Foxo3 Is Essential for the Regulation of Ataxia Telangiectasia Mutated and Oxidative Stress-mediated Homeostasis of Hematopoietic Stem Cells*[S]

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Unchecked accumulation of reactive oxygen species (ROS) compromises maintenance of hematopoietic stem cells. Regulation of ROS by the tumor suppressor protein ataxia telangiectasia mutated (ATM) is critical for preserving the hematopoietic stem cell pool. In this study we demonstrate that the Foxo3 member of the Forkhead Box O (FoxO) family of transcription factors is essential for normal ATM expression. In addition, we show that loss of Foxo3 leads to defects in hematopoietic stem cells, and these defects result from an overaccumulation of ROS. Foxo3 suppression of ROS in hematopoietic stem cells is mediated partly by regulation of ATM expression. We identify ROS-independent modulations of ATM and p16^{INK4a} and ROS-mediated activation of p53/p21$^{CIP1/WAF1/Sdi1}$ tumor suppressor pathways as major contributors to Foxo3-null hematopoietic stem cells defects. Our studies demonstrate that Foxo3 represses ROS in part via regulation of ATM and that this repression is required for maintenance of the hematopoietic stem cell pool.

Reactive oxygen species (ROS)[3] such as superoxide anions and hydrogen peroxide are by-products of oxidative metabolism and are involved in many signaling processes (1). However, excess accumulation of ROS resulting from defects in ROS scavenging is believed to impact cellular aging and the senescence process (1), whereas the ability to withstand oxidative stress has been correlated with enhanced longevity in several species (1). In agreement with deleterious effects of ROS, abnormal ROS accumulation has been implicated in the pathogenesis of various diseases, including bone marrow failure and disorders with high susceptibilities to malignancies such as ataxia telangiectasia, Fanconi anemia, as well as malignancies of hematopoietic tissues (2–5).

Regulation of ROS by ATM is essential for hematopoietic stem cell self-renewal, because loss of ATM leads to depletion of the hematopoietic stem cell pool and bone marrow failure in old mice in a ROS-dependent manner (2). ATM serine/threonine protein kinase is a critical enzyme in the regulation of stress response to DNA damage, specifically double strand DNA break (6). Loss of function mutations in ATM is associated with ataxia telangiectasia that is an autosomal disorder characterized by neuronal degeneration, immunodeficiencies, genomic instability, predisposition to lymphomas and other malignancies, and extreme sensitivity to ionizing radiation (7). Although ROS are found accumulated in several cell types and tissues of ataxia telangiectasic patients (8) and antioxidant enzymes are either up-regulated or down-regulated in ATM mutant cells, the mechanism of ATM regulation of ROS is not clear (6, 9).

The forkhead homolog DAF-16 is a major mediator of defense against oxidative stress in *Caenorhabditis elegans* (10). FOXO1, FOXO3a, FOXO4, and FOXO6 are the human homologs of DAF-16 and belong to the forkhead FoxO family of winged helix transcription factors. Although FOXO6 is predominantly expressed in the brain, FOXO1, FOXO3a, and FOXO4 are ubiquitous. As with DAF-16 in *C. elegans*, FoxO transcription factors are critical regulators of oxidative stress in mammals (11, 12). In particular, activated FOXO3a up-regulates ROS scavenging enzymes in cultured cells (13, 14) and is required for optimum expression of these enzymes in primary mouse erythroid cells (12). FOXO3a responds to cellular stress (including but not limited to oxidative stress) by inducing cell cycle arrest at G_1-S and G_2-M checkpoints, repair of damaged DNA, and apoptosis via up-regulation of genes that control these processes (reviewed in Ref. 15).

FoxO proteins are negatively regulated by the highly conserved PI 3-kinase/AKT signaling pathway (15). In response to

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures," Fig. 1, and Table 1.
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[3] The abbreviations used are: ROS, reactive oxygen species; ATM, ataxia telangiectasia mutated; MEF, mouse embryonic fibroblast; WT, wild type; IRES, internal ribosome entry site; FCS, fetal calf serum; LTC-IC, long term culture-initiating cell; NAC, *N*-acetyl-L-cysteine; 5-FU, 5-fluorouracil; IL, interleukin; BrdU, bromodeoxyuridine; QRT, quantitative real time; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; SOD, superoxide dismutase; shRNA, short hairpin RNA; DAPI, 4′,6-diamidino-2-phenylindole; CDK, cyclin-dependent kinase; PI, phosphatidylinositol; HSC, hematopoietic stem cell(s); LT-HSC, long term HSC; ST-HSC, short term HSC; BM, bone marrow.

factors such as hematopoietic cytokines (16–19) or oncogenic stimuli such as BCR-ABL oncoprotein (19), the AKT family of serine/threonine protein kinases phosphorylates nuclear FoxO and mediates their translocation to the cytosol where FoxO is unable to induce the expression of their transcriptional targets (16).

In response to oxidative stress, phosphorylation of FoxO mediated by mammalian sterile 20-like kinase-1 (MST1) (20) and Ral/Jun N-terminal kinase-dependent pathway results in FoxO nuclear translocation and activation (21). Identification of FoxO at sites of chromosomal translocations in human tumors, including acute myeloid leukemias, suggested a role for these factors in tumorigenesis.

Under certain conditions of stress, such as when food is scarce, DAF-16 is activated to promote the dauer formation. We found the characteristics of the dauer that is in "juvenile form, resilient, long lived, and reproductively immature" (10), to resemble in many respects the "quiescent" state of stem cells. Because quiescence is (*a*) a major characteristic of stem cells, (*b*) reminiscent of dauer formation, and (*c*) protected from apoptosis by antioxidant function of FoxO homolog DAF-16, we postulated that the Foxo3 (the mouse homolog of FOXO3a) transcription factor that is the most highly expressed FoxO in the bone marrow (12) may protect mammalian stem cells from oxidative stress.

Recently, conflicting data as to the function of Foxo3 in the regulation of hematopoietic stem cell activity have been reported (11, 22). Studies by Tothova *et al.* (11) found a loss of hematopoietic stem cell activity only when all three Foxo1, Foxo3, and Foxo4 were deleted in mice. These authors did not find any hematopoietic stem cell abnormalities as a result of loss of Foxo3 alone (11). Conversely, Miyamoto *et al.* (22) found that Foxo3 is essential for the maintenance of hematopoietic stem cell activity. However, at steady state, these authors did not detect a significant defect of hematopoietic stem cell frequency in young mice (22). Here we provide our data showing that Foxo3 is essential for the regulation of hematopoietic stem cell frequency and activity in young mice. We further demonstrate the following: (*a*) Foxo3 is critical for the regulation of oxidative stress in hematopoietic stem cells, and (*b*) Foxo3 modulation of oxidative stress mediates Foxo3 regulation of hematopoietic stem cell activity. Our *in vivo* and *ex vivo* data demonstrate that loss of Foxo3 results in ROS accumulation that mediates activation of $p53/p21^{CIP1/WAF1/Sdi1}$ and in alterations of hematopoietic stem cell cycling, specifically exit from quiescence and G_2/M arrest.

Furthermore, we demonstrate that Foxo3 is required for the expression of ATM gene that is an essential factor for hematopoietic stem cell self-renewal. Using genetic loss and gain of function approaches by RNA interference and retroviral transduction of primitive hematopoietic stem cells, we demonstrate that ATM deficiency participates in perturbations of ROS regulation in Foxo3-null hematopoietic stem cells.

These novel findings establish Foxo3 as a physiological regulator of oxidative stress in hematopoietic stem cells, demonstrate that Foxo3 modulation of ATM regulates oxidative stress in hematopoietic stem cells, and provide insight into the mechanism of Foxo3 regulation of stem cell cycling. We discuss what

we believe is the source of discrepancies between our results and previous reports (11, 22) and present our model of Foxo3 regulation of hematopoietic stem cells.

EXPERIMENTAL PROCEDURES

Mice—The generation and genotyping of mice were performed as described previously (23). Briefly, heterozygotes $Foxo3^{+/-}$ mice (129×FVB/n) were backcrossed to FVB/n (F3), and progenies (that were Cre^-) were intercrossed with littermates to generate the experimental cohort (23). 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—Mononuclear cells from blood, spleen, and bone marrow were prepared using lympholyte M (Cederlane, Hornby, Ontario, Canada). $ATM^{-/-}$ and wild type (WT) mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated bovine serum.

Retroviral Constructs and Plasmids—The full-length human ATM cDNA from pMAT1 (24) was inserted upstream of the IRES in the XhoI site of MSCV-IRES-GFP (MIG) vector to construct MIG-ATM. MIG-Foxo3 was described previously (19), to construct pL4-shRNA Foxo3, synthesized shRNA oligomers targeting Foxo3 were annealed and cloned into EcoRI/XhoI site downstream of H1 RNA polymerase III promoter of pL4 (Invitrogen) vector.

Flow Cytometry and Hematopoietic Stem Cell Isolation—For Lin^- $Sca-1^+$ $c-kit^{high}$ (LSK) cells, total bone marrow cells were preincubated with 5% rat serum, c-Kit-APC, and Sca-1-PE (BD Biosciences) and biotinylated hematopoietic multilineage monoclonal antibody mixture (StemCell Technologies) containing CD5 (lymphocytes), CD11b (leukocytes), CD19 (B cells), CD45R (lymphocytes), 7-4 (neurophils), Ly-6G-Gr-1 (granulocytes), TER119 (erythroid cells) antibodies to remove mature cells and incubated with PECyc7-streptavidin secondary antibody. In addition to LSK staining, total bone marrow cells were stained with FITC-CD34 and PECyc5-Flk2 antibodies (eBiosciences) to subfractionate the long term and short term HSC (Lin^- $Sca1^+$ $c-Kit^{high}$ $CD34^{-/low}$ $Flk2^{low}$ and Lin^- $Sca1^+$ $c-Kit^{high}$ $CD34^+$ $Flk2^{low}$), respectively. Cell sorting was performed using Influx (Cytopeia, Inc.) equipped with Spigot software version 53.8.

Measurement of Intracellular ROS—An aliquot of cells (5×10^5) first stained for LSK was used for ROS measurement. Cells were resuspended in pre-warmed phosphate-buffered saline, 2% FCS and loaded with 5 μM 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) incubated in the dark for 20 min at 37 °C, 5% CO_2. The oxidative conversion of 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate to its fluorescent product is measured immediately by flow cytometry. In these experiments, bone marrow cells incubated with H_2O_2 (500 μM) were used as positive controls.

Long Term Culture-initiating Cell (LTC-IC) Assay—Long term cultures were initiated with bone marrow cells (1×10^5) cocultured on preestablished S17 stromal feeders in MyeloCult M5300 (StemCell Technologies) containing freshly added hydrocortisone (10^{-6} M) and carried as described previously

(25). *N*-Acetyl-L-cysteine (NAC) (100 μM) was added weekly in specified experiments.

