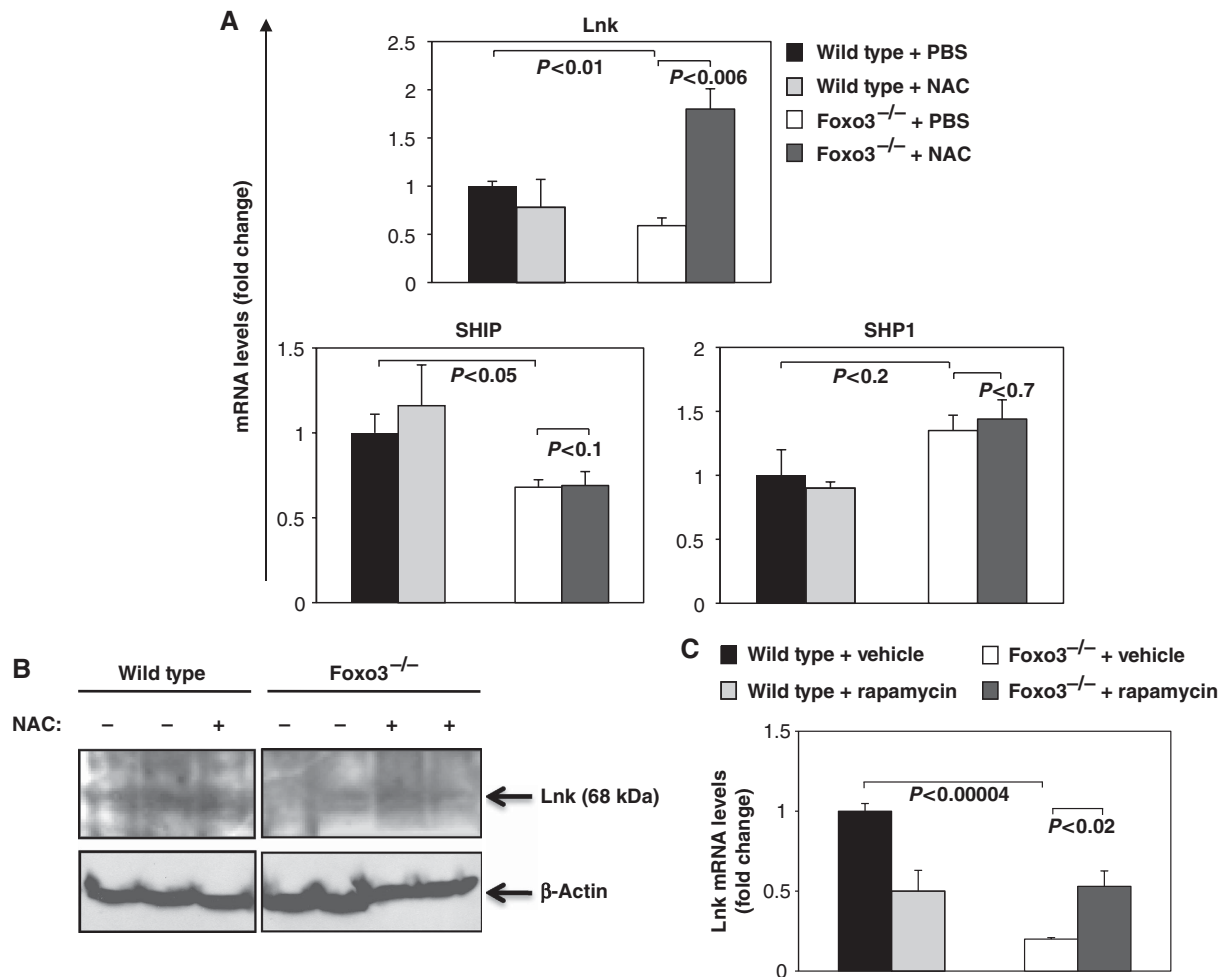


Figure 9 Regulation of Lnk expression is mediated by ROS in Foxo3^{-/-} primitive myeloid progenitor cells. (A) QRT-PCR gene expression analysis of regulators of cytokine signalling in Lin⁻ IL7Rα⁻ Sca-1⁻ c-Kit⁺ bone marrow cells from mice (*n* = 3) treated *in vivo* for 3 consecutive days with NAC (100 mg/kg) or PBS. One representative of three independent experiments is shown. (B) Western blot analysis of endogenous Lnk protein in total bone marrow cells isolated from wild-type and Foxo3^{-/-} mice daily treated with NAC (100 mg/kg) or PBS *in vivo* for 3 days. A representative immunoblot of two independent experiments is shown. (C) QRT-PCR analysis of Lnk expression in total bone marrow cells isolated from wild-type and Foxo3^{-/-} mice and treated *in vitro* with rapamycin (2 μM) or vehicle for 24 h (*n* = 3).

