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Aging-like Phenotype and Defective Lineage Specification in SIRT1-Deleted Hematopoietic Stem and Progenitor Cells

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SUMMARY

Aging hematopoietic stem cells (HSCs) exhibit defective lineage specification that is thought to be central to increased incidence of myeloid malignancies and compromised immune competence in the elderly. Mechanisms underlying these age-related defects remain largely unknown. We show that the deacetylase Sirtuin (SIRT)1 is required for homeostatic HSC maintenance. Differentiation of young SIRT1-deleted HSCs is skewed toward myeloid lineage associated with a significant decline in the lymphoid compartment, anemia, and altered expression of associated genes. Combined with HSC accumulation of damaged DNA and expression patterns of age-linked molecules, these have striking overlaps with aged HSCs. We further show that SIRT1 controls HSC homeostasis via the longevity transcription factor FOXO3. These findings suggest that SIRT1 is essential for HSC homeostasis and lineage specification. They also indicate that SIRT1 might contribute to delaying HSC aging.

INTRODUCTION

Adult stem cells maintain tissue homeostasis by regenerating damaged or lost cells during their lifetime. The decline of the regenerative capacity of stem cells with age compromises tissue integrity and may promote organ failure and diseases of aging (Liu and Rando, 2011). This age-related decline in tissue function is considered to be at the root of overall organismal aging. Whether mechanisms that control aging of stem cells influence organismal longevity is unknown. Identifying regulators of stem cell aging is of major significance for public health because such regulators may contribute to promote healthy aging and be valuable therapeutic targets to combat disorders of aging like cancer and Parkinson's disease.

Hematopoietic stem cells (HSCs) are the most extensively studied model of stem cell aging. Although it has been known for decades that HSC age (Harrison, 1983), and the properties of aged HSCs have been greatly characterized, the mechanisms that govern HSC aging have only begun to be defined. HSC aging leads to a paradoxical increase in the stem cell pool and decline in stem cell function (Morrison et al., 1996; Sudo et al., 2000). One of the prominent modifications of HSC properties with age is their biased differentiation toward myeloid lineage at the expense of their lymphoid potential (Challen et al., 2010; Dykstra et al., 2011; Rossi et al., 2005). These age-associated modulations of the composition of HSC progenies lead to defective adaptive immune response. Similarly, the age-related increased incidence of myeloid malignancies, including acute myeloid leukemias, myelodysplasias, and myeloproliferative neoplasms, may be related to the enhanced generation of myeloid skewed HSC progenies. Aging of HSCs is also associated with increased onset of anemia. Although defects in the DNA damage repair program, increased tumor suppressor function, loss of polarity, and epigenetic deregulation have all been implicated in HSC aging, the mechanisms underpinning the age-associated alterations of HSC lineage specification remain largely unknown (Chambers et al., 2007; Dykstra and de Haan, 2008; Florian et al., 2012; Rossi et al., 2005).

The NAD-dependent protein silent information regulator 2 (Sir2) is a deacetylase for histones and other proteins and a key regulator of life span in several organisms. Sirtuin (SIRT)1 of the Sirtuin family is the closest homolog of yeast Sir2 in mammals and has critical functions in the

regulation of metabolism, genome stability, DNA repair, chromatin remodeling, and stress response (Guarente, 2011; Haigis and Sinclair, 2010). SIRT1 coordinates pluripotency, differentiation, and stress response in mouse embryonic stem cells (ESCs) (Han et al., 2008). Whether SIRT1 regulates adult stem cells particularly in the hematopoietic system has been a matter of debate (Leko et al., 2012; Li et al., 2012; Narala et al., 2008; Singh et al., 2013; Yuan et al., 2012). Despite recent advances in understanding SIRT1 regulation of malignant and stressed hematopoiesis, whether SIRT1 has any function in the control of adult HSC homeostasis or aging remains unknown.

The study of SIRT1 in adult mice and during aging has been hampered by the developmental defects and perinatal death of germline SIRT1 knockout mice (Cheng et al., 2003; McBurney et al., 2003). Using a recently developed adult tamoxifen-inducible SIRT1 knockout mouse model (Price et al., 2012), we show that SIRT1 is essential for the self-renewal and homeostatic maintenance of the HSC pool. Importantly, we show that loss of SIRT1 is associated with anemia and a significant expansion of the myeloid compartment, specifically granulocyte-monocyte progenitors (GMPs), at the expense of the lymphoid compartment. These phenotypic alterations are concomitant with significant modulations of expression of transcription factors implicated in the generation of GMPs and common lymphoid progenitors (CLPs). Notably, we show that the longevity transcription factor FOXO3 mediates SIRT1 homeostatic effects in HSCs. These unexpected results indicate that young SIRT1-deleted HSCs have several overlapping features with normal aged HSCs. Altogether, our studies identify SIRT1 as a key regulator of HSC maintenance under homeostasis. In addition, the evidence supports an essential function for SIRT1 in the regulation of HSC lineage specification. Overall, our findings suggest that SIRT1 might be implicated in delaying HSC aging.

RESULTS

Loss of SIRT1 Compromises Hematopoietic Stem Cell Function at the Steady State

To address the potential function of SIRT1 in hematopoietic stem and progenitor cells (HSPCs), we first analyzed SIRT1 expression. As predicted by previous studies (Deneault et al., 2009), SIRT1 transcript was increased in Lin$^-$SCA-1$^+$C-KIT$^+$ (LSK) cells enriched for HSCs and in Lin$^-$SCA$^-$1$^+$C-KIT$^+$ (c-Kit$^+$) hematopoietic multipotential progenitors as compared to total bone marrow (BM) cells (Figure S1A available online). However, the level of SIRT1 transcript in LSK CD48$^-$CD150$^+$ that is highly enriched for long-term HSCs (LT-HSCs), and in all hematopoietic progenitor cells surveyed, was relatively similar (Figure S1B). SIRT1 protein was also readily detected in the nucleus of HSPCs (Figure S1C). Next, we used sirtinol, a pharmacological inhibitor of SIRT1 (Grozinger et al., 2001), to evaluate whether SIRT1 has any functions in HSCs. A 3 week in vivo injection of sirtinol led to a significant decrease of the frequency and the total number of LSK cells (Figures S1D and S1E) in the treated animals, including the LSK CD48$^-$CD150$^+$ cell subset (Figures S1F and S1G).

To circumvent potential off-target effects of pharmacological inhibitors of SIRT1 and investigate the function of SIRT1 more directly, we used a tamoxifen-inducible SIRT1 deletion mouse model (Price et al., 2012). The SIRT1 catalytic domain was conditionally deleted from 6- to 8-week-old floxed SIRT1$^{\Delta ex4}$; Cre-ERT2 mice by intraperitoneal tamoxifen injection over 5 days. The regimen led to the expression of a truncated protein that was detected as a lower molecular weight SIRT1 4 weeks after tamoxifen treatment in the BM and spleen (Figure S2A). At the same time, the truncated SIRT1 protein was detected in highly purified HSPCs (Figure S2B). Notably, and in contrast to Singh et al. (2013), this regimen did not significantly modulate the BM cellularity (Figure S2C) overtime whether or not Cre was present (Figure S2D), enabling us to examine the effects of loss of SIRT1 function on HSPCs under homeostatic conditions.