Long Term Repopulation Assay—In these experiments Foxo3$^{+/-}$ (CD45.1, H2-Kq$^+$) mice backcrossed onto C57BL6 (CD45.1, H2-Kb$^+$ purchased from NCI, National Institutes of Health) (N5) were used. Wild type littermates were used as controls. Lethally irradiated (12 gray as a split dose) congenic C57BL6-CD45.2 mice (Charles River Laboratory) were reconstituted with intravenous injections of donor total bone marrow cells from 8- to 10-week-old wild type control (CD45.1, 2×10^5 cells) or Foxo3$^{-/-}$ (also CD45.1, 1×10^6 cells) and 2×10^5 competitor bone marrow cells (CD45.2). Reconstitution of donor-derived cells were distinguished from host cells by the expression of CD45.1 *versus* CD45.2 antigens (BD Biosciences). For the experiment in Fig. 2*e*, Foxo3$^{+/-}$ (CD45.1) mice backcrossed onto C56BL6 (CD45.1, F10) were used. FACS-sorted 100 Lin$^-$ c-kit^{ihigh} Sca-1$^+$ CD34$^-$ bone marrow cells from wild type and Foxo3$^{-/-}$ mixed with 2×10^5 total bone marrow of congenic C57BL6 –CD45.2 were injected into lethally irradiated (12 gray as a split dose) congenic C57BL6 –CD45.2.

N-Acetyl-L-Cysteine Treatment—Mice were injected intraperitoneally with 100 mg/kg body weight (Sigma) in phosphate-buffered saline solution, pH 7.4, every day for the indicated time period. For cultured cells, NAC (100 μM) was used for the indicated time.

Retroviral Production and Transduction of Bone Marrow Mononuclear Cells—Retroviral supernatants were produced as described previously (26). Bone marrow mononuclear cells were isolated from WT and Foxo3$^{-/-}$ (8–10 week-old) littermates treated 4 days previously with 5-fluorouracil (5-FU) (150 mg/kg, Sigma) using lympholyte-M (Cedarlane). Cells were prestimulated for 2 days in Iscove's modified Dulbecco's medium containing 15% heat-inactivated FCS supplemented with IL-6 (10 ng/ml), IL-3 (6 ng/ml), and stem cell factor (100 ng/ml) (PeproTech EC), after which cells were resuspended in retroviral supernatants (multiplicity of infection of 10) for 2 consecutive days, as described previously (27), and plated on retronectin-coated dishes in Iscove's modified Dulbecco's medium, 15% FCS containing the same factors. Forty eight hours after the initiation, live GFP-positive cells were FACS-sorted and lysed, and total RNA was isolated for gene expression analysis.

Cell Cycle Analysis—Mice were injected intravenously with 2 mg of 5-bromo-2-deoxyuridine (BrdU). At 19 h post-injection, bone marrow cells were collected, stained for LSK, fixed, and stained with 7-amino-actinomycin D and anti-BrdU antibody (Pharmingen) for analysis. The G_0/G_1 analysis was performed as described (28), and FACS-sorted LSK populations were collected in Hanks' buffered salt solution medium containing 10% FCS, 1 g/liter glucose, 20 mM Hepes, pH 7.2, 50 μg/ml verapamil. Cells were then washed; Hoechst 33342 (20 μg/ml, Invitrogen) was added, and cells were immediately incubated at 37 °C for 45 min after which pyronin Y (1 μg/ml, Sigma) was added. Cells were incubated for another 15 min at 37 °C, washed in cold medium, and resuspended in medium containing 1 μg/ml of propidium iodide. Samples were immediately analyzed by flow cytometry (BD Biosciences LSRII).

RNA Isolation and Quantitative Real Time (QRT)-PCR—RNA isolation and QRT-PCR were conducted as described (12). In brief, total RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized using SuperScript (Invitrogen), QRT-PCRs were performed using SYBR® Green JumpStart^{TM} Taq ReadyMix (Sigma) in duplicate (Light Cycler 2.0, Roche Diagnostics). Gene-specific primers span the intron-exon boundary by Primer Express 2.0 (ABI). Relative quantification was achieved by normalization against endogenous β-actin. For primer sequence, see supplemental Table 1. In NIH 3T3 and MEF experiments, TRIzol (Invitrogen catalog number 15596-026) was used for total RNA extraction.

Western Blot Analysis—Lineage-negative WT, Foxo3-deficient bone marrow cells or cells transfected with pL-4 plasmid encoding for shRNA targeting Foxo3 and harvested after 96 h (time required for RNA interference effect to take place) were lysed in Laemmli buffer (5 ml of 4× Tris-HCl, pH 6.8, 4 ml of glycerol, 0.8 g of SDS, 0.4 ml of β-mercaptoethanol, 0.01 g of bromphenol blue), resolved on 6.5% SDS-PAGE, and transferred to nitrocellulose membranes that were incubated with the indicated antibodies as follows: 1) FOXO3a (Upstate Biotechnology Inc., 07-702, 1:200); 2) ATM (GeneTex, GTX70103, 1:500), and 3) JAK2 (Upstate Biotechnology Inc., 06-255, 1:500). Band intensities were quantified by ImageQuant TL software (Amersham Biosciences).

Immunofluoresence Staining—LSK cells were FACS-sorted and cytospun onto glass slides fixed with 4% paraformaldehyde. After washing with phosphate-buffered saline, cells were permeabilized using 3% bovine serum albumin, 0.4% Triton X-100 and then incubated in 1% bovine serum albumin incubated overnight with the following: 1) anti-FOXO1 (Santa Cruz Biotechnology), 2) anti-FOXO3a (Upstate Biotechnology Inc.), 3) active anti-caspase-3 (Promega), or 4) anti-FOXO4 (Santa Cruz Biotechnology) antibodies (1:250 dilution for each). Cells were washed and stained with Alexa Fluor 594 anti-rabbit IgG antibody (1:400). For ATM-phosphoserine 1981 staining, LSK cells were incubated with anti-ATM phosphoserine 1981 mouse monoclonal antibody (Novus Biologicals) 1:500 dilution in 1% goat serum. Cells were washed and stained with Alexa Fluor 594 anti-mouse IgG antibody (1:1000). Nuclei were stained with Vectashield-DAPI (Vector Laboratories). Images were captured using Nikon EclipseE600 microscope and processed with Photoshop software. Data were quantified by Colocalizer Pro.2 software and Overlap coefficient according to Manders *et al.* (50). ATM phosphorylation signal was quantified using Velocity software.

RESULTS

ROS-mediated Hematopoietic Stem Cell Deficiency in Foxo3-null Mice—Foxo3 is the most highly expressed FoxO in the bone marrow (12). Nuclear localization is often used as a surrogate assay for FoxO activity. Hematopoietic stem cells are highly limited in numbers, and only few cells can be obtained from each mouse (average of 5000 cells from two mice). We analyzed FoxO expression by both QRT-PCR and immunostaining in hematopoietic stem cells. Analysis of FoxO subcellular localization showed that although both Foxo1 and Foxo3
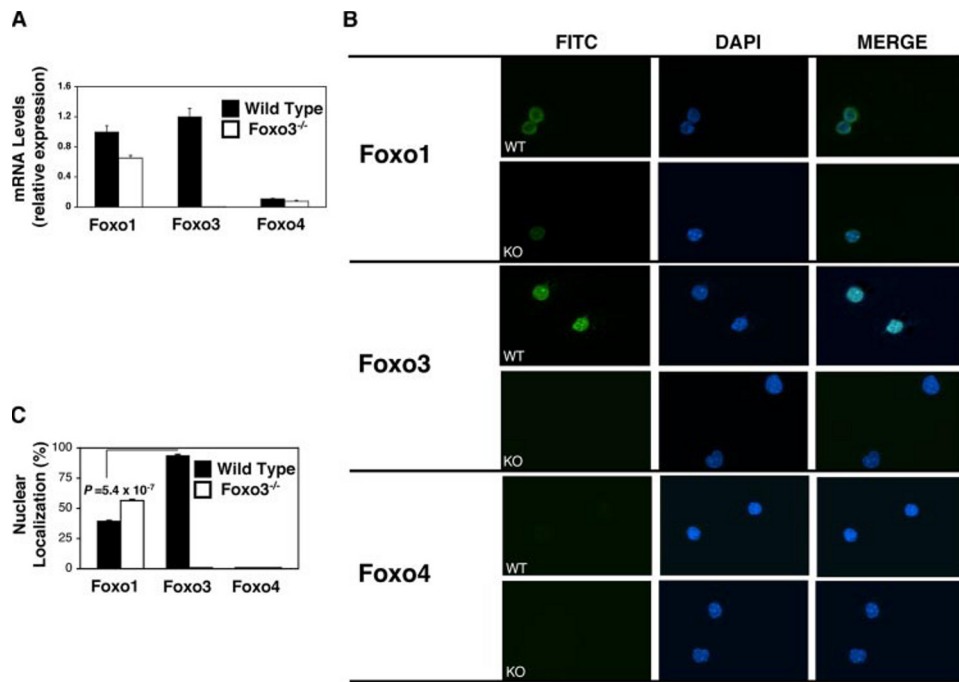
FIGURE 1. **Foxo3 is the principal active FoxO in hematopoietic stem cells.** A, QRT-PCR analysis of FoxO expression in WT and Foxo3$^{-/-}$ LSK cells. Relative quantification was achieved by normalization against endogenous β-actin. B, FoxO immunostaining of primitive hematopoietic stem (LSK) cells. Cells are FACS-sorted and immunostained with anti-FOXO1, anti-FOXO3a, or anti-FOXO4 antibodies and counterstained with DAPI. C, quantification of nuclear colocalization with DAPI as measured by Colocalizer Pro.2 software and Overlap coefficient according to Manders et al. (50).
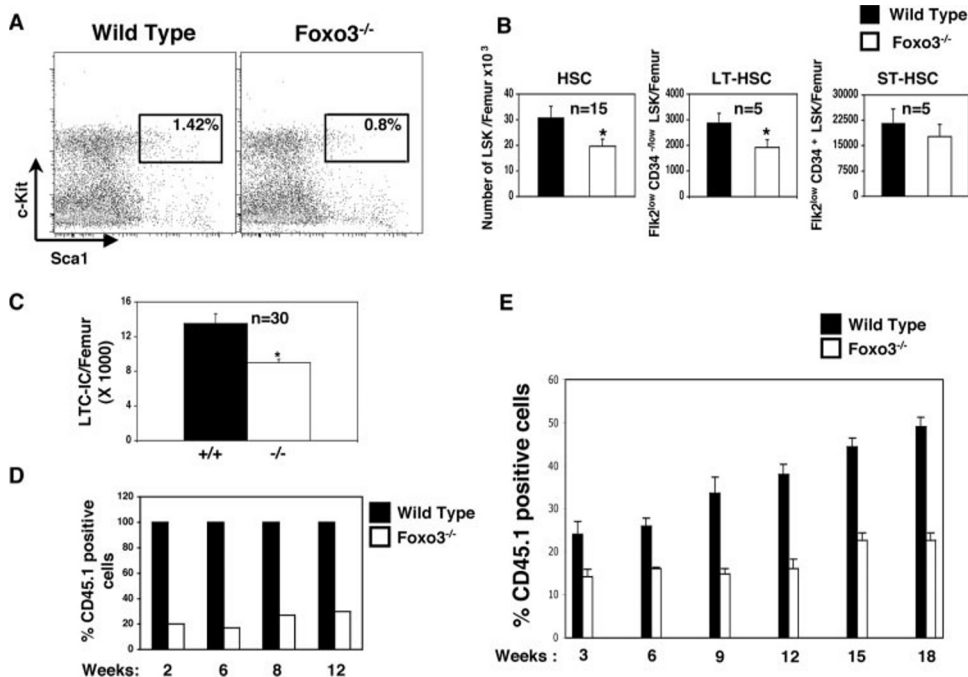