expression, although this effect was dependent on H₂O₂ concentration (Supplementary Figure 8). Given ROS-mediated activation of mTOR signalling (Figure 4), the effect of ROS on Lnk (Figure 9A, B and Supplementary Figure 8) and the impact of Lnk on AKT/mTOR signalling (Figure 8D and Supplementary Figure 7), we asked whether mTOR signalling has any impact on the expression of Lnk. As shown in Figure 9C, rapamycin treatment increased Lnk expression in bone marrow cells to some extent, suggesting that mTOR signalling participates in the regulation of Lnk expression. The mTOR regulation of Lnk expression might be through its control of ROS (Supplementary Figure 9). These results suggest that intracellular ROS concentrations are critical for the regulation of expression of the negative regulator of cytokine signalling Lnk.

Discussion

Excessive ROS concentrations lead to abnormal proliferation, growth and malignancies (Neumann *et al*, 2003; Ito *et al*, 2004). Here, we have identified deregulation of sensitivity of

cellular signalling to physiological ROS as an additional mechanism by which ROS compromise normal growth and lead to malignancies. Our key finding is that overaccumulated ROS in Foxo3^{-/-} mice exert a critical role in expansion of primitive HP cell compartment and the regulation of myeloproliferation in these mice. We have shown that Foxo3^{-/-} mice display a myeloproliferative syndrome characterized by splenomegaly, abnormal enhanced production of primitive HPs in hematopoietic organs, extramedullary hematopoiesis, high sensitivity of HPs to cytokines and significant increase in the production of white blood cells. This phenotype is reminiscent of myeloproliferative disorders and suggests that Foxo3 has a role in these diseases. In addition, sensitivity of the Foxo3^{-/-} myeloproliferative phenotype to ROS scavenger NAC suggests that this compound may have some effect in myeloproliferative diseases in general.

Overaccumulation of ROS is the principal mediator of the amplification of Foxo3^{-/-} primitive HP pool, as ROS scavenger NAC normalized the number of Foxo3^{-/-}-derived CFU-S_{d12} and the cycling of Foxo3^{-/-} primitive myeloid progenitors, and ameliorated the myeloproliferative syndrome phenotype of Foxo3^{-/-} mice (Figures 3–6). One could

hypothesize that potential DNA damage in ROS-hi subpopulation (Figure 3) of Foxo3^{-/-} Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cells may contribute to acquisition of growth-enhancing mutations in this cell population, leading to malignant progression.

Our results suggest that ROS regulation of Lnk is an integral part of balancing cytokine receptor signalling by regulating their sensitivity to various modulations of physiological ROS. Relative decrease of Lnk expression as a result of loss of Foxo3 leads to enhanced AKT/mTOR signalling pathway, increased cycling and expansion of Foxo3-mutant HP cells (Figure 8D and Supplementary Figure 7). Several recent reports establish a strong link between alteration of Lnk expression and myeloproliferative syndromes in mouse and man (Baran-Marszak *et al*, 2010; Bersenev *et al*, 2010; Oh *et al*, 2010), further highlighting the potential importance of ROS regulation of Lnk signalling pathway in understanding the underlying mechanism of myeloproliferative syndromes.

ROS are generated by oncoproteins and several cytokine and growth factor stimuli (Sundaresan *et al*, 1995; Thannickal and Fanburg, 1995; Irani *et al*, 1997; Sattler *et al*, 1999, 2000; Vafa *et al*, 2002; Zhu *et al*, 2006). Although the exact mechanism of generation of ROS by cytokine receptor signalling in non-phagocytic cells is not known, it is believed that by modifying the function of many signalling proteins, ROS participate in normal cellular signalling and regulate cell proliferation (reviewed in Thannickal and Fanburg, 2000; Ghaffari, 2008). Here, we have shown that, in addition to its known targets such as tyrosine phosphatase 1B (PTPB1, PTPN1) and PTEN (Hecht and Zick, 1992; Barford *et al*, 1994; Barrett *et al*, 1999a, 1999b; Meng *et al*, 2002, 2004; Savitsky and Finkel, 2002; Finkel, 2003; Seo *et al*, 2005), ROS regulate the expression of the adaptor protein Lnk. While ROS regulated the activity of these phosphatases, ROS control Lnk through modulation of expression of its transcript. Although the exact mechanism of this control is not known, ROS are known to modulate the expression and activity of several transcription factors including the AP1 complex (Thannickal and Fanburg, 2000). Lnk regulatory regions contain several evolutionarily conserved AP1 sites, suggesting that ROS might regulate Lnk expression through modulation of AP1 activity.