The effect of SIRT1 deletion on the HSC compartment of *Sirt1fl/fl;Cre*$^+$ (Δ/Δ) mice was monitored at different time points after tamoxifen treatment using both *Sirt1fl/fl;Cre*$^-$ (fl/fl) and *Sirt1WT/WT;Cre*$^+$ (WT/WT) mice as controls. Loss of SIRT1 function in young adult mice resulted in a gradual increase in the total number and the frequency of both LSK cells (Figure 1A, top panel; Figure S2E) and LT-HSC (LSK CD48$^-$CD150$^+$) (Figure 1A, bottom panel; Figure S2F) in the BM. In addition, the total number and frequency of multipotent progenitors (MPPs; LSK FLK2$^+$ CD34$^+$) and lymphoid multipotent progenitor (LMPP; LSK FLK2^{high}CD34$^+$) cells also increased in SIRT1-deleted mice without significant effects on the short-term HSC (ST-HSC; LSK FLK2$^-$CD34$^+$) (Figures 1B and S2G–S2H). These results were intriguing and suggested that SIRT1-deficiency compromised the HSC compartment at the steady state.

To evaluate whether SIRT1 has cell-autonomous functions, we injected 100 highly purified SIRT1-deficient LT-HSCs into lethally irradiated congenic-recipient mice along with 200,000 recipient BM cells in an in vivo competitive repopulation assay. Whereas control cells gave rise to strong chimerism and multilineage reconstitution in all 12 transplanted recipients, the long-term repopulation ability of SIRT1-deficient HSCs was 2.5-fold reduced as compared to controls (Figure 1C). The analysis of recipient mice 16 weeks after the primary transplantation showed a significant decrease in the frequency and total number of donor-derived LSK cells (Figure 1D), suggesting that

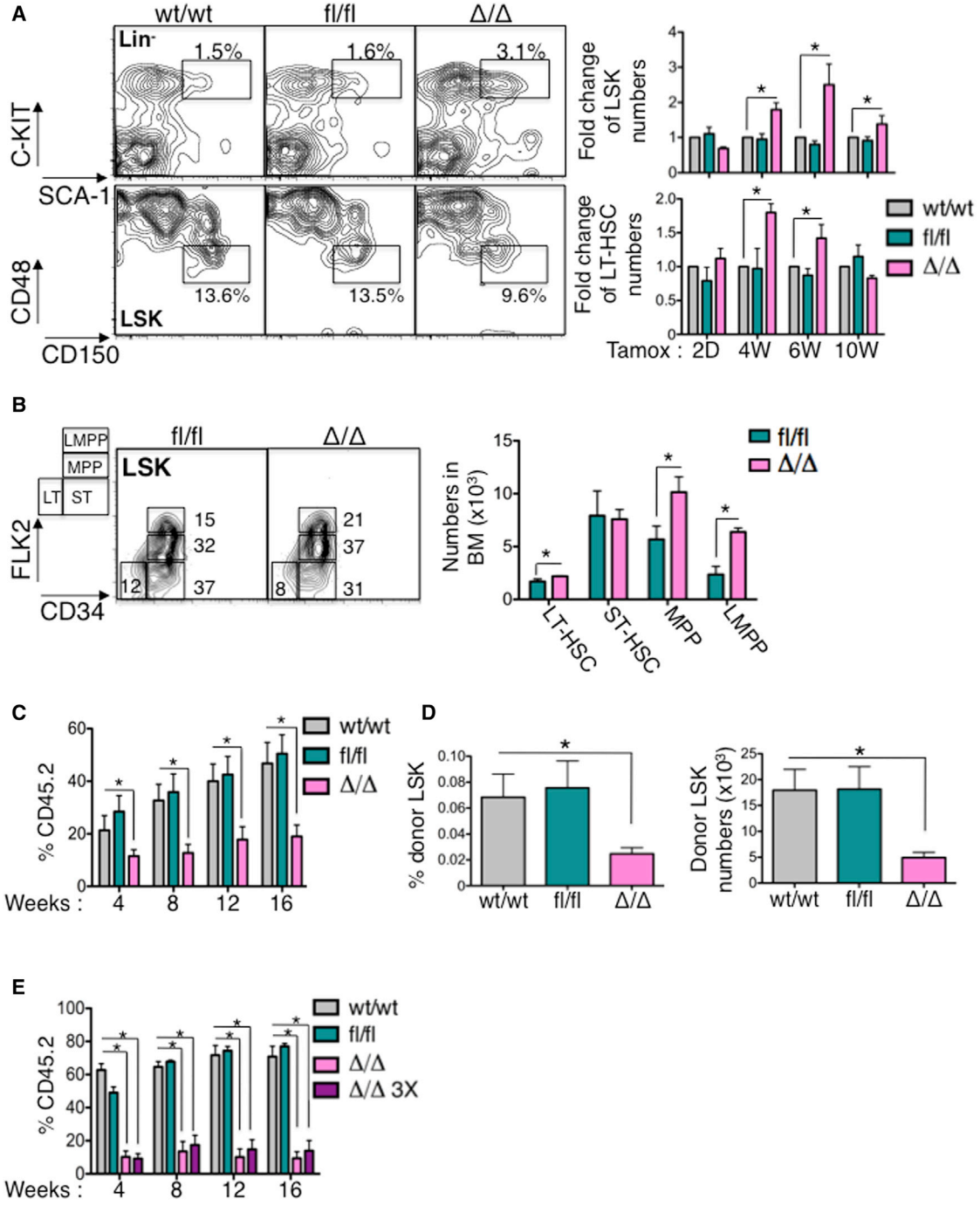

Figure 1. Conditional Deletion of SIRT1 Compromises Homeostatic HSC Function

(A) Representative fluorescence-activated cell sorting (FACS) plots of LSK cells (left upper panel) and LSK $CD48^{-}CD150^{+}$ (LT-HSC) (left lower panel) frequencies from *Sirt1WT/WT* (WT/WT; cre^{+}), *Sirt1fl/fl* (fl/fl; cre^{-}), and *Sirt1*Δ/Δ (Δ/Δ, cre^{+}) BM (see Figures S2E and, S2F). Fold change of total BM LSK (right upper panel) and LT-HSC (right lower panel) numbers normalized to *Sirt1WT/WT* controls set at one (= 25,000 LSK and 4,000 LT-HSC; n = 6 mice for each group and time points; d, days; w, weeks after tamoxifen [Tamox] treatment).

(B) CD34 versus FLK2 expression analysis of LSK cells. Representative FACS plots of frequency of LT-HSC (LT) and ST-HSC (ST), MPP, and LMPP within LSK cells (left panel) are shown. Total numbers of each population in the BM (right panel) 8 weeks after tamox treatment are shown (n = 3 mice).