FIGURE 2. **Foxo3$^{-/-}$ HSC are functionally defective.** A, flow cytometry analysis of live BM cells gated to exclude doublets and mature cells as defined by their FSC and SSC characteristics. WT and Foxo3$^{-/-}$ lineage negative BM cells were analyzed for c-Kit versus Sca-1 expression (LSK) (contain all hematopoietic stem cell activity). Frequency of LSK cells is noted. B, total numbers of LSK cells ($p < 0.02$, $n = 15$), LSK cells that are CD34^{low} Flk2^{low}, enriched for LT-HSC ($p < 0.04$, $n = 5$) (48), and LSK cells that are CD34$^+$ Flk2^{low} enriched for ST-HSC (49) in WT and Foxo3$^{-/-}$ mice. C, total numbers of LTC-IC-derived colonies were measured after 6 weeks in culture of WT ($+/+$) and Foxo3$^{-/-}$ ($-/-$) mice ($p < 6.2 \times 10^{-10}$, $n = 30$). D, % of CD45.1 peripheral blood in lethally irradiated CD45.2 mice. Results shown are percentage of control. E, CD34$^-$ Lin$^-$ c-kit^{lhigh} Sca-1$^+$ (100 cells) from Foxo3$^{-/-}$ (backcrossed to C56BL6, F10, CD45.1) or wild type C57BL6 (Cd45.1) mixed with 2×10^5 total bone marrow (CD45.2) were transplanted into lethally irradiated recipients (CD45.2), and the contribution to the peripheral blood formation of (CD45.1) cells was evaluated ($n = 5$).

are expressed at similar levels in hematopoietic stem cells (Fig. 1A), Foxo3 is almost entirely nuclear (over 95% nuclear as compared with 40% nuclear for Foxo1, see Fig. 1, B and C) suggesting that Foxo3 is presumably highly active (and is the most active FoxO) in hematopoietic stem cells. Importantly, the expression of Foxo1 was not up-regulated, and its subcellular localization was only increased by 15% in Foxo3-null hematopoietic stem cells (Fig. 1). Foxo4 transcript or protein was barely detectable in hematopoietic stem cell compartment (Fig. 1). These results suggest that Foxo3 is the prevalent active FoxO in hematopoietic stem cells, and Foxo1 or Foxo4 activity does not fully substitute for loss of Foxo3.

Thus, to address the potential evolutionary role of FoxO in the regulation of quiescence of hematopoietic stem cells, we focused on Foxo3 and investigated the hematopoietic stem cell compartment in Foxo3$^{-/-}$ mice. We found a significant suppression of Foxo3-deficient hematopoietic stem cell numbers and activity (Fig. 1). In contrast to Miyamoto et al. (22), we found that the frequency (Fig. 2A) and the size (Fig. 2B) of the Foxo3-null hematopoietic stem cell compartment was significantly reduced as compared with the wild type. The hematopoietic compartment that is depleted from mature cells (called lineage-negative population) and expresses cell surface markers Sca1 and high levels of c-Kit receptor (Lin$^-$ Sca1$^+$ c-Kit^{high}, LSK) encompasses all stem cell activity, and this population was substantially reduced in young Foxo3-deficient mice as compared with wild type littermates (Fig. 2). Hematopoietic stem cell (LSK) compartment is heterogeneous and consists of distinct populations of long term HSC (LT-HSC) and short term (ST-HSC) repopulating HSC (29). LT-HSC (Lin$^-$ Sca1$^+$ c-Kit^{high} CD34$^{-/low}$ Flk2^{low}) have extensive self-renewing potential and give rise to ST-HSC (Lin$^-$ Sca1$^+$ c-Kit^{high} CD34$^+$ Flk2^{low}) with more restricted self-renewal
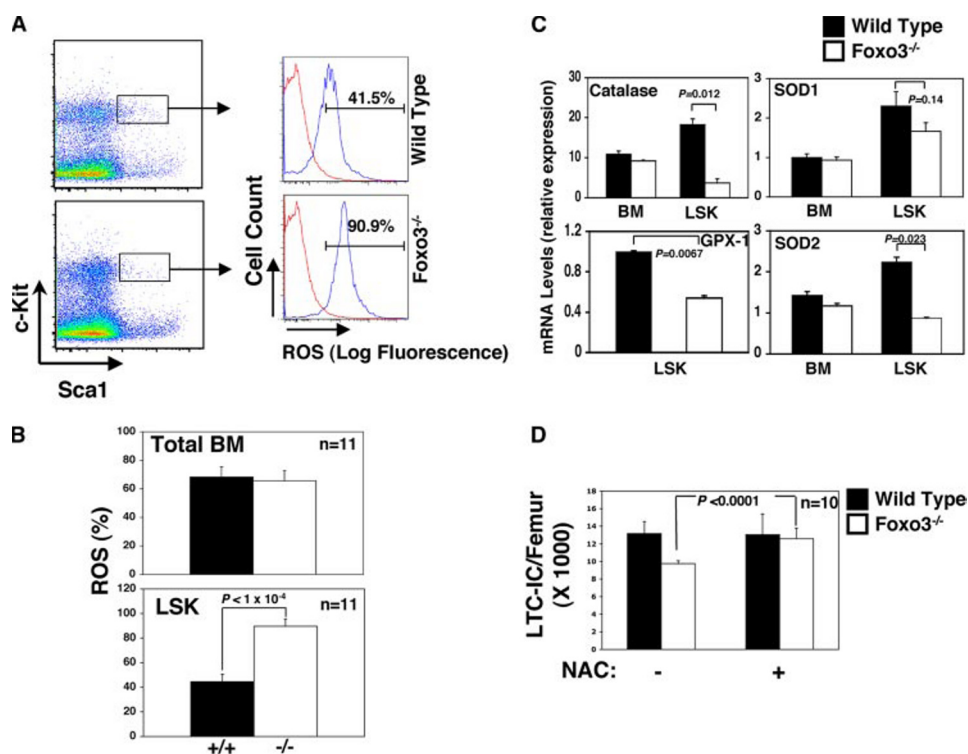
FIGURE 3. **Defects in Foxo3-null hematopoietic stem cell activity are mediated by oxidative stress.** *A,* flow cytometry analysis of ROS concentrations in WT and Foxo3$^{-/-}$ LSK cells (ROS overlay on unstained). *B,* quantification of ROS measurements in WT and Foxo3$^{-/-}$ total BM (*top*) and in LSK cells (*bottom*) from *A*. Results are shown as mean ± S.E. ($p < 0.0001$, $n = 11$). *C,* QRT-PCR analysis of antioxidant enzymes in RNA extracted from freshly isolated live LSK cells. Results are shown as mean ± S.E. of at least three cDNAs, each derived from pooled LSKs from 2 to 3 WT and Foxo3$^{-/-}$ mice. *D,* LTC-IC-derived colonies were measured in the presence and absence of 100 μM NAC added at each half-media change to long term cultures ($p < 0.0001$, Student's *t* test, $n = 10$). *E,* CFU-S-derived colonies were measured 12 days after injection of 10^5 WT or Foxo3-deficient bone marrow cells into lethally irradiated hosts, in the presence or absence of NAC (100 μM) injected intraperitoneally daily. One representative of two independent experiments is shown ($n = 5$ in each group).

potentials. Foxo3-deficient LT-HSC were the most highly affected in the hematopoietic stem cell compartment (Fig. 2*B*, $p < 0.02$) suggesting that the self-renewing capacity of Foxo3-null hematopoietic stem cell was compromised. This was further supported by the suppression of Foxo3$^{-/-}$ hematopoietic stem cell functional activity. The ability of Foxo3-deficient hematopoietic stem cell in generating progenitors after 4 weeks in long term culture (LTC-IC assay) *in vitro* (25) was notably decreased as compared with their wild type counterparts (Fig. 2*C*, $p < 6.2 \times 10^{-10}$, $n = 30$). Similarly, the ability of Foxo3-deficient hematopoietic stem cells to repopulate *in vivo* lethally irradiated animals over several weeks was also severely compromised (Fig. 2*D*) suggesting that the suppression of stem cell activity was intrinsic to Foxo3-null hematopoietic stem cells. We futher evaluated the long term repopulating ability of Foxo3-null HSC by using highly purified HSC from Foxo3$^{-/-}$ mice backcrossed to C57BL6 mice (F10) in our *in vivo* competitive repopulation assay. We transplanted 100 CD34$^-$ Lin$^-$ c-kit^{ihigh} Sca-1$^+$ that are highly enriched in long term HSC from either wild type or Foxo3$^{-/-}$ (backcrossed to C56BL6, F10, CD45.1) along with 2 × 10^5 total bone marrow (CD45.2) into lethally irradiated recipients (CD45.2) and compared the contribution to peripheral blood formation of these cells with that of wild type (CD45.1) cells. As shown in Fig. 2*E*, these experiments confirmed that Foxo3-null hematopoietic stem cells are highly

compromised in their long term ability to reconstitute multiline blood formation in lethally irradiated recipient mice. Together these results strongly indicate that Foxo3$^{-/-}$ mice are markedly deficient in their hematopoietic stem cell compartment (Fig. 2), specifically in cells with long term repopulating ability (Fig. 2*B*).

We next asked whether the defect in Foxo3-null hematopoietic stem cell compartment resulted from abnormalities in scavenging ROS (Fig. 3). Using a hydrogen peroxide-sensitive probe, we found ROS to be highly accumulated in Foxo3-deficient populations of hematopoietic cells containing all stem cells (LSK cells) (Fig. 3, *A* and *B*). Increased ROS concentrations were specific to Foxo3$^{-/-}$ hematopoietic stem cells (LSK), because accumulation of ROS was similar in total WT and Foxo3-deficient bone marrow cells (Fig. 3*B*, compare *top* and *bottom panels*).

In agreement with these results, several antioxidant enzymes, including catalase and mitochondrial superoxide dismutase 2 (SOD2, MnSOD), which are known FoxO targets, and glutathione peroxidase 1 (GPX-1), which is not

known to be regulated by FoxO, were profoundly and specifically down-regulated in Foxo3-null LSK cells as analyzed by QRT-PCR (Fig. 3*C*). These results suggest that Foxo3 is the chief regulator of these antioxidant enzymes in hematopoietic stem cells, and potential activity of Foxo1 and/or Foxo4 does not fully complement loss of Foxo3 in hematopoietic stem cells.

Importantly, reducing ROS using NAC (100 μM) that is a generic ROS scavenger rescued the Foxo3-deficient hematopoietic stem cell phenotypes and the numbers of hematopoietic stem cell-generated colonies over a 4-week period in culture (LTC-IC assay, Fig. 3*D*). These findings demonstrate that abnormal accumulation of ROS in Foxo3-null hematopoietic stem cells leads to the defective activity of hematopoietic stem cells. These combined findings strongly point to the deleterious impact of unchecked ROS as a result of loss of Foxo3 on inducing defective hematopoietic stem cell activity.