Interestingly, it was reported recently, while this paper was under preparation, that in *Drosophila*, Lnk negatively regulates lifespan via control of oxidative stress (Slack *et al*, 2010). In addition, these studies suggested that dFoxo represses Lnk expression directly. These findings are consistent with our results here, given that Lnk functions as a positive regulator of insulin receptor signalling and PI3-kinase in *Drosophila*, in contrast to its roles in mammals (Werz *et al*, 2009).

The high sensitivity of AKT and mTOR phosphorylation (Figure 4) in Foxo3^{-/-} myeloid progenitor compartment to the administration of anti-oxidants (Figures 5–6) suggests that the expansion of this population is by AKT/mTOR amplification and is mediated by ROS. ROS-mediated amplification of mTOR signalling pathway is consistent with the redox modulation of the interaction of mTOR with its regulator raptor (Sarbasov and Sabatini, 2005) and its repression of oxidative stress in hematopoietic cells (Chen *et al*, 2008). Collectively, these findings strongly suggest that loss of Foxo3 amplifies ROS-mediated cytokine signalling, in particular AKT/mTOR signalling pathway, in primitive myeloid progenitor cells *in vivo*. Importantly,

the short period of anti-oxidant treatments in all these experiments (Figures 4–6, 8) strongly suggests that a hematopoietic stem cell effect is unlikely.

AKT protein kinases are frequently activated in a broad array of tumours, including haematological malignancies (reviewed in Bellacosa *et al*, 2005; Bhaskar and Hay, 2007; Manning and Cantley, 2007). Here, we found a specific over-activation of AKT/mTOR signalling in response to cytokines that contributes significantly to the enhanced generation of primitive myeloid progenitors in Foxo3-deficient mice (Figures 3–6). In contrast to the overactivation of AKT/mTOR signalling, we did not detect any significant increase in phosphorylation of STAT5 (Ser 726 or Tyr 694), another major target of cytokine signalling, in response to cytokines in Foxo3-mutant cells (Figure 4A). One potential explanation might be that STAT5 follows a distinct phosphorylation kinetic in wild-type lineage-negative bone marrow cells, as compared with AKT or mTOR.

The phenotype in Foxo3^{-/-} mice resembles human myeloproliferative disorders that include chronic myeloid leukemia (CML), polycythemia vera, essential thrombocythemia and primary myelofibrosis. Constitutive suppression of Foxo3 is critical for growth and survival of cells transformed by BCR-ABL, the key mediator of CML pathogenesis (Ghaffari *et al*, 2003; Komatsu *et al*, 2003). Our present findings support the notion that constitutive suppression of Foxo3 activity by oncoproteins in myeloproliferative disorders such as by BCR-ABL in CML (Ghaffari *et al*, 2003; Komatsu *et al*, 2003; Naka *et al*, 2010) or, potentially, by JAK2V617F in polycythemia vera, may be a significant contributor to the pathogenesis of these disorders. In addition, our results in Foxo3-deficient mice recapitulate the myeloproliferative phenotype of the triple Foxo-deficient mice (Tothova *et al*, 2007). Together, these findings indicate that Foxo3 is the principal FoxO operating in hematopoietic stem, as we and others have previously reported (Miyamoto *et al*, 2007, 2008; Yalcin *et al*, 2008), and in progenitor cells, as shown in this study, and have an essential non-redundant function in hematopoietic homeostasis. In agreement with this, Foxo3 was recently shown to be critical for the maintenance of leukaemic stem cells in CML (Naka *et al*, 2010). Despite similarities in our findings, there are few distinctions between our results and that of Miyamoto *et al* (2007) on Foxo3 regulation of hematopoietic stem and progenitor cells, including lack of Foxo3^{-/-} myeloproliferation in Miyamoto *et al*'s report. These discrepancies are likely due to distinct mouse strains used. As previously reported (Dejean *et al*, 2009), loss of Foxo3 did not lead to lymphoproliferation at the steady state (Table 1, Figure 1 and Supplementary Figures 1 and 10), suggesting that the lymphoproliferative phenotype observed in Foxo3 gene-trap mice (Lin *et al*, 2004) might be due to the strategy or, as has been previously suggested (Dejean *et al*, 2009) to the mixed strain used to generate these mice.