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SIRT1-deleted HSCs have a cell-autonomous functional defect. These results were associated with a profound decline in HSC self-renewal (Figure 1E). Equal numbers of total bone marrow cells from both control and SIRT1-deficient primary recipients were transplanted into lethally irradiated secondary recipients. Whereas control cells exhibited a normal ability to mediate long-term repopulation, the reconstitution of SIRT1Δ/Δ cells was greatly reduced in ten secondary recipients 4 to 16 weeks after transplantation (Figure 1E). In addition, injection of three times as many SIRT1-deficient HSCs as control cells did not significantly improve reconstitution in secondary transplants (Figure 1E), suggesting that SIRT1-defective HSCs are highly compromised in their self-renewal ability. Altogether, these findings may indicate that HSCs expanded transiently in numbers in response to loss of SIRT1 but were unable to maintain their function overtime (Figures 1A–1E).

SIRT1 Maintains HSC Quiescence at the Steady State In Vivo

To obtain further insight into the expansion and subsequent loss of HSC function, we examined HSC cell-cycle status. Although there was no effect of SIRT1 deletion on the cell-cycle distribution of total BM, BM cells depleted of all mature cells (lineage-negative cells, Lin^-) and c-Kit^+ cells (Figures 2A and S2I), there was a significant increase in the fraction of SIRT1-deleted LT-HSCs and LSK cells that incorporated in vivo the nucleotide analog bromodeoxyuridine (BrdU) compared to their wild-type (WT) counterparts (Figure 2A). In addition, the cycling fraction of SIRT1-deleted MPP (LSK $FLK2^+CD34^+$) (Figure 2B) and SIRT1-deleted lineage-restricted progenitors (LRP; LSK $CD48^+CD150^-$) (Yilmaz et al., 2006) were significantly increased (Figures 2A and S2I). Altogether, these results suggest that the dividing fraction of SIRT1-deleted HSPCs, including LT-HSCs had increased ($p < 0.05$), compared to more restricted progenitors.

In agreement with these findings, a significant fraction of SIRT1-defective LSK cells exited quiescence (G0) as compared to wild-type cells and showed increased expression of KI67 that marks proliferating cells (Figure 2C). Staining with Hoechst 33342 and Pyronin Y for DNA/RNA content led to similar results from LT-HSCs and HSPCs as compared to c-Kit^+ cells (Figure S2J). These abnormalities of SIRT1-deleted HSPC cycling were associated with altered expression of genes critical for the regulation of HSC dormancy versus cycling, including *p27* (*Cdkn1b*), *p21* (*Cdkn1a*), *CyclinG2* (*Ccng2*), and *CyclinD1* (*Ccnd1*) (Passegué et al., 2005). Specifically, the expression of p27 and Cyclin G2 that is associated with the maintenance of quiescence (Cheng et al., 2000; Yalcin et al., 2008) was reduced, whereas *CyclinD1*'s expression that is specifically increased with HSC differentiation was highly enhanced in SIRT1-deleted LT-HSCs (Figure 2D). These expression patterns were relatively distinct from the ones observed in c-Kit^+ cells (Figure 2D). Loss of SIRT1 was also associated with a slight, but significant, decrease in apoptosis of LSK cells (ANNEXIN V^+ cells) ($n = 6$, $p < 0.05$) (Figure 2E). Consistent with this, the expression of *Bim* and *Bax*, two critical mediators of apoptosis, was significantly downregulated (Figure S2K). However, the altered apoptotic rate was also seen in SIRT1Δ/Δ BM, Lin^-, and c-Kit^+ cells (Figure 2E), suggesting that SIRT1 regulation of apoptosis of hematopoietic cells is not limited to the HSPC compartment.

Together, these data indicate that SIRT1 has a significant impact on the cycling status of HSPC compartments. In particular, they suggest that SIRT1-defective HSCs exit quiescence and enter the cell cycle to maintain a functionally declining HSC pool.

SIRT1 Is Essential for the Myeloid versus Lymphoid Lineage Specification

Loss of SIRT1 function was associated with noticeable anomalies in the peripheral blood (PB) cell counts (Figure 3A; Table S1). SIRT1-deficient mice displayed anemia, significantly decreased absolute and relative numbers of lymphocytes, and increased numbers of neutrophils, monocytes, and eosinophils within 14 weeks after tamoxifen treatment (Table S1). Blood abnormalities were associated with lineage-specific defects (Figure 3A) in SIRT1Δ/Δ BM (Figure S3A) and spleen (Figure S3B). In particular, BM was enriched for colony-forming unit-spleen (CFU-S) multipotent progenitors (Figure 3B). Consistent with these results, the myeloid colony-forming cells were significantly increased in SIRT1-deleted BM (Figure S3C). In addition, anemia was accompanied with a significant defect in terminal erythroid maturation in SIRT1-deleted BM (Figures S4A and S4B) and in the BM erythroblasts derived from SIRT1-deleted HSC donors (Figures S4C and S4D).

Consistent with the PB count of SIRT1-deleted mice and defective differentiation of SIRT1-deleted HSCs (Table S1), frequencies of Gr-1^+ Mac-1^+ ($Ly6G^+CD11b^+$) myeloid cells were increased, whereas frequencies of $CD4^+$ $CD8^+$, $CD3^+$

(C) The contribution of transplanted SIRT1-deleted LT-HSC (CD45.2) to PB of recipient mice (CD45.1) in a long-term competitive repopulation assay is shown (n = 12 mice in each group).
(D) Frequency and total number of donor LSK (CD45.2) cells 16 weeks posttransplantation in the BM of recipient mice (n = 6 mice).
(E) Self-renewal analyzed by secondary transplantation of total BM cells from primary recipients (Δ/Δ 3X, three times as many BM cells from $SIRT1^{\Delta/\Delta 3X}$; n = 10 mice in each group). All data are expressed as mean ± SEM (*$p < 0.05$).