To evaluate whether loss of Foxo3 has any specific impact on hematopoietic stem cell quiescence, G$_0$ distribution of WT and Foxo3-deficient hematopoietic stem cells (LSK) was compared using measurements of RNA *versus* DNA contents. These studies showed that loss of Foxo3 results in a significant reduction of the proportion of bone marrow hematopoietic stem cells (LSK) in G$_0$ that is concomitant with the increased proportion of these cells in S/G$_2$/M suggesting that Foxo3 promotes hematopoietic stem cell quiescence (Fig. 4, *A* and *B*). These results suggested
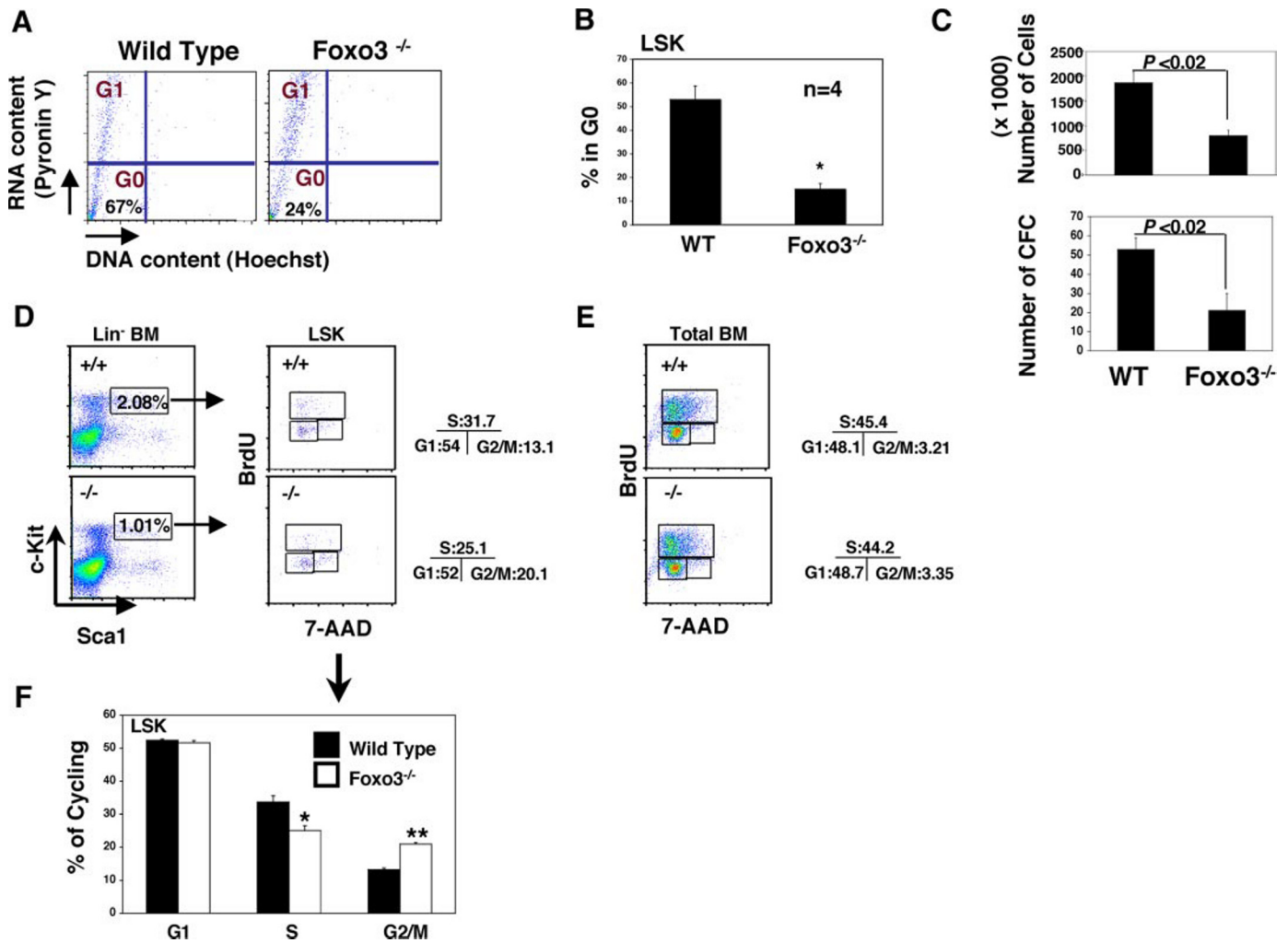
FIGURE 4. **Decreased quiescence and impaired G_2/M transition in Foxo3-deficient hematopoietic stem cells.** *A,* FACS-sorted LSK cells are stained with pyronin Y and Hoechst and analyzed by flow cytometry for G_0/G_1 distribution. Plot of percentage of wild type and Foxo3$^{-/-}$ LSK cells in G_0 (negative for both DNA-binding Hoechst and RNA-binding pyronin Y). *B,* mean ± S.E. of experiments in *A* is shown (*, $p < 0.02$, Student's *t* test, pool of 2–3 WT and Foxo3$^{-/-}$ mice for each experiment, $n = 4$). *C,* wild type and Foxo3$^{-/-}$ 1000 LSK cells (in triplicate) were FACS-sorted and incubated in culture in the presence of steel factor (100 ng/ml), IL-6 (10 ng/ml), IL-3 (10 ng/ml), and Flk2 (5 ng/ml), and cells (*upper panel*) and colony-forming cells (*lower panel*) generated after 5 days were counted, and results are shown as mean ± S.E. *D,* mice were injected with BrdUrd; after 19 h bone marrow (*BM*) was extracted, and FACS-sorted and -gated wild type and Foxo3$^{-/-}$ LSK cells (*left panel*) were analyzed for BrdUrd uptake (*right panel*). A representative cell cycle distribution is shown ($n = 8$). *E,* as control, wild type and Foxo3$^{-/-}$ total bone marrow from *D* were analyzed for BrdUrd uptake (*left panel*), and a representative cell cycle distribution is shown (*right panel*). *F,* graph of results from *D* shown as mean ± S.E., *, $p < 0.002$; **, $p < 3.06 \times 10^{-8}$, $n = 8$. *7-AAD,* 7-amino-actinomycin D.

that hematopoietic stem cells are in cycle and are producing progenitors. However, despite these results, Foxo3-deficient hematopoietic stem (LSK) cells cultured under optimum growth factor conditions showed significantly reduced production of total cells (Fig. 4*C, upper panel*) and progenitors (Fig. 4*C, lower panel*). To further investigate this observation, the cell cycle distribution of the hematopoietic stem cell compartment using the nucleotide analog BrdU uptake *in vivo* was performed. As shown in Fig. 4*E,* loss of Foxo3 did not significantly impact cell cycle distribution in the whole bone marrow using BrdU uptake. In contrast, Foxo3 deficiency resulted in an altered increase in G_2/M phase and decreased proliferation as measured by reduced incorporation of BrdU into the S phase of Foxo3-deficient hematopoietic stem cells (LSK, Fig. 4, *D* and *F*). These results were in sharp contrast to the observations reported by Miyamoto *et al.* (22). In contrast to its impact on cell cycle distribution,

loss of Foxo3 did not appear to significantly alter the apoptotic rate of hematopoietic stem cells as measured by annexin-V binding of freshly isolated LSK cells (data not shown) or by the analysis of caspase 3 cleavage in FACS-sorted LSK cells *ex vivo* (supplemental Fig. 1). Together these studies suggest that loss of Foxo3 results in loss of quiescence of hematopoietic stem cells that is concomitant with alterations in the ability of hematopoietic stem cells to complete their cycling.

ROS-mediated Activation of p53 Tumor Suppressor Pathways in Foxo3-deficient Hematopoietic Stem Cells—To obtain insight into the Foxo3-null hematopoietic stem cell cycle alterations, we analyzed transcript levels of candidate molecules previously implicated in hematopoietic stem cell cycling and/or known to be transcriptional targets of Foxo3. This analysis indicated a significant reduction in expression of Foxo3 targets the cyclin-dependent kinase (CDK) inhibitor 1B *Cdkn1b* (p27^{KIP1})
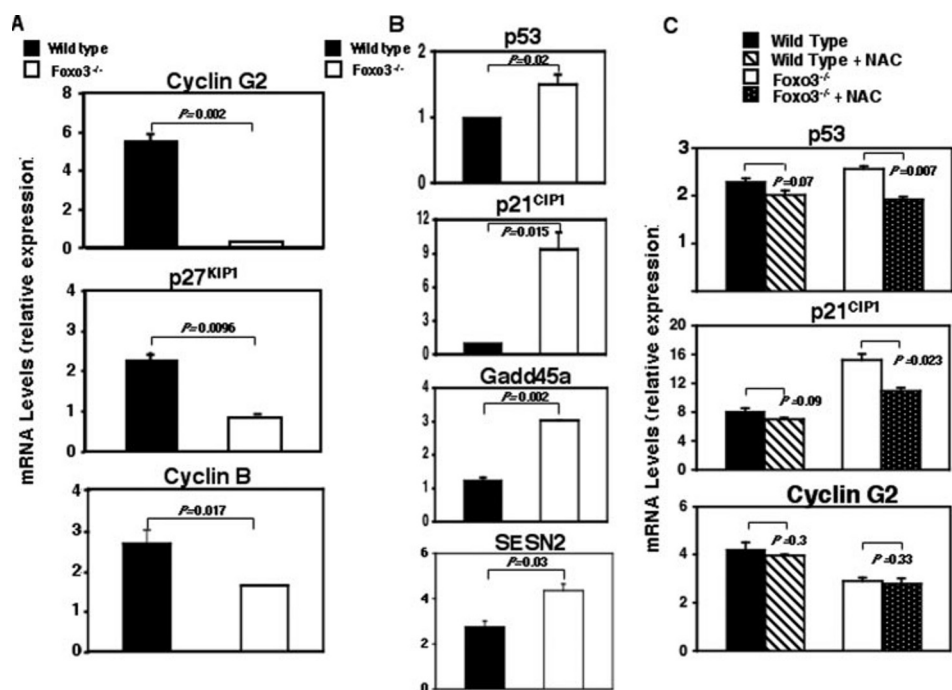
FIGURE 5. ROS-mediated and -independent alterations of tumor suppressor pathways in Foxo3$^{-/-}$ hematopoietic stem cells. *A,* QRT-PCR analysis of some of cell cycle regulators that are direct target of FoxO in wild type and Foxo3$^{-/-}$ hematopoietic stem (LSK) cells. Results are shown as mean ± S.E. of analysis of three cDNAs each generated from a pool of two to three WT and Foxo3$^{-/-}$ mice. *B,* QRT-PCR analysis of antioxidant gene expression in wild type and Foxo3$^{-/-}$ hematopoietic stem (LSK) cells. Results are shown as mean ± S.E. of analysis of three cDNAs each generated from a pool of two to three WT and Foxo3$^{-/-}$ mice. *C,* wild type and Foxo3$^{-/-}$ mice were treated *in vivo* with or without NAC (100 mg/kg) every day for 5–7 days. BM LSK were then isolated and incubated with NAC *ex vivo* for an additional 2 days after which total RNA was isolated for QRT-PCR analysis. Results are shown as mean ± S.E. of analysis of at least three cDNAs each generated from pool of two to three wild type and Foxo3$^{-/-}$ mice.

transcript (17), *Ccng2* (cyclin G2) (30), and *Ccnb1* (cyclin B) (31) in Foxo3-null hematopoietic stem cells (LSK, Fig. 5*A*). Expression of p27^{KIP1} and cyclin G2, both important regulators of hematopoietic stem cell cycling (28, 32), was profoundly down-regulated in Foxo3-deficient hematopoietic stem (LSK) cells (Fig. 5*A*). Similarly, Foxo3 transcriptional target cyclin B that is required for completion of cell cycle and mitosis was significantly reduced in Foxo3$^{-/-}$ LSK cells (Fig. 5*A*). These results strongly suggest that Foxo3 is a key transcriptional regulator of p27^{KIP1}, cyclin G2, and cyclin B in hematopoietic stem cells, and potential functional Foxo1 (or Foxo4) does (do) not complement for loss of Foxo3 in the regulation of these genes.