On the basis of these results, we propose a working model that postulates that, in contrast to hematopoietic stem cells in which Foxo3 is constitutively active (Yamazaki *et al*, 2006; Yalcin *et al*, 2008), in HPs transcriptional activity and nuclear localization of Foxo3 are modulated by cytokine signalling and ROS. In turn, Foxo3 balances the regulation of cytokine signalling and ROS (see Figure 10). This model projects that ROS amplification of cytokine signalling participates in the activation of HP cells, producing more ROS that will mobilize

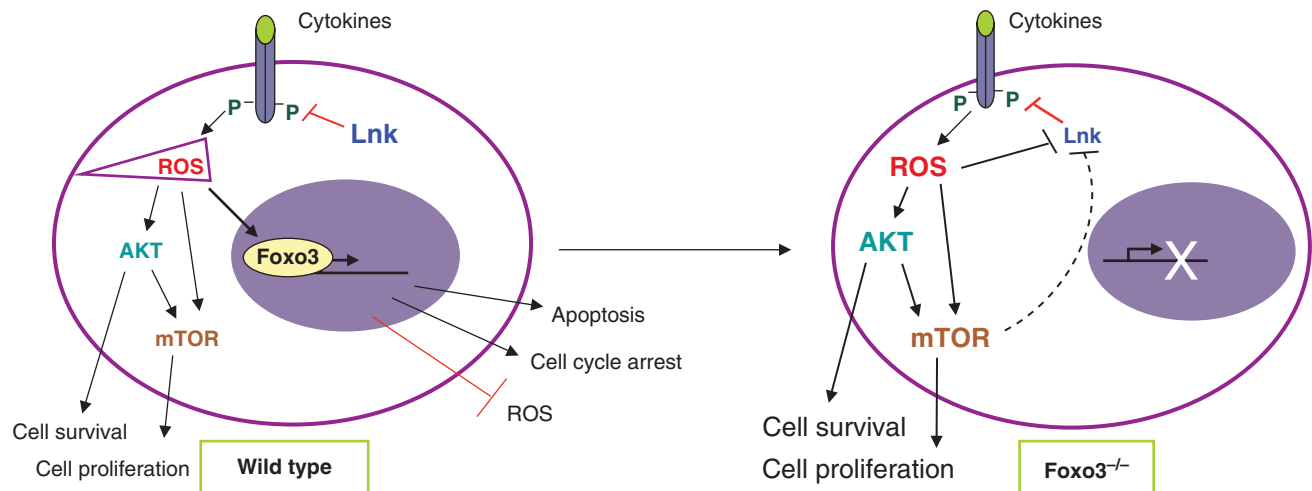


Figure 10 Model for Foxo3 regulation of hematopoietic progenitor cell activity. In hematopoietic progenitors, cytokine receptor signalling generates ROS that further activate receptor signalling (AKT/mTOR), leading to cell proliferation, increased production of ROS and ultimate induction of Foxo3 nuclear localization once ROS are accumulated above certain threshold. Signalling is modulated by cytokine receptor regulators including Lnk, a negative regulator. In Foxo3^{-/-} hematopoietic progenitors, decreased expression of Lnk, associated with significant increase in ROS accumulation, enhance cytokine-mediated signalling, leading to myeloproliferation.

Foxo3 to the nucleus once accumulated above a certain threshold. Indeed, it is known that ROS accumulation stimulates nuclear localization of FoxO via activation of multiple kinases (Greer and Brunet, 2008). Thus, according to this model, nuclear Foxo3 will in turn suppress ROS and/or induce apoptosis/cell cycle arrest in the progenitor population to maintain hematopoietic homeostasis.