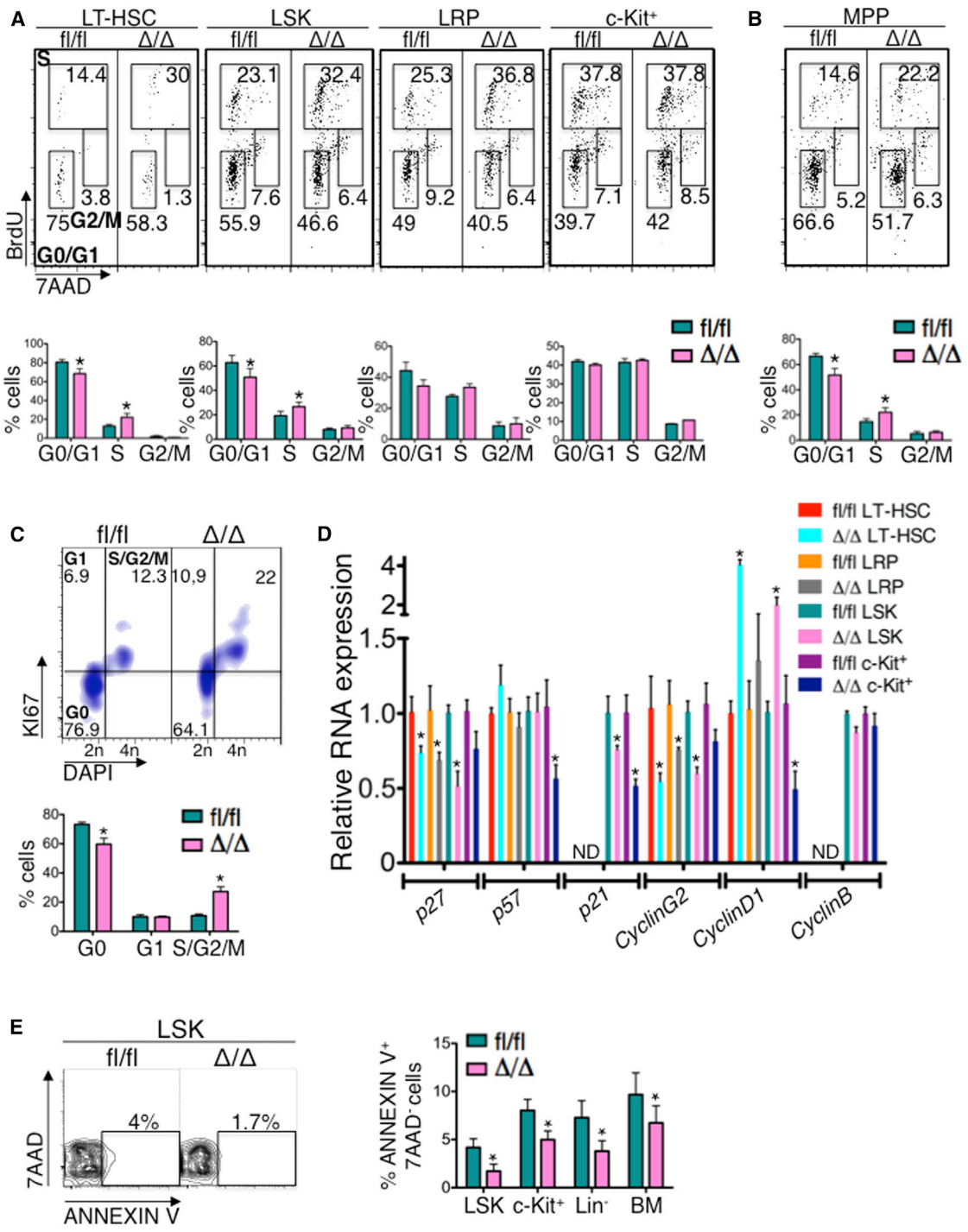

Figure 2. Conditional Deletion of SIRT1 Compromises HSC Quiescence

(A and B) Representative FACS plots (upper panel) and mean values (lower panel) of cell-cycle distribution of BrdU incorporation of LT-HSC, LSK, LSK $CD48^{+}CD150^{-}$ (lineage-restricted progenitors, LRP; n = 3 mice) and c-Kit^{+} (n = 6 mice per genotype) (A) and of LSK $FLK2^{+}CD34^{+}$ MPP cells (n = 3 mice per genotype) (B).

(C) Representative FACS plots (upper panel) and mean values (lower panel) of cell-cycle distribution by KI67/DAPI staining of LSK cells (n = 6 mice per genotype).

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T lymphocytes were reduced in the PB of both primary recipients of SIRT1-deleted HSC donors (Figures 3C and S5A) and of secondary recipients at 16 weeks posttransplantation (Figures 3D and S5B). These results altogether reflect cell-autonomous defects of HSC differentiation. Similar abnormalities were found in the BM (Figure S5C) and spleen (Figure S5D) of the primary recipients. To further delineate whether the SIRT1-deleted T and B lymphoid abnormalities were HSPC driven, we isolated SIRT1-deficient LSK cells 4 weeks after tamoxifen injections and measured their potential to produce lymphoid cells when cultured on stromal OP9-DL1 and OP9 monolayers that support T and B cell growth, respectively (Schmitt and Zúñiga-Pflücker, 2002; Vieira and Cumano, 2004). The OP9 stromal cell line supports B cell growth under defined in vitro condition (Vieira and Cumano, 2004), whereas the OP9 line ectopically expressing the NOTCH ligand Delta-like-1 protein supports the differentiation of hematopoietic progenitors into T cells (Schmitt and Zúñiga-Pflücker, 2002). In agreement with the in vivo findings, the potential of SIRT1-deficient HSPCs to generate T cells in vitro was significantly compromised without noticeable effect on cell viability (Figures 3E, S6A, and S6B; data not shown). Similarly, the B cell differentiation potential was also impaired, albeit more mildly and with a slightly different kinetic (Figures 3F, S6C, and S6D). These combined findings strongly suggest that SIRT1-deleted HSC differentiation is altered generating increased myeloid and decreased lymphoid cells both in vivo and in vitro.

In support of this notion, SIRT1-deficient mice exhibited splenomegaly (Figure 4A). The splenic architecture was noticeably disorganized with regional disruption of the white pulp and a concomitant increase in hematopoietic elements in the red pulp that consisted mostly of myeloid cells (Figure 4B). In contrast, the white pulp was composed of a large number of pale, poorly defined lymphoid nodules (Figure 4B). The total number of LSK cells in the spleen was also increased (Figure 4C). Consistent with these results, spleen histology revealed abundant extramedullary hematopoiesis associated with hemosiderin deposition in the red pulp (Figure 4B).

In search for the source of lineage abnormalities, we examined the hematopoietic progenitor compartment. Unexpectedly, the myeloid-biased HSC differentiation observed in SIRT1-deficient hematopoietic organs, was associated with a significant and specific increase in the GMP compartment in the BM detectable 10 weeks after tamoxifen treatment, whereas the size of common myeloid progenitor (CMP) and megakaryocyte/erythroid progenitor (MEP) compartments did not change (Figures 5A and 5B). These surprising alterations were further associated with a progressive 2-fold decrease in the frequency of CLP overtime (Figure 5B). Remarkably, the mild but specific enhanced production of GMP associated with anemia and decreased CLP characterizes the age-associated alterations of HSC differentiation (Rossi et al., 2005, 2007a).