Unexpectedly, however, the expression of another Foxo3 transcriptional target CDK inhibitor *Cdkn1a* (p21$^{CIP1/WAF1/Sdi1}$), with critical functions in the repression of hematopoietic stem cell cycling (33), was robustly up-regulated in Foxo3$^{-/-}$ hematopoietic stem (LSK) cells (Fig. 5*B*). p21$^{CIP1/WAF1/Sdi1}$ is an essential regulator of G_1 and G_2 checkpoints in response to oxidative stress and DNA damage either as part of a transcriptional program activated by p53 tumor suppressor or independently (34, 35). Up-regulation of p21$^{CIP1/WAF1/Sdi1}$ was associated with a significant up-regulation of p53 expression (Fig. 5*B*) suggesting that enhanced expression of p21$^{CIP1/WAF1/Sdi1}$ was mediated by p53 activation in Foxo3-deficient hematopoietic stem cells. In addition, known stress-response targets of activated p53, such as the DNA repair gene growth arrest and DNA damage-inducible 45α (Gadd45a), also a transcriptional target of Foxo3, and

the antioxidant gene sestrin 2 (Sesn2), were significantly up-regulated in Foxo3-deficient as compared with WT hematopoietic stem cells (Fig. 5*B*), further supporting that p53 is activated as part of an antioxidative stress response in Foxo3-deficient hematopoietic stem cells.

To evaluate the role of ROS in alterations of Foxo3-deficient hematopoietic stem cells (LSK), the gene expression pattern of hematopoietic stem cells treated with a ROS scavenger was examined. Treatment with NAC for 5 days *in vivo* followed by 2 days *in vitro* resulted in a significant down-regulation of both p21$^{CIP1/WAF1/Sdi1}$ and p53 expression in Foxo3-deficient but not WT LSK cells (Fig. 5*C*), further demonstrating that up-regulation of p21$^{CIP1/WAF1/Sdi1}$ transcription in Foxo3-null hematopoietic stem cells was mediated by an antioxidant response of activated p53. As anticipated, scavenging ROS in Foxo3-null hematopoietic stem cells did not rescue the expression of direct targets of Foxo3 such as cyclin G2 (Fig. 5*C*) or p27^{KIP1} (data not shown) that were highly repressed in Foxo3-deficient hematopoietic stem cells (Fig. 5*A*). These results confirmed that cyclin G2 and p27^{KIP1} are direct targets of Foxo3, and their expression is not mediated by ROS. Although the basal levels of cyclin G2 (Fig. 5*A*) or p21$^{CIP1/WAF1/Sdi1}$ (Fig. 5*B*) in wild type LSK were higher in *ex vivo* cultured cells as compared with levels found in freshly isolated wild type LSK (Fig. 5*B*, likely because of conditions in culture as compared with *in vivo*), the notable reduction upon scavenging ROS was only seen with p21$^{CIP1/WAF1/Sdi1}$ transcript but not with cyclin G2 and only in Foxo3-null and not in wild type cells (Fig. 5*C*).

ATM Lies Downstream of Foxo3—ATM is an essential regulator of stress response and ROS-induced DNA damage that is critical for hematopoietic stem cell self-renewal (2, 6). ATM activates p53 via multiple pathways, including direct phosphorylation. To our surprise and in contrast to the observed up-regulation of p53 expression, ATM transcript expression was significantly and unexpectedly down-regulated in Foxo3-null hematopoietic stem cells (Fig. 6*A*, ~2-fold reduction, $p = 0.0035$). Importantly, this reduction in transcript expression was associated with similar suppression of ATM protein expression in primitive Foxo3-deficient hematopoietic cells (Fig. 6*B*, over 2-fold reduction) suggesting that ATM lies downstream of Foxo3. The 2-fold reduction of ATM expression in Foxo3-null primitive hematopoietic cells is of major significance because ATM is required for genomic stability and ATM$^{+/-}$ mice exhibit an altered stress response phenotype
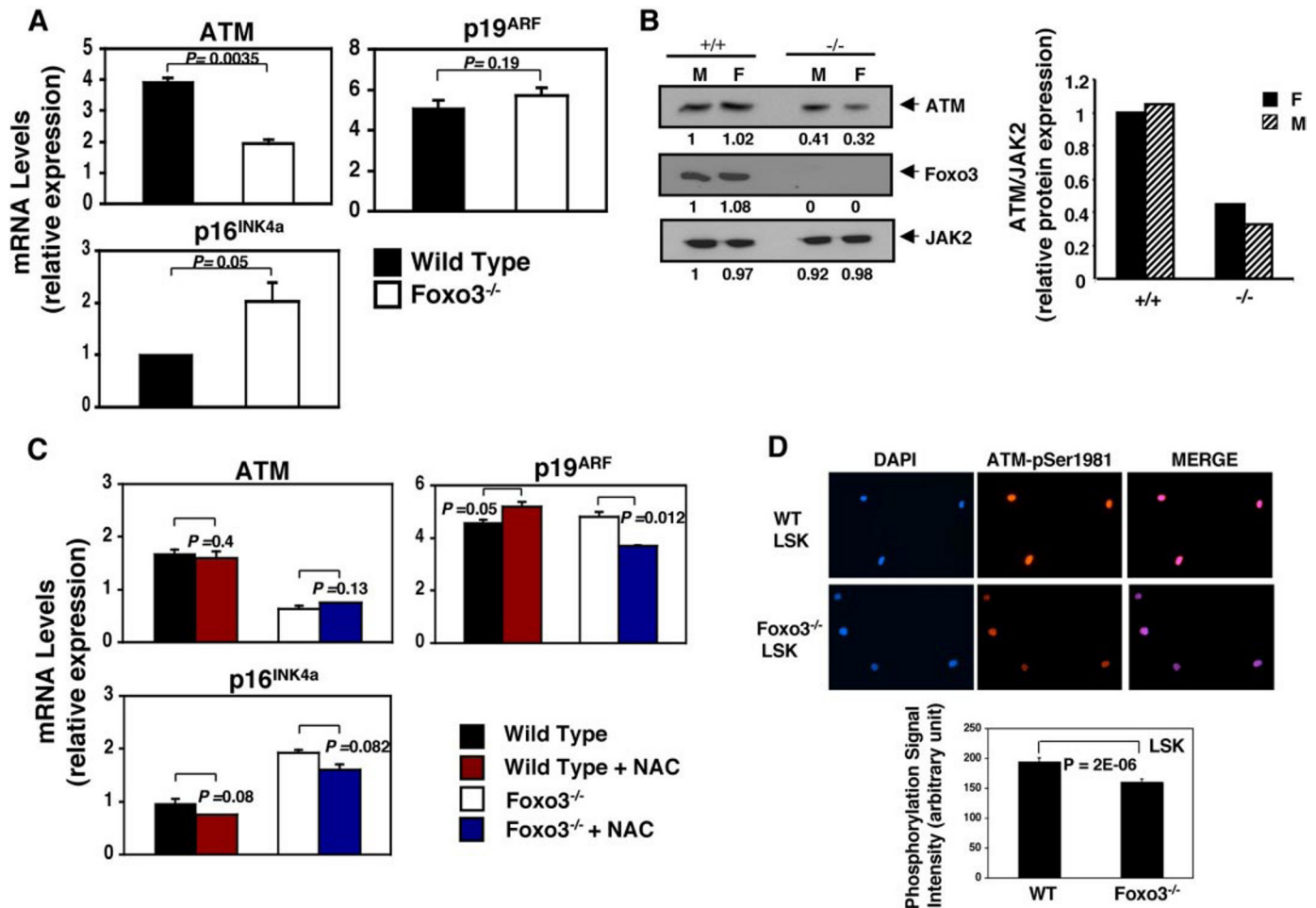
FIGURE 6. **ROS-independent repression of ATM in Foxo3-null primitive hematopoietic cells.** *A,* QRT-PCR analysis of ATM, p16^{INK4a}, and p19^{ARF} in wild type and Foxo3$^{-/-}$ hematopoietic stem (LSK) cells. Results are shown as mean ± S.E. of analysis of three cDNAs each generated from a pool of two to three WT and Foxo3$^{-/-}$ mice. *B,* Western blot analysis of ATM in lineage-negative bone marrow (*BM*) cells. JAK2 was used as a loading control. Band quantification (using ImageQuant TL, *right panel*) normalized to 1 (for WT male) is shown *below* each lane. *M,* male; *F,* female. *C,* QRT-PCR analysis of same genes as in *A* after *in vivo* NAC treatment as in Fig. 5C. Results are shown as mean ± S.E. of analysis of at least three cDNAs each generated from pool of two to three wild type and Foxo3$^{-/-}$ mice. *D,* immunostaining of ATM-phospho-Ser-1981 in wild type and Foxo3-null LSK cells at the steady state (*top*) and quantification of phosphorylation signal in LSK cells by Volocity software (*bottom*); data are shown as mean ± S.E. collected from *n* = 20 cells.

(36) suggesting that loss of half of ATM may participate in the defective Foxo3-null hematopoietic stem cell activity or stress response. ATM-deficient hematopoietic stem cells in old mice exhibit an up-regulation of both p16^{INK4a} and p19^{ARF} tumor suppressors (2) that are generated from alternative open reading frames of the *INK4a* locus. p16^{INK4a} and p19^{ARF} inhibit cell cycle progression through inhibition of inhibitors (therefore activation) of retinoblastoma and p53 proteins, respectively. Although in Foxo3-deficient hematopoietic stem cells, p16^{INK4a} expression was significantly up-regulated, the expression of p19^{ARF} remained unchanged (Fig. 6A).

In contrast to the expression of p53/p21$^{CIP1/WAF1/Sdi1}$, the levels of ATM or p16^{INK4a} transcripts in Foxo3-null hematopoietic stem cells did not change significantly in response to the NAC antioxidant treatment (Fig. 6C). These results further confirmed that reduced ATM expression may be a direct result of loss of Foxo3 and not ROS accumulation in primitive hematopoietic stem cells. In agreement with a functional ATM downstream of Foxo3 in hematopoietic stem cells, the phosphorylation of ATM serine 1981 that is a marker of ATM kinase activity (37) was slightly albeit significantly reduced at the

steady state in Foxo3-null hematopoietic stem cell-containing population of LSK cells as compared with their wild type counterparts (Fig. 6D).

NAC treatment led to a significant down-regulation of p19^{ARF} in Foxo3-deficient hematopoietic stem cells (LSK; Fig. 6C). This was highly unexpected because p19^{ARF} transcript levels were similar in WT and Foxo3-null hematopoietic stem cells (Fig. 6A). These findings suggest that ROS mediate regulation of p19^{ARF} expression in Foxo3-deficient but not in WT hematopoietic stem cells. Because p19^{ARF} inhibits the degradation of p53 protein through sequestration of MDM2 (38), these results suggest that ROS may induce activation of p53 antioxidant function, both directly by stimulating the expression of p53 transcript and indirectly through up-regulation of p19^{ARF}. These combined findings suggest that ROS accumulation leads to activation of p19^{ARF}/p53 signaling pathway resulting in up-regulation of p21$^{CIP1/WAF1/Sdi1}$.