Our combined results indicate that abnormal expression of Lnk coupled with accumulation of ROS leads to amplified cytokine signalling, in particular AKT/mTOR signalling pathway, and altered hematopoietic homeostasis in Foxo3^{-/-} mice. Thus, modulations of ROS accumulation significantly amplify alterations of cytokine signalling and may lead to malignancies. These findings may be critical in understanding the role of anti-oxidant pathways in promoting malignancies and cellular aging.

Materials and methods

Mice

The generation and genotyping of mice were performed as previously described (Castrillon *et al*, 2003). Progenies, aged 8–12 weeks, of Foxo3^{+/-} mice (129 × FBV/n) backcrossed to FVB/n were intercrossed with littermates to generate the experimental cohort (F6). In transplantation experiments, Foxo3^{+/-} mice backcrossed 10 generations onto C57BL6 were used. Wild-type littermates were used as controls in all experiments. Protocols were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine.

Cells

Blood samples were collected and lineage-negative cells were separated using a mouse progenitor cell enrichment kit (StemCell Technologies, Canada), as previously described (Marinkovic *et al*, 2007; Yalcin *et al*, 2008). Bone marrow mononuclear cells were isolated using lympholyte M density separation medium according to manufacturer's protocol (Cederlane, Hornby, Ontario, Canada).

In vitro clonogenic progenitor assay

Myeloid clonogenic assays were performed as previously described (Ghaffari *et al*, 2006; Zhao *et al*, 2006; Yalcin *et al*, 2008). Cells (5×10^6 peripheral blood, 5×10^5 spleen and 3×10^4 bone marrow cells) were cultured in MethoCult 3234 (StemCell Technologies)

containing 50 ng/ml rat stem cell factor (SCF), 10 ng/ml IL6, 10 ng/ml IL3 and 3 U/ml Epo (all from PeproTech EC, Rocky Hill, NJ, USA). Colonies were counted after 8–10 days.

Colony-forming unit spleen assay day 12

Bone marrow (1×10^5) cells from 8 to 10-week-old wild-type or Foxo3^{-/-} littermates or GFP-positive transduced cells were isolated and intravenously injected into recipient C57BL6 mice (Charles River Laboratory) previously subjected to 10 Gy irradiation. Recipient spleens were excised 12 days later, fixed in Telleyesniczky's solution and macroscopic spleen colonies were counted as described (Till and Mc, 1961).

Flow cytometry and cell sorting

Antibody staining and flow cytometry analysis were previously described (Marinkovic *et al*, 2007; Yalcin *et al*, 2008). For CMP isolation (Lin⁻ IL-7R α ⁻ Sca-1⁻ c-Kit⁺ Fc γ R^{low}CD34⁺) (Akashi *et al*, 2000), cells were stained with anti-c-Kit (BD Biosciences), anti-FcR (eBioscience) and anti-CD34 (eBioscience) antibodies directly conjugated with fluorescein isothiocyanate, phycoerythrin or allophycocyanin, and with biotinylated multi-lineage monoclonal antibody cocktail (StemCell Technologies) as well as biotinylated antibodies against IL-7R (eBioscience) and Sca-1 (BD Biosciences) visualized with PECyc7-Streptavidin or Pacific Blue-Streptavidin.

To measure intracellular AKT phosphorylation, Lin⁻ IL-7R α ⁻ Sca-1⁻ c-Kit⁺ cells were fixed with fix/permeabilization buffer (BD Biosciences) and incubated with 1:50 dilution of anti-pSer473 AKT antibody (Cell Signaling Technology). To measure protein phosphorylation in response to Lnk expression, transduced cells were starved *in vitro* in Iscove's modified Dulbecco's medium (IMDM) with 0.1% FCS for 2 h, stimulated with IL-3 (10 ng/ml) and fixed, and optimum phosphorylation was detected by flow cytometry (15 and 45 min for pmTOR/pS6 and pAKT, respectively). Cells were incubated with 1:100 dilution of anti-pSer473 AKT, pSer2448 mTOR and pSer235/236 S6 antibodies (Cell Signaling Technology). Samples were washed and protein phosphorylation was analysed in GFP⁺-gated cells by flow cytometry.

Cell proliferation and apoptosis assays

Mice were injected intravenously with 2 mg of BrdU. At 19 h post injection, bone marrow Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cells were fixed and stained with anti-BrdU antibody (BD Biosciences) for flow cytometric analysis of cell proliferation.