Hematopoietic lineage abnormalities of SIRT1-deleted HSCs (Figures 3, 4, and 5; Figures S3–S6; Table S1) were associated with alterations in the expression of several transcription factors implicated in the generation of GMPs, including C/EBPα, PU.1, GATA-1, and GATA-2 (Iwasaki et al., 2006) (Figure 5C). Similarly, expression of lymphoid specification transcription factors IKAROS and GATA-3 was significantly reduced in SIRT1-deleted c-Kit$^+$ multipotent progenitors (Figure 5C). Key genes (Rothenberg and Hogan, 2006) associated with eosinophil production, including *Gata2*, Interleukin 5 receptor α (*Il5ra*), and major basic protein 1 (*Mbp1*), were abnormally expressed in BM myeloid progenitors (Figure 5D). Interestingly, *Il5ra* and *Mbp1* were highly upregulated in MEPs, where these genes are normally undetectable (Figure 5D). Collectively, these findings suggest that SIRT1 has a critical function in HSC lineage specification, in particular in the control of myeloid versus lymphoid lineage commitment.

Young SIRT1-Deleted HSCs Recapitulate the Main Features of Aged HSCs

We further examined whether SIRT1-deleted HSCs exhibit age-associated features. DNA damage is chief among molecular processes leading to cellular aging, including aging of HSCs (Rossi et al., 2007b). Freshly isolated SIRT1-deficient HSPCs exhibited accumulation of damaged DNA overtime as indicated by increased phosphorylation of histone H2AX that marks the response to DNA damage (Figure 6A). These abnormalities were associated with increased length of comets generated by DNA breaks in gel electrophoresis (Figure 6B), suggesting that loss of SIRT1 results in relative loss of DNA repair potential of HSPCs. Accumulation of reactive oxygen species (ROS) has been associated with an aging phenotype in many cell types (Dykstra and de Haan, 2008). SIRT1-deficient HSPCs exhibited increased levels of ROS measured by flow cytometry in freshly isolated cells using the oxidative stress-sensitive CM-H_2DCFDA probe (Figure 6C). ROS

(D) qRT-PCR analysis of cell-cycle regulators. Results are relative to *Sirt1fl/fl* set at one in each population (results are from three independent experiments each based on three replicates of one pool of three mice).

(E) Representative FACS plots (left panel, LSK) and frequency (right panel) of apoptotic cells (ANNEXIN V$^+$ 7AAD$^-$) in the BM (n = 6 mice per genotype). In all experiments, SIRT1-deleted cells were isolated 4 weeks after tamox treatment. All data are expressed as mean ± SEM (*p < 0.05).

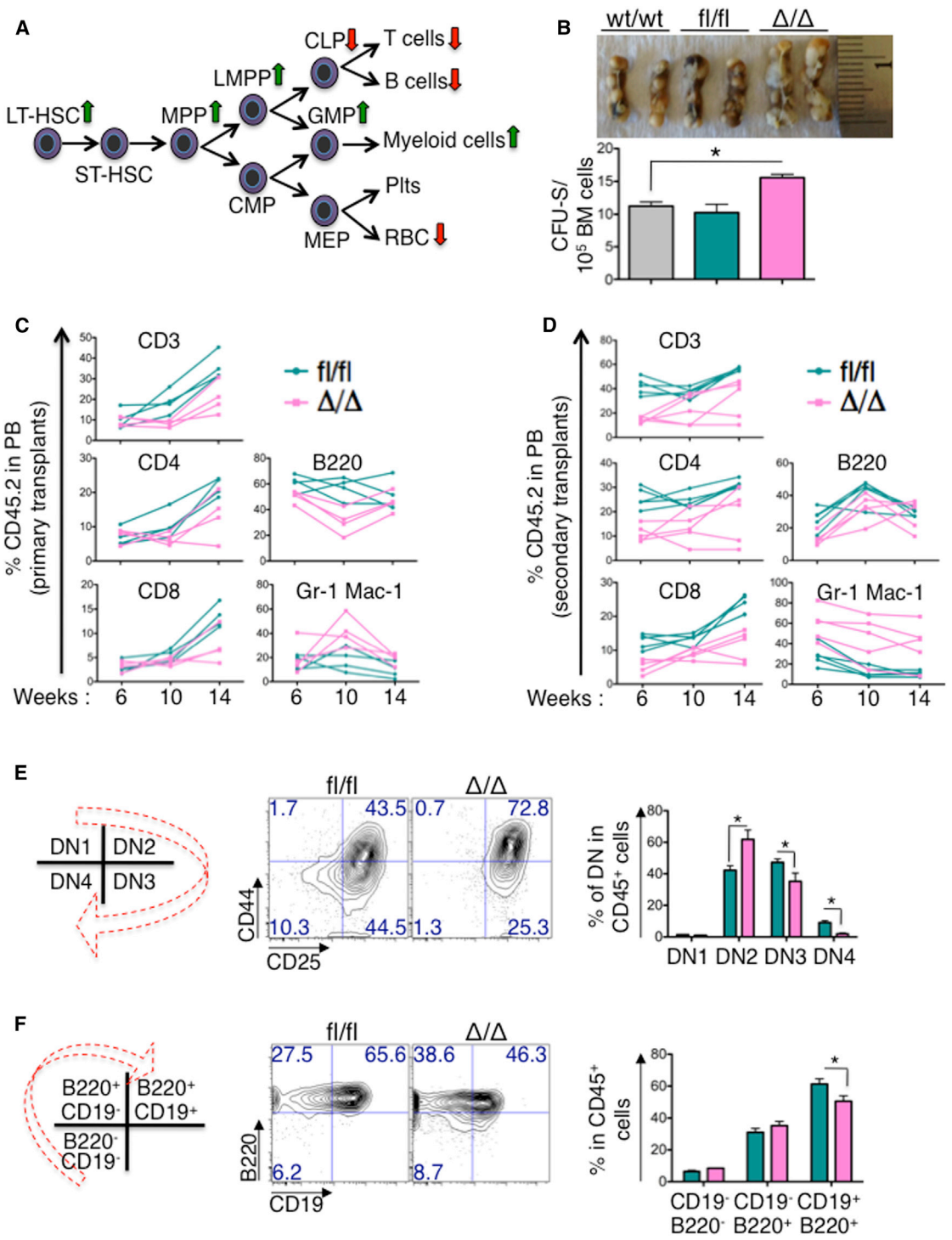

Figure 3. SIRT1-Deleted HSCs Generate In Vivo Myeloid-Biased and Lymphoid-Defective Progenies

(A) Impact of SIRT1 deficiency on the hematopoietic lineages is shown. Green and red arrows indicate increased and decreased cell populations, respectively.

(B) Representative spleens with CFU-S-derived colonies (upper panel) and mean values of BM CFU-S_{d12} frequency (lower panel). One representative of three independent experiments (n = 5 mice in each group) is shown.

(C) Frequency of donor ($CD45.2^+$)-derived multilineage mature cells in the PB of primary transplants (n = 4), at indicated time points posttransplantation.