Foxo3 Is Required for Normal ATM Expression—We next asked whether ATM expression is modulated by Foxo3 and thus examined the expression of ATM in Foxo3-deficient primitive hematopoietic cells expressing Foxo3 ectopically. WT and
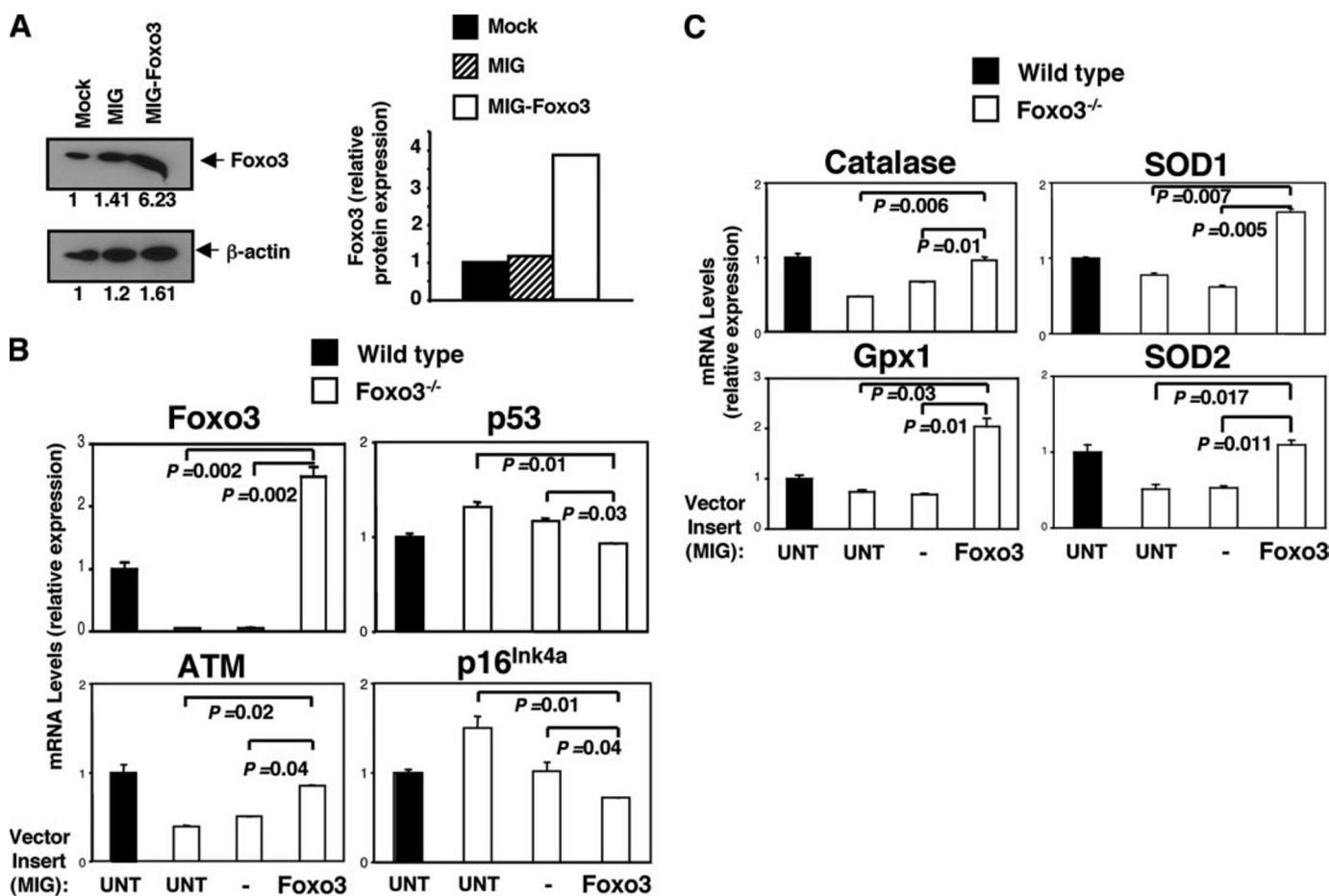
FIGURE 7. **Ectopic expression of Foxo3 rescues expression of ATM and antioxidant genes in Foxo3-null primitive hematopoietic cells.** *A,* Western blot analysis of retrovirally produced Foxo3 in transduced NIH 3T3 cells. Cells (4×10^5) were transduced with $^1/_2$ dilution of retroviral supernatant in the presence of Polybrene (4 μg/ml), and protein lysates were prepared 48 h later. Band quantification (using ImageQuant TL) normalized to 1 (for mock transduction) is shown *below* each lane. *Bar graphs* of relative protein expression values are shown (below each Western blot). *B,* 5-FU-treated BM mononuclear cells were transduced with the bicistronic MSCV-IRES-GFP encoding for Foxo3 (MIG-Foxo3). QRT-PCR analysis of gene expression in FACS-sorted GFP$^+$ cells is shown. *UNT,* untransduced. *C,* QRT-PCR analysis of antioxidant enzyme gene expression in cells from *B.* Results shown as mean $\pm$ S.E. of analysis of three cDNAs each generated from transduced cells of pool of four to five 5-FU-treated WT and Foxo3$^{-/-}$ mice.

Foxo3-null bone marrow mononuclear cells highly enriched in primitive hematopoietic cells as a result of a 4-day treatment with a high dose of 5-FU (that ablate proliferating hematopoietic cells (25, 39)) were isolated and transduced with the bicistronic retroviral vector MSCV-IRES-GFP encoding for Foxo3 (Fig. 7*A*). Retroviral expression of Foxo3 and not the vector control (Fig. 7*B*) resulted in a significant up-regulation of ATM transcript in GFP$^+$-transduced Foxo3-null primitive hematopoietic cells suggesting that Foxo3 regulates ATM expression. Expression of Foxo3 also induced significant down-regulation of p53 and p16^{INK4a} (Fig. 7*B*) without modifying the expression of p19^{ARF} (data not shown) in Foxo3$^{-/-}$ primitive hematopoietic cells.

As anticipated ectopic retroviral expression of Foxo3 and not the vector control induced the expression of SOD2 and catalase (Fig. 7*C*) in Foxo3-null primitive hematopoietic cells. In addition, overexpressed Foxo3 strongly and significantly induced the expression of SOD1 (cytoplasmic SOD, CuZn-SOD) and GPX-1 in primitive hematopoietic cells (Fig. 7*C*) suggesting that expression of these antioxidant enzymes is also regulated by Foxo3. The mechanism through which Foxo3 induces SOD1 and GPX1 is not known.

To further examine Foxo3 regulation of ATM, we tested ATM expression in NIH 3T3 cells overexpressing Foxo3 by both QRT-PCR and Western blot. As in Foxo3-null primitive hematopoietic cells, ectopic expression of Foxo3 and not the vector control in NIH 3T3 cells resulted in a significantly enhanced ATM expression as measured by both QRT PCR (Fig. 8*A*) and Western blot (data not shown). Importantly, targeted silencing of Foxo3 expression by RNA interference using two distinct shRNAs resulted in significant reduction of expression of both ATM transcript (Fig. 8*B*) and protein (Fig. 8*C*) in NIH 3T3 cells. A 50% reduction in Foxo3 expression resulted in over 25% reduced expression of ATM (Foxo3 shRNA3), whereas 80% reduction of Foxo3 resulted in 36% suppression of ATM protein (Foxo3 shRNA4). However, because less than 100% transfection efficiency may underestimate these results, we repeated these experiments and FACS-sorted GFP$^+$ (all expressing shRNA targeting Foxo3) NIH 3T3 cells for analysis (Fig. 8*D*). These experiments further confirmed our previous findings and showed that over 95% reduction in Foxo3 expression leads to 70% reduction in ATM transcript expression further demonstrating that Foxo3 is required for ATM expression (Fig. 8*D*).
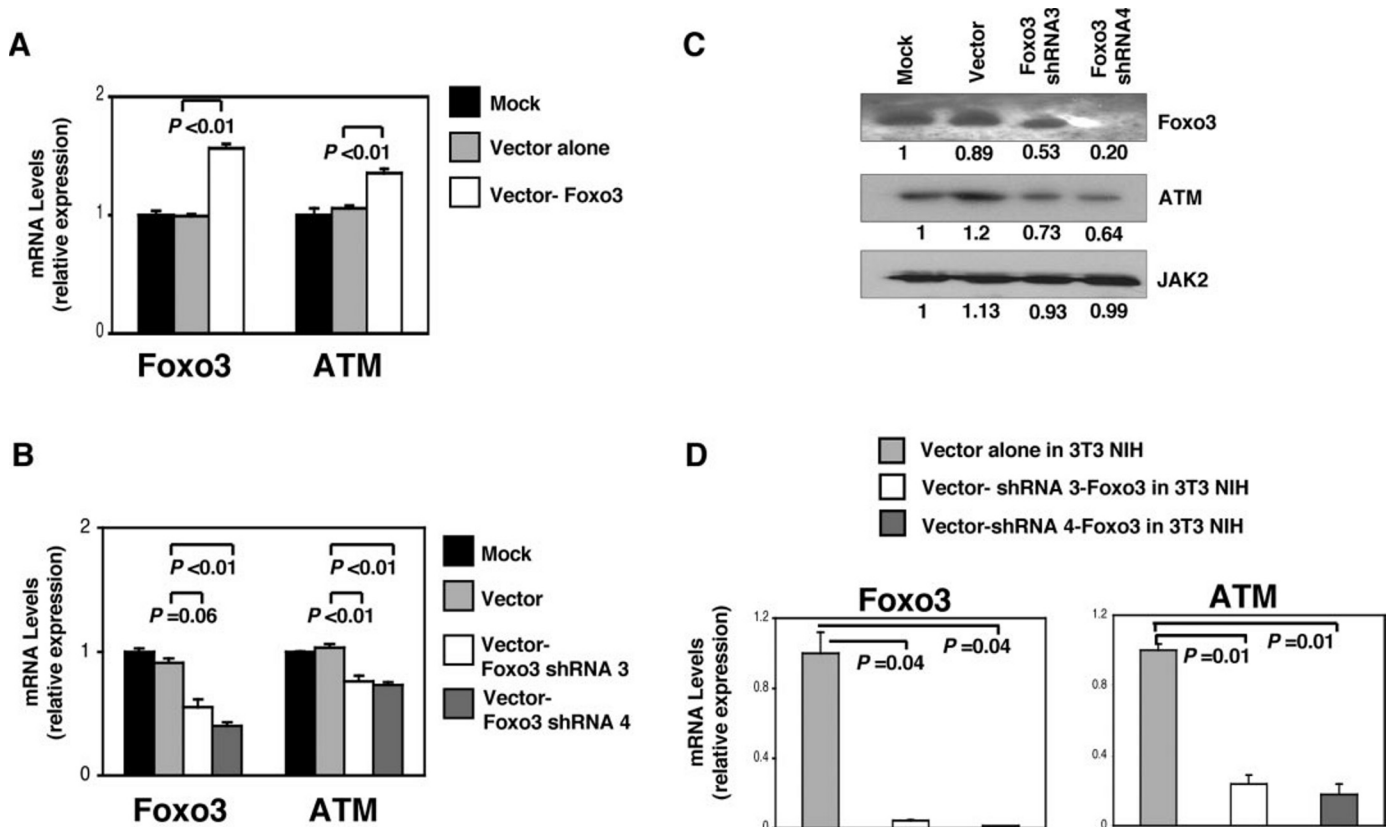
FIGURE 8. **Foxo3 is required for the regulation of ATM expression.** *A,* QRT-PCR analysis of ATM gene expression in NIH 3T3 cells ectopically expressing MIG-Foxo3 or MIG control ($n = 3$). *B,* QRT-PCR analysis of ATM in NIH 3T3 cells transfected 72 h earlier with PL4-shRNA-Foxo3 ($n = 3$) to silence Foxo3; shRNA3 and -4 are two distinct shRNAs targeting Foxo3. *C,* Western blot analysis of cells in *B,* and band quantification (using ImageQuant TL) normalized to 1 (for mock transfection) is shown *below* each lane. *D,* 96 h after calcium phosphate transfection, GFP$^+$ NIH 3T3 cells expressing shRNA targeting Foxo3 were FACS-sorted and analyzed by QRT-PCR for Foxo3 and ATM expression.