Freshly isolated Lin⁻ IL7R α ⁻ Sca-1⁻ c-Kit⁺ cells were assayed for annexin-V (BD Biosciences) binding to measure apoptotic cells.

Measurement of intracellular ROS

ROS were measured as previously described (Marinkovic *et al*, 2007; Yalcin *et al*, 2008).

RNA isolation and QRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized using SuperScript (Invitrogen). Quantitative RT-PCR was performed using SYBR Green JumpStart *Taq* ReadyMix (Takara) in duplicates using ABI Prism 7900 HT Cyclor (Applied Biosystems). Gene-specific primers spanning intron-exon boundary were designed by Primer Express 2.0 (ABI) and subjected to BLAST analysis to ensure the primer specificity. The PCR cycle parameters were as follows: 95°C for 10" followed by 45 cycles at 95°C for 5", 60°C for 34" and 72°C for 30". Relative quantification was achieved using the sequence detection system software (Applied Biosystems) and a fractional cycle number at which threshold fluorescence was obtained (threshold cycle, CT); for each analysis, comparative CT method for quantification of the target genes relative to β -actin as the reference was used. Results shown as fold change are relative to wild type controls. Primer sequences are listed in Supplementary Table 1.

Western blot analysis

Freshly isolated lineage-negative bone marrow cells were starved *in vitro* for 2 h in IMDM with 0.1% FCS and then stimulated with IL-3 (20 ng/ml) for the indicated time points. Cells were harvested and lysates were prepared in 1 \times RIPA lysis buffer (20 mM sodium phosphate, 300 mM sodium chloride, 4 mM EDTA) containing 2% sodium deoxycholate, 2% NP-40, 0.2% SDS, 400 μ M sodium orthovanadate, 0.2% β -mercaptoethanol, 2 mM PMSF and 100 mM sodium fluoride and protease cocktail inhibitors (Roche). Samples were run on SDS-PAGE, blotted and probed with following antibodies at 1:1000 dilutions: anti-pSer473 AKT, anti-AKT, anti-pThr389 S6Kinase, anti-S6Kinase, anti-pSer2448 mTOR, anti-mTOR and anti-pTyr694 STAT5 (all from Cell Signaling Technology) and anti-Lnk (M-20; Santa Cruz). Anti-pSer726 STAT5 (Upstate Biotechnology) and anti-STAT5 (BD Biosciences) were used at final concentrations of 1:500 and 1:200, respectively.

Histology

Spleen and liver tissues from 11-week-old wild-type or FoxO3^{-/-} littermates were collected and paraffin embedded after 10% formalin fixation. Sections (8–10 μ m) were stained with hematoxylin and eosin for histology analysis.

In vivo treatment with N-acetyl-L-cysteine or rapamycin

Mice received intraperitoneal administration of 100 mg/kg of body weight NAC (Sigma, St. Louis, MO, USA) in PBS (pH 7.4) for the indicated time period, as previously described (Yalcin *et al*, 2008). Cultured cells were incubated with NAC (100 μ M) for the indicated

time period. Rapamycin (Sigma) was administrated intraperitoneally (4 mg/kg in PBS containing 5% Tween 80, 5% PEG 400 and 4% ethanol) for 5 days a week (Yilmaz *et al*, 2006).

For *in vitro* rapamycin treatment, total bone marrow cells isolated from wild-type and Foxo3^{-/-} mice were cultured with rapamycin (final concentration 2 μ M) or vehicle for 24 h after which mRNA was isolated for QRT-PCR analysis.

Retroviral production and transduction of 5-FU-treated bone marrow mononuclear cells

Retroviral supernatants were produced as previously described (Zhao *et al*, 2006). Bone marrow mononuclear cells isolated from mice treated 4 days previously with 5-FU (150 mg/kg, Sigma) were pre-stimulated for 2 days in IMDM containing 15% heat-inactivated FCS supplemented with IL-6 (10 ng/ml), IL-3 (6 ng/ml) and SCF (100 ng/ml; PeproTech EC), after which cells were resuspended in retroviral supernatants (multiplicity of infection of 10) for 2 consecutive days and plated on retronectin-coated dishes in IMDM-15% FCS containing the same factors. At 48 h after initiation, live GFP-positive cells were FACS sorted and used for experiments.

Statistical analysis

The unpaired two-tail Student's *t*-test was used for all experiments. A *P*-value of <0.05 was considered to be significant.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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