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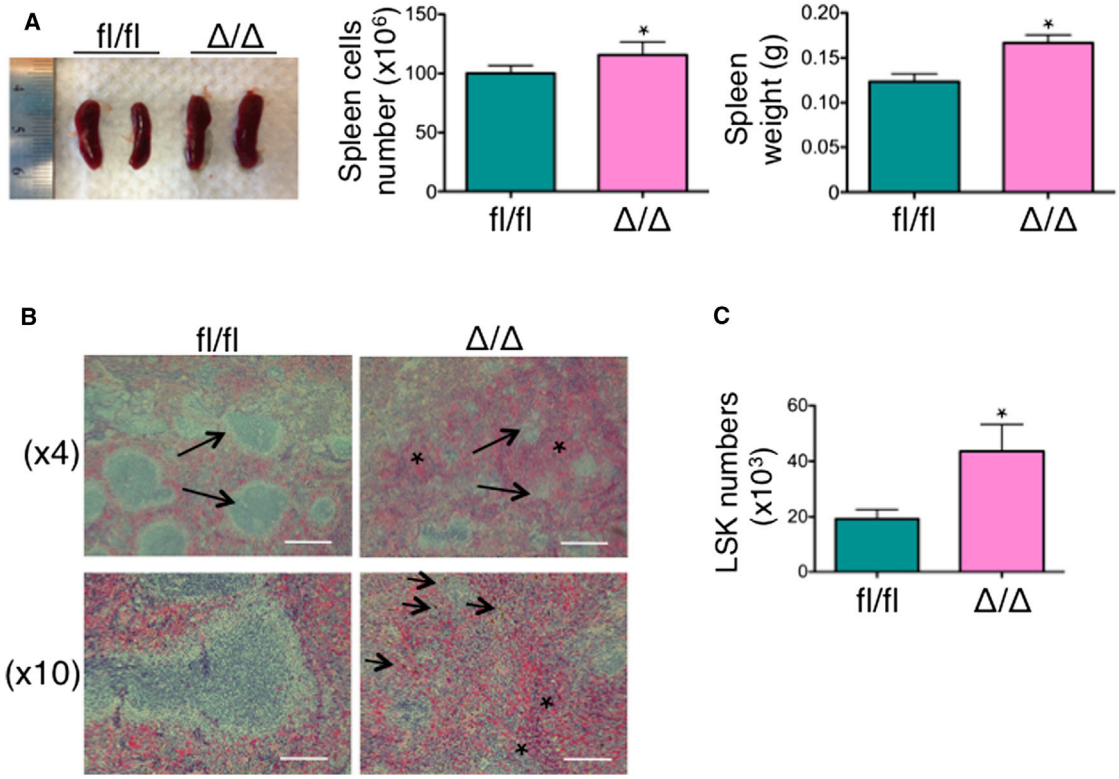

Figure 4. Extramedullary Hematopoiesis in SIRT1-Deleted Mice

(A) Representative spleens (left panel) and mean values of cellularity (mid panel) and weight (right panel), 4 weeks after tamox treatment are shown (n = 6 mice per genotype).

(B) Representative hematoxylin and eosin (H&E) staining of paraffin-embedded sections of spleen 8 weeks after tamox treatment at distinct magnifications. Stars and arrows indicate the red and white pulps, respectively. Scale bar, 200 μm (upper panel). Stars and arrows show myeloid cell increase and hemosiderin deposition in the red pulp, respectively. Scale bar, 20 μm (lower panel).

(C) Total number of LSK cells in the spleen 4 weeks after tamox treatment (n = 6 mice in each group). All data are expressed as mean ± SEM (*p < 0.05).

accumulation was associated with decrease of several antioxidant transcripts (Figure 6D) 4 weeks after tamoxifen treatment, indicating that SIRT1 is required for the maintenance of HSPC redox homeostasis. We also examined the expression of a number of molecules that are modulated with age in HSCs. Among these, the levels of LT-HSC expression of integrin αIIb (*itga2b*, CD41), a classic platelet marker, and the CD150 Slam protein that marks LT-HSCs increase with age (Beerman et al., 2010; Gekas and Graf, 2013). Similarly, CD41 was highly upregulated on SIRT1-deleted LT-HSCs (Figure 6E). The frequency of SIRT1-deleted LT-HSC subset expressing CD41 also increased to 79.4% ± 2.3% from 64.4% ± 3.2% on wild-type controls (p < 0.011, n = 3 mice) (Figure 6E). Notably, the increase of CD41 levels was the highest on $CD150^{high}$-expressing LSK cells (Figure S7A), further supporting the aging-like phenotype of SIRT1-deleted HSCs. Like on old (16 months) wild-type LSK cells, the expression of P-SELECTIN protein (Chambers et al., 2007; Rossi et al., 2005) was increased on the surface of young SIRT1Δ/Δ LSK cells (Figure 6F). Expression of several other genes also highly modulated with age in HSCs, including *Sox4*, *Fos*, and *Pml* (Rossi et al., 2005), was similarly altered in young SIRT1-deficient HSCs and old controls (Figure 6G). In line with a potential

(D) Frequency of donor ($CD45.2^+$)-derived multilineage mature cells in the PB of secondary transplants (n = 5).

(E and F) Representative FACS plots of T (E) and B (F) cell differentiation of LSK cells cultured in vitro for 16 days on OP9-DL1 and OP9 stromal lines. Frequencies of T cells within the $CD4^-$ and $CD8^-$ double-negative (DN) cells positive for CD45 (E, right panel) and B cells within CD45-positive cells (F, right panel) are shown; one representative of two independent experiments (n = 3 technical replicates). The schematic progression of the T and B cell differentiation is shown (same as the third panel of Figure S6). Data expressed as mean ± SEM (*p < 0.05).

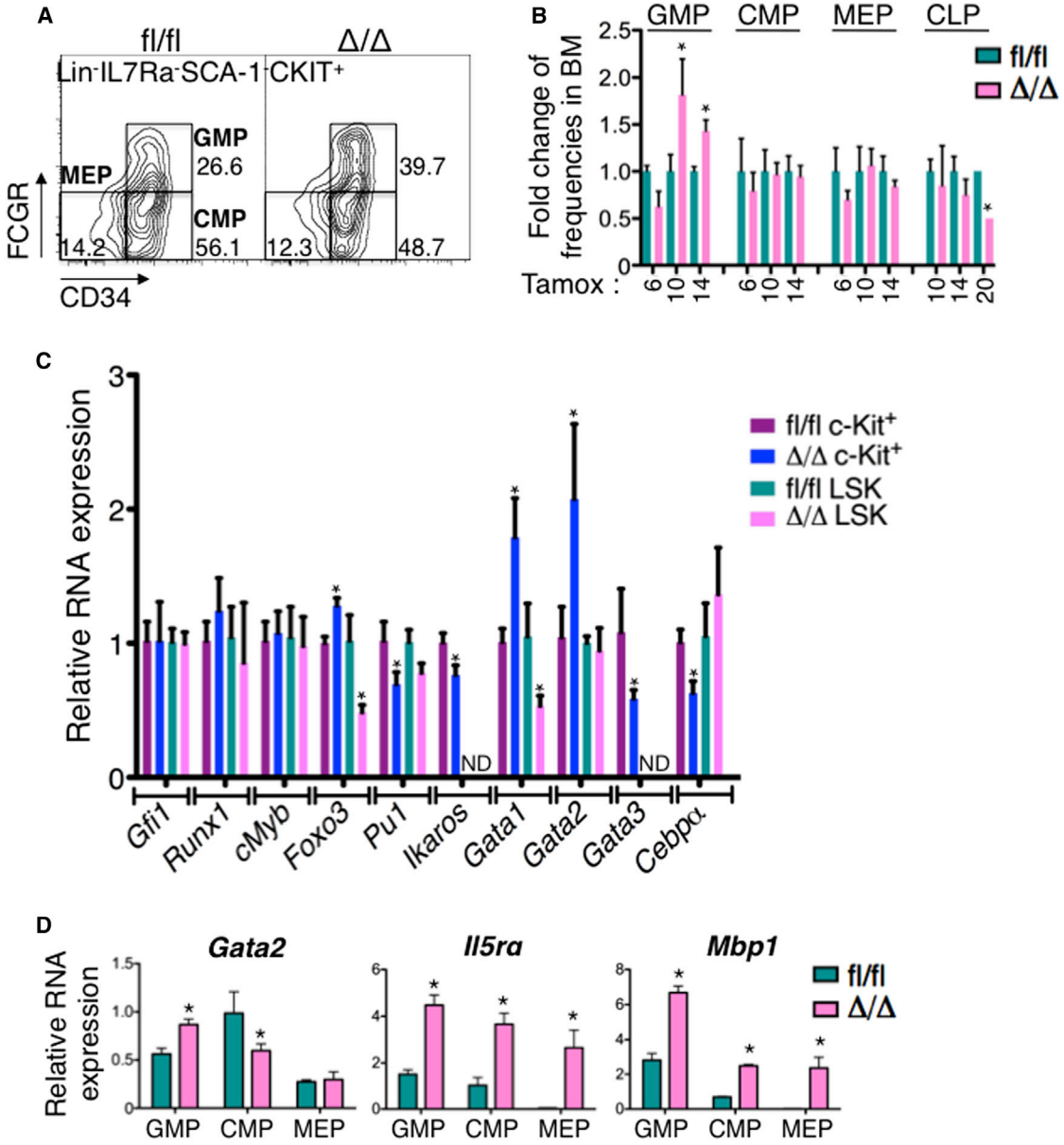

Figure 5. SIRT1-Deleted HSPCs Are Altered in Their Lineage Specification

(A) Representative FACS plots of GMP, CMP, and MEP 10 weeks after tamox treatment.

(B) GMP, CMP, MEP, and CLP frequencies in the BM after tamox treatment (n = 6 mice per group).

(C) qRT-PCR analysis 10 weeks (20 weeks in the case of IKAROS and GATA-3) after tamox treatment. Results are relative to *Sirt1fl/fl* (n = 6 from two independent experiments). nd, not done.

(D) Gene expression analysis of eosinophil markers in GMP, CMP, and MEP by qRT-PCR (normalized to β-actin) 14 weeks after tamox treatment. Results are relative to *Sirt1fl/fl* c-Kit$^+$ (n = 3 replicates from one pool of three mice). All data are expressed as mean ± SEM (*p < 0.05).

contribution of defective SIRT1 to HSC aging, expression of SIRT1 in the old versus young HSPCs was reduced 2-fold (Figure 6H).

One of the main features of aging is a declining immune system associated with impairment in T cell production and function (Linton and Dorshkind, 2004; Rossi et al., 2007a). We found that despite the significant decrease in the CD4$^+$ T cell population (Figures 2 and 3; Figures S3, S5, and S7A), the total number of CD4$^+$ CD25$^+$ FOXP3$^+$ regulatory T cells (Treg cells) remained unaltered by the loss of

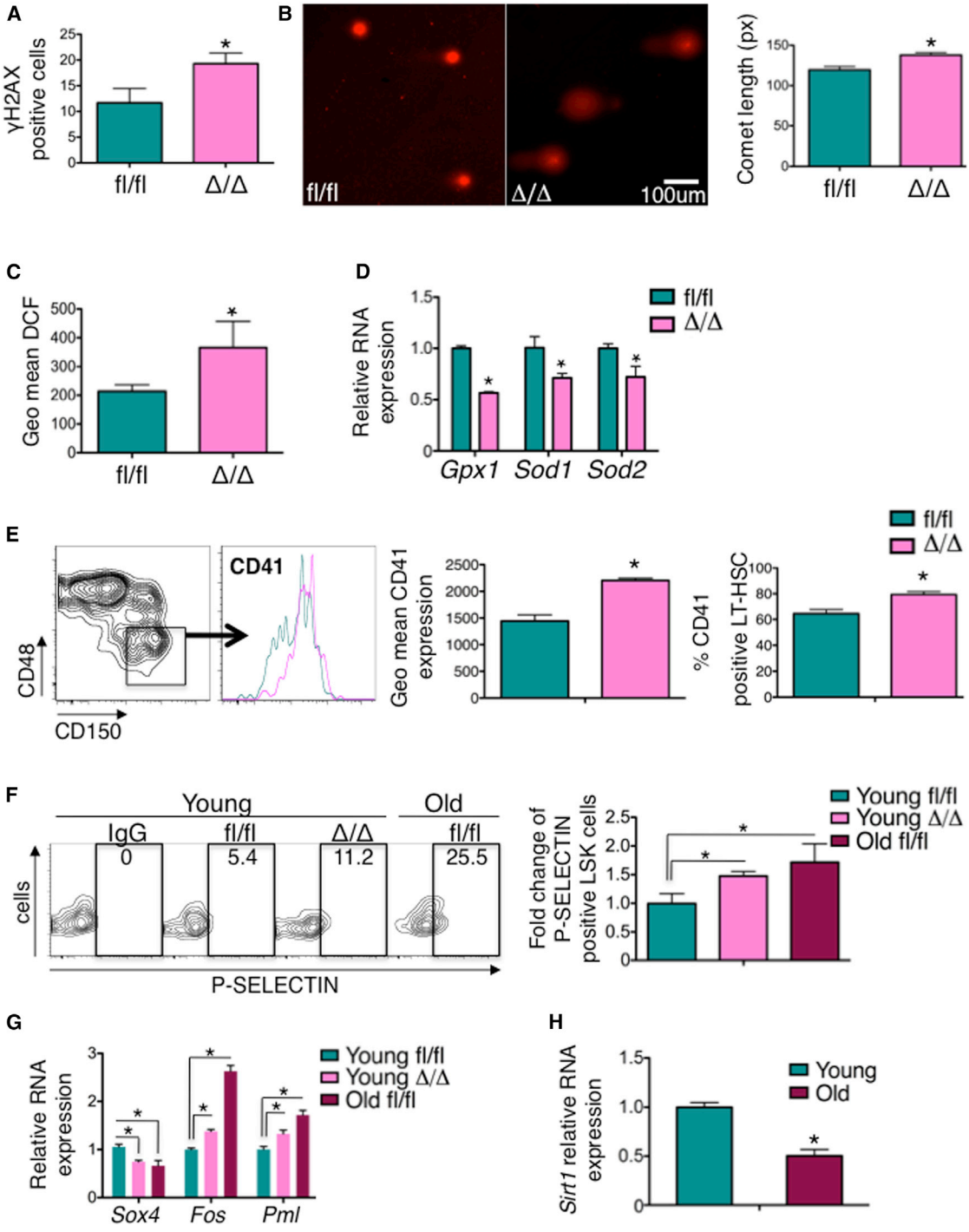

Figure 6. Young SIRT1-Deleted HSPCs Exhibit Main Features of Old HSPCs

(A) Frequency of γH2AX-positive cells within LSK compartment of freshly isolated bone marrow 38 weeks after tamox treatment is shown (n = 3 mice per genotype).

(B) Comet length of damaged DNA measured in pixels in freshly isolated LSK cells from (A) (left panel). Representative micrographs are shown (right panel) (n = 3 replicates per group).

(C) Endogenous ROS measured by CM-H_2DCFDA in LSK cells isolated from mice 4 weeks after tamox treatment (n = 9 mice per genotype).

(D) qRT-PCR analysis of antioxidant enzymes glutathione peroxidase 1 (*Gpx1*), superoxide dismutase (*Sod*) 1 and 2, 4 weeks after tamox treatment. Results are relative to *Sirt1fl/fl* (n = 6 replicates from two independent experiments).

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SIRT1 (Figure S7B), indicating that the frequency of T regs was increased in the peripheral blood of SIRT1-deleted mice (Figures S7C and S7D). Similar alterations are observed in some of the disorders of aging (Kordasti et al., 2007).

Collectively, these findings suggest that loss of SIRT1 leads to disparate and considerable changes in HSC maintenance and lineage specification and may promote an aging-like phenotype in young HSPCs.

FOXO3 Is Required for SIRT1 Regulation of HSC Activity

SIRT1's impact on HSCs may be via several mediators. One of the potential mechanisms through which SIRT1 may contribute to the HSC phenotype is by deacetylation of a number of targets, including FOXO3, p53, and HIF1α (Haigis and Sinclair, 2010). Among these, we focused on FOXO3 as the hematopoietic phenotypes of SIRT1Δ/Δ and *Foxo3*$^{-/-}$ mice are similar in many respects (Figures 1, 2, 3, 4, 5, and 6; Figures S3, S5, S7E, and S7F), specifically in the HSC and myeloid compartments, despite conditional versus germline deletion of *Sirt1* versus *Foxo3*, respectively (Hedrick et al., 2012; Marinkovic et al., 2007; Miyamoto et al., 2007; Yalcin et al., 2008, 2010). Furthermore, although FOXO3 is a known SIRT1 substrate (Brunet et al., 2004; Motta et al., 2004), the outcome of SIRT1 deacetylation of FOXO3 is likely context dependent and unknown in HSCs. Lastly, FOXO3, like SIRT1, is an evolutionarily conserved regulator of organismal longevity (Eijkelenboom and Burgering, 2013; Zhang et al., 2011a).

We reasoned that if FOXO3 was the mediator of SIRT1's impact on HSCs, then inhibition of SIRT1 would not affect significantly the *Foxo3*$^{-/-}$ HSC compartment. Whereas sirtinol inhibition of SIRT1 in vivo compromised the frequency of LSK, LT-HSCs, and the capacity for long-term competitive repopulation in WT mice (Figures 7A and 7B) as we had previously observed (Figures S1D–S1G), administration of sirtinol to *Foxo3*$^{-/-}$ mice did not affect their LSK or LT-HSC compartments (Figure 7A) or the competitive repopulation ability of *Foxo3*$^{-/-}$ HSCs (Figure 7B), suggesting that FOXO3 is required for the inhibitory effects of sirtinol on HSCs.

Similarly, we hypothesized that if FOXO3 is a SIRT1 substrate in HSPCs, then mice lacking both FOXO3 and SIRT1 in HSPCs should exhibit a phenotype similar to that observed in FOXO3-deficient HSPCs. Indeed, *Foxo3*$^{-/-}$/*Sirt1*Δ/Δ and *Foxo3*$^{-/-}$ HSPC phenotypes were remarkably similar in that they were both reduced in numbers (Figure 7C) and in the quiescent fraction (Figure 7D), further suggesting that SIRT1 is an upstream activator of FOXO3 in HSPCs. We next asked whether SIRT1 deacetylation directly modulates FOXO3's activity in HSPCs. In addition to acetylation/deacetylation, phosphorylation by AKT kinase represses FOXO3's function by promoting its nuclear exit (Eijkelenboom and Burgering, 2013; Zhang et al., 2011a). However in HSPCs, FOXO3 is mostly in the nucleus even when pAKT is constitutively active (Lee et al., 2010; Yalcin et al., 2008) (data not shown), suggesting that additional mechanisms are involved. In contrast to wild-type cells, FOXO3's localization in SIRT1-deficient HSPC nuclei was significantly reduced (Figure 7E). FOXO3's transcriptional activity was also decreased in SIRT1-deficient HSPCs as illustrated by reduced expression of a number of FOXO3's direct targets, including *p27* (*Cdkn1b*), *Bnip3*, *CyclinG2* (*Ccng2*), *Sod2*, and *Bim*, and by modulation of *CyclinD1* (CCND1) (Figures 2D, 6D, and S7F). In agreement with these results, the nuclear localization of an ectopically expressed FOXO3 that is mutated to mimic constitutive acetylation (FOXO3 5KQ, 5 lysine residues mutated to glutamine) was significantly reduced in BM mononuclear cells (Figure 7F).

These combined results strongly suggest that FOXO3 is a substrate of SIRT1 in HSCs and that SIRT1 deacetylation is key in promoting FOXO3's retention in HSC nuclei and maintaining FOXO3 in an active form in these cells. They also support the notion that reduced FOXO3 proapoptotic function (Figures 2E and S2K) may protect SIRT1-deleted HSCs from apoptosis despite elevated ROS (Figures 2E, 6C, and 6D). Collectively, these findings indicate that SIRT1-FOXO3 constitutes a regulatory pathway controlling HSC maintenance.

DISCUSSION

SIRT1 Is a Critical Regulator of Homeostatic Adult HSCs with Potential Functions in Delaying the HSC Aging

Our key finding is that SIRT1-deficient HSCs recapitulate within a temporal window some of the main features of

(E) Representative FACS plots (left panel) of levels of CD41 on LSK CD48^{-}CD150^{+} (middle panel) and frequency of LSK CD48^{-}CD150^{+} cells expressing CD41 (right panel) measured 4 weeks after tamox treatment (n = 3 mice per genotype).
(F) Representative FACS plots (left panel) of P-SELECTIN expression in LSK cells 6 weeks after tamox treatment, and in 16 months old LSK. Fold change of P-SELECTIN-positive LSK cells (