Foxo3 and ATM Regulate the Expression of Antioxidant Enzymes Independently—Although some ATM-null cells and tissues, including ATM$^{-/-}$ hematopoietic stem cells (2), accumulate ROS, depending on tissues and cells, antioxidant enzymes are up- or down-regulated in ATM-null cells, and the exact mechanism of ATM regulation of ROS is unknown (6, 8, 9). Thus, we examined whether relative loss of ATM contributes to the deficiency of antioxidant gene expression observed in Foxo3-null primitive hematopoietic cells (Fig. 3*C*). To this end, retroviral supernatant highly expressing ATM was prepared (Fig. 9*A*) and used to transduce Foxo3-null primitive hematopoietic cells (Fig. 9*B*). Ectopic retroviral expression of ATM and not the vector control corrected the transcript level of p16^{INK4a}, without much impact on p19^{ARF} or p53 transcript expression in Foxo3-null primitive hematopoietic cells suggesting that ATM may regulate p16^{INK4a} expression. These results further suggest that up-regulation of p16^{INK4a} expression is the result of the relative loss of ATM expression in Foxo3-null primitive hematopoietic cells. Interestingly, retroviral expression of ATM restored the expression of antioxidant enzymes in GFP$^+$ Foxo3-null primitive hematopoietic cells (Fig. 9*C*) suggesting that ATM is sufficient for the expression of SOD1, SOD2, catalase, and GPX1 in the absence of Foxo3.

We next asked whether ATM is required for Foxo3 regulation of antioxidant enzymes. ATM-deficient or WT MEFs (40) retrovirally expressing either empty vector or Foxo3 or ATM were examined for gene expression of antioxidant enzymes. As shown in Fig. 10, SOD2 was significantly down-regulated in ATM-deficient MEFs suggesting that ATM is required for SOD2 expression. Ectopic expression of either Foxo3 or ATM induced marked up-regulation of SOD1, SOD2, and GPX-1 in ATM-deficient MEFs suggesting that Foxo3 regulation of these antioxidant enzymes does not require ATM expression (Fig. 10). Up-regulation of the expression of these enzymes by over-expression of ATM suggests, however, that although ATM is only required for normal expression of SOD2, ATM may also participate in the regulation of expression of SOD1, catalase, and GPX-1 antioxidant enzymes.

These combined findings demonstrate that Foxo3 is required for the regulation of ATM expression. In addition, these findings strongly suggest that both ATM-mediated and independent pathways regulate oxidative stress in Foxo3-null primitive hematopoietic stem cells.

DISCUSSION

Foxo3 Is a Physiological Regulator of Oxidative Stress in Hematopoietic Stem Cells—Oxidative stress is broadly defined as an imbalance between the production of oxygen radicals and the ability to detoxify ROS to prevent oxygen radical-induced injuries. Although the deleterious impact of ROS accumulation on HSC has begun to be explored, little is known about the
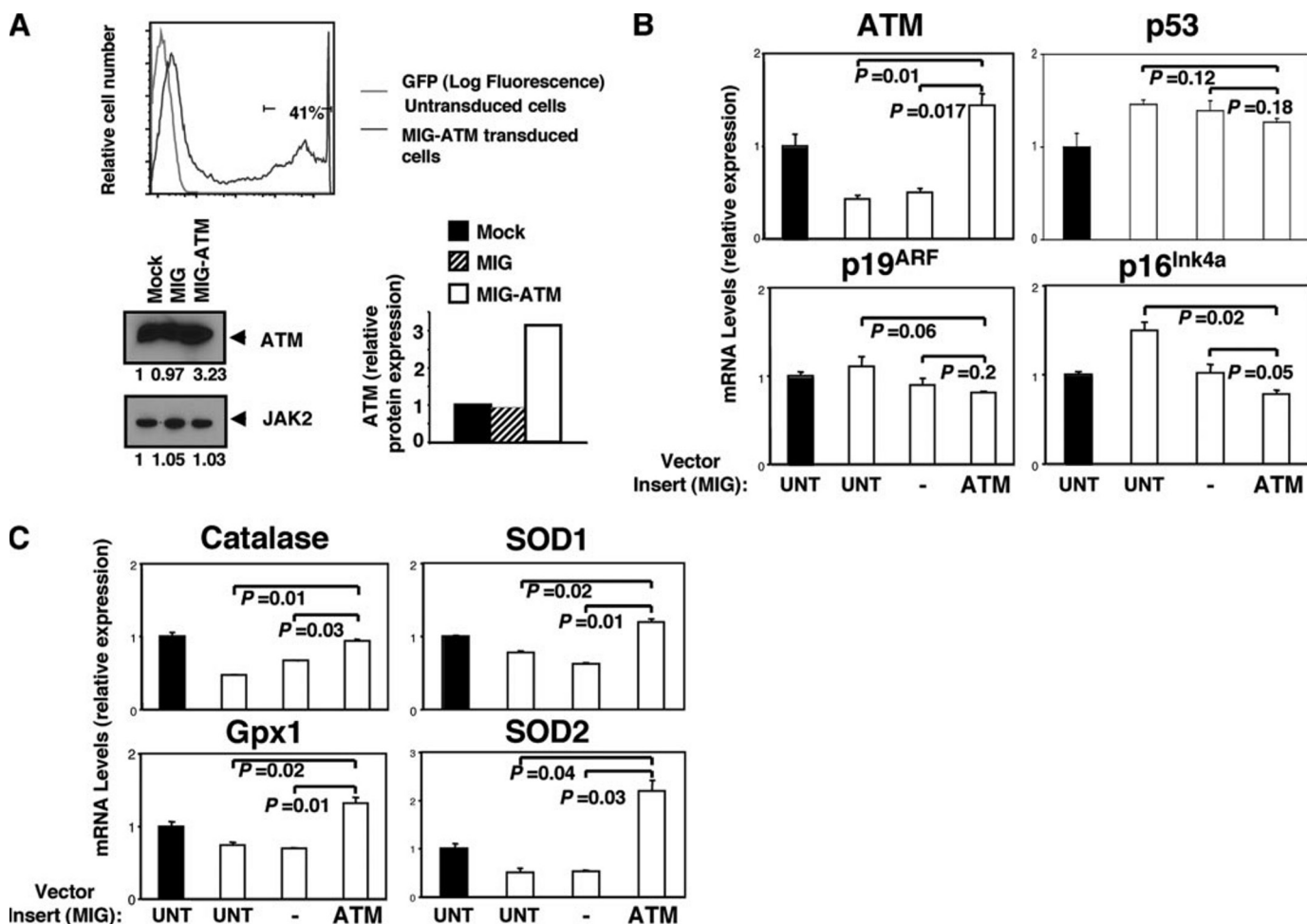
FIGURE 9. **ATM rescues expression of antioxidant enzymes in Foxo3-null primitive hematopoietic cells.** *A,* representative flow cytometry histogram of GFP expression in 5-FU-treated bone marrow mononuclear cells transduced with the bicistronic MIG-ATM vector (*left panel*). Western blot analysis of retrovirally produced ATM in transduced NIH 3T3 cells (*right panel, top*) and quantitative measurements of ATM protein expression are shown as *bar graph* (*right panel, bottom*). Band quantification (using ImageQuant TL) normalized to 1 (for mock transduction) is shown *below* each lane. *B,* QRT-PCR analysis of gene expression in FACS-sorted retrovirally transduced GFP-positive 5-FU-treated bone marrow mononuclear cells. Results shown as mean ± S.E. (as in Fig. 7). *C,* QRT-PCR analysis of antioxidant enzyme gene expression in cells from *B,* SOD, GPX-1. *UNT,* untransduced.
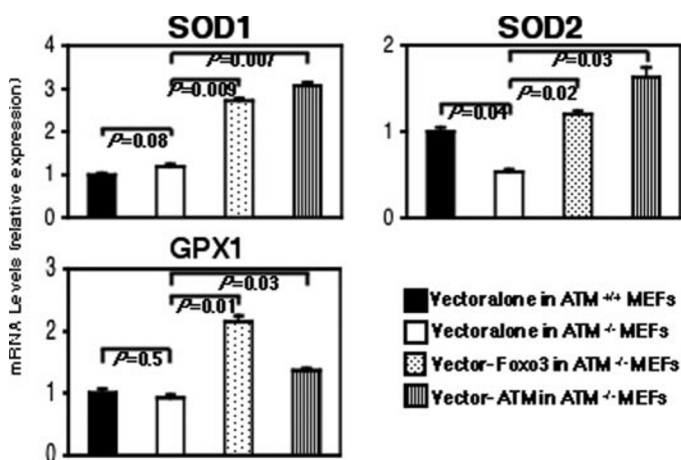
FIGURE 10. **Foxo3 and ATM regulate independently expression of antioxidant enzymes.** *A,* wild type and ATM$^{-/-}$ MEFs were transfected using Lipofectamine with MIG-Foxo3 or MIG-ATM or empty MIG vector; GFP$^+$ MEFs were FACS-sorted 48 h later, and expression of antioxidant enzymes was analyzed by QRT-PCR. Results shown as mean ± S.E. of two independent experiments each performed in triplicate.

source of ROS and signaling pathways governing the regulation of ROS in hematopoietic stem cells.

Our key finding is that Foxo3 is a physiological regulator of oxidative stress that is essential for the regulation of hematopoietic stem cell fate. Foxo3 is required for the expression of ATM and antioxidant enzymes. By regulating expression of ATM tumor suppressor and antioxidant enzymes, Foxo3 maintains ROS below a harmful threshold.

Foxo3 Is Essential for Normal ATM Expression—FoxO functions parallel those associated with p53 tumor suppression, including induction of apoptosis, cell cycle arrest, and antioxidative stress function. In addition, Foxo3 and p53 share a growing list of common targets such as Fas ligand, PUMA, SOD2, Gadd45, and p21$^{\text{CIP1/WAF1/Sdi1}}$ (as in our studies) and undergo similar phosphorylation, acetylation, and ubiquitination that regulate their activities, albeit in a nonsimilar fashion (reviewed in Ref. 41). Cross-talk between Foxo3 and p53 has also been observed in model culture systems (42, 43). Here we have shown that in Foxo3-null hematopoietic stem cells, p53 antioxidant response that is acti-

vated and in contrast to the studies by Miyamoto *et al.* (22), we observed a robust p53-mediated up-regulation of p21$^{\text{CIP1/WAF1/Sdi1}}$. Activation of p53/p21$^{\text{CIP1/WAF1/Sdi1}}$ may shield Foxo3-deficient hematopoietic stem cells from damages that may cause clonal expansion of this population. Activation of p53 in response to oxidative stress in Foxo3-null hematopoietic stem cells may suggest that p53 and Foxo3 play complementary functions counterbalancing each other as gatekeepers in hematopoietic stem cells.

ATM serine/threonine protein kinase is a critical enzyme in the regulation of stress response to DNA damage. ATM activation enhances p53 activity through several pathways, including direct phosphorylation of p53. ATM serine/threonine protein kinase is activated in response to stress, especially stress-induced by double strand DNA break that may also be ROS-induced (6, 44). Activation of ATM involves its kinase domain and is not known to affect its level of expression (6). Activated ATM in turn results in accumulation and activation of p53 tumor suppressor.

Thus, down-regulation of ATM and ATM phosphoserine 1981 in Foxo3$^{-/-}$ hematopoietic stem cells was not anticipated (Fig. 6) and clearly is not mediated by ROS (Fig. 6C). The over 2-fold reduction of ATM expression in Foxo3-null hematopoietic stem cells is of significance because ATM$^{+/-}$ mice display a compromised stress response (36). Our results demonstrate that Foxo3 is essential for the regulation of ATM expression (Figs. 6–8) as shown by the following: (*a*) ATM transcript and protein are highly and specifically down-regulated in Foxo3-null hematopoietic stem cells (Fig. 6); (*b*) ATM activity (ATM phosphoserine 1981) is also down-regulated; (*c*) down-regulation of ATM expression in Foxo3-null hematopoietic stem cells is ROS-independent; (*d*) overexpression of Foxo3 rescues the expression of ATM in Foxo3-deficient primitive hematopoietic cells (Fig. 7) and enhances ATM expression in NIH 3T3 cells (Fig. 8A); and (*e*) shRNA silencing of Foxo3 in NIH 3T3 cells results in significant down-regulation of ATM transcript and protein (Fig. 8, *B–D*).

Furthermore, our results show that the relative ATM deficiency participates in accumulation of ROS in Foxo3-null LSK cells because of the following: (*a*) overexpression of ATM rescues expression of antioxidant enzymes in Foxo3-deficient primitive hematopoietic cells (Fig. 9C) as does ectopic expression of Foxo3 in these cells (Fig. 7C); (*b*) ATM like Foxo3 is required for full SOD2 expression (Fig. 10); and (*c*) ATM and Foxo3 enhance the expression of several antioxidant genes independently (Figs. 7, 9, and 10).

As in ATM$^{-/-}$ (2), p16^{INK4a} was significantly up-regulated in Foxo3-deficient hematopoietic stem cells (Fig. 6A). However, in contrast to ATM$^{-/-}$ hematopoietic stem cells (2), enhanced expression of p16^{INK4a} was not mediated by ROS (Fig. 6C). This difference may be due to age-related regulation of ROS; ROS-mediated up-regulation of p16^{INK4a} was observed in old (24-week-old) ATM$^{-/-}$ hematopoietic stem cells, whereas ROS-independent up-regulation of p16^{INK4a} was detected in young (8–12-week-old) Foxo3$^{-/-}$ hematopoietic stem cells in our studies (Fig. 6A). In Foxo3-deficient hematopoietic stem cells, ROS also mediated the pregulation of p19^{ARF} that inhibits the degradation of p53 (Fig. 6C).

These data suggest that in Foxo3-deficient hematopoietic stem cells, multiple ROS-mediated signaling pathways lead to the up-regulation and activation of p53.
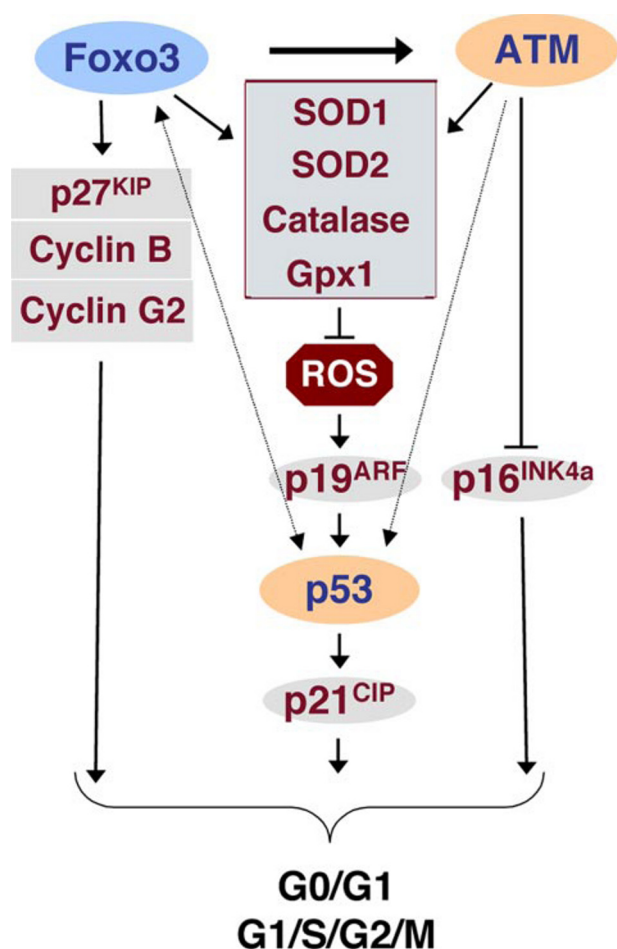
Although ROS accumulation mediated the activation of p53/p21$^{\text{CIP1/WAF1/Sdi1}}$ in both Foxo3-null hematopoietic stem cells (Fig. 6) and in a subset of erythroid precursor cells (12), its impact on cell cycling of these populations was distinct. Loss of Foxo3 led to relative loss of quiescence and a mitotic delay (altered G$_2$/M phase) in hematopoietic stem cells as compared with a G$_1$ arrest in erythroid precursor cells (that are about to exit the cell cycle and differentiate) resulting in reduced erythroid cell differentiation (12). These findings suggest that Foxo3 plays distinct functions in different cellular contexts (hematopoietic stem cell as compared with erythroid cell) and may itself be regulated differently in these cells.

We found that Foxo3-null LSK cells display a delay in the G$_2$/M phase of cell cycle (Fig. 4, *D* and *F*) consistent with the notion that FOXO3a activity is required in G$_2$/M for cell cycle completion (31). These findings may result from loss of Foxo3 directly, from accumulation of ROS, or from reduced expression and/or activity of ATM in Foxo3-null LSK cells. Induced nuclear localization of active FOXO3a fused to the ligand-binding domain of estrogen receptor in Rat-1 fibroblasts also resulted in a delay in the G$_2$/M phase of cell cycle (45) further supporting that the function of FoxO may vary with cellular context and conditions.

Foxo3 Is an Essential Regulator of Hematopoietic Stem Cell Fate—We showed that Foxo3 is the principal active FoxO (Foxo4 expression is barely detectable and Foxo1 is mostly cytoplasmic and therefore presumably functionally mostly repressed) in hematopoietic stem cells (Fig. 1). Our studies determined that loss of Foxo3 alone results in severe reduction of expression of FoxO direct targets in hematopoietic stem cells such as cell cycle and antioxidant enzyme genes (Figs. 3C and 5), demonstrating the essential function of Foxo3 in the regulation of these genes in hematopoietic stem cells. In addition, we found substantial accumulation of ROS in Foxo3-null hematopoietic stem cells (Fig. 3) similar to what was observed in FoxO-null hematopoietic stem cells (11). We further demonstrated that this ROS accumulation mediates the loss of hematopoietic stem cell numbers and function in young Foxo3-null animals (Fig. 3). Moreover, in contrast to Tothova *et al.* (11) and as anticipated, we did not find up-regulation of expression of direct targets of FoxO such as p27^{KIP1} and cyclin G2 in response to NAC treatment (Fig. 5). Whether the differences in gene regulation in response to antioxidant treatment indicate distinct regulation of FoxO direct targets in Foxo3-null as compared with FoxO-null hematopoietic stem cells require further investigation.

Together these findings strongly argue that Foxo3 (and not other FoxO) is the principal regulator of oxidative stress and cell cycle in hematopoietic stem cells. In addition, our studies demonstrate that the function of Foxo3 in the modulation of oxidative stress is essential for determining the fate of hematopoietic stem cells. These findings have significant consequences beyond stem cells of hematopoietic origin as they suggest potential important function for Foxo3 in other stem cell systems such as oocytes, where loss of Foxo3 leads to their

FIGURE 11. **Model for Foxo3/ATM regulation of stem cell cycling and self-renewal.** This model is based on data derived from studies with primary mouse cells and MEF cells.

pendent mechanisms and lead to G_0 exit and G_2/M arrest in Foxo3-null hematopoietic stem cells (Figs. 5 and 6).

Foxo3-deficient hematopoietic stem cells exhibit a phenotype less pronounced but similar to hematopoietic stem cells with deficiency in phosphatase and tensin homology that is a negative regulator of PI 3-kinase/AKT (46, 47). Foxo3- and FoxO-deficient hematopoietic stem cells display distinct cell cycle and apoptosis phenotypes (Fig. 4) (11, 22). It is noteworthy that although we cannot rule out additional loss through apoptosis, we did not detect significant apoptosis in freshly isolated or short term cultured Foxo3-deficient hematopoietic stem cells (supplemental Fig. 1). These combined findings indicate that relative levels of ROS and FoxO activity have important impacts on hematopoietic stem cell fate. Thus, tight regulation of phosphatase and tensin homology/PI 3-kinase/Fox signaling pathway is essential for preserving hematopoietic stem cell pool and homeostasis.

Our studies suggest a model in which Foxo3 together with ATM, p53, and p16^{INK4a} provide a strong tumor suppressor network in protecting stem cells from damage that may undermine their genomic stability and result in their clonal expansion (model Fig. 11). Sustained activation of these pathways may alternatively result in senescence leading to the defects seen in Foxo3-null hematopoietic stem cells derived from aged mice (22). Future elucidation of the mechanisms of regulation of Foxo3 in hematopoietic stem cells, in particular potential interactions of Foxo3 with coactivators and corepressors, as well as identification of Foxo3 targets in stem cells, will be of fundamental importance for better understanding of aging of hematopoietic stem cells and malignant transformation in cancer stem cells.

Note Added in Proof—While this manuscript was under review, a new report showing that Foxo3 directly interacts with ATM and regulates its activity in response to DNA damage was published (51). These findings further the link between Foxo3 and ATM.

depletion (23), and likely Foxo3 regulation of oxidative stress in nonhematopoietic stem cells.

The discrepancy between our study and studies reported by Tothova *et al.* (11) may be due to the timing of experiments relative to the excision of the knock-out alleles (4 weeks after excision) in the work of Tothova *et al.* (11). Because we observe a clear phenotype only after 8–9 weeks of age, we believe it may take this long for loss of Foxo3 (and accumulation of ROS in Foxo3-null HSC) to make a deleterious impact on hematopoietic stem cell function. Analysis of conditional Foxo3$^{-/-}$ mice in the studies of Tothova *et al.* (11) only 4 weeks after excision may not reveal the effect of loss of Foxo3 in hematopoietic stem cells. However, differences in the mouse strain (mixed $129 \times$ FVB *versus* mixed $129 \times$ C57BL6) may explain some of the divergence in our findings of Foxo3-null hematopoietic stem cell phenotype as compared with Miyamoto *et al.* (22). In addition to the findings by Miyamoto *et al.* (22), we found substantial ROS accumulation in Foxo3-null hematopoietic stem cells (Fig. 3, *A* and *B*) derived from young mice. We demonstrated that ROS mediates the repressed hematopoietic stem cell activity in Foxo3-null mice (Fig. 3D). We further identified several tumor suppressor pathways that are highly altered in Foxo3-null hematopoietic stem cells via ROS-dependent and -inde-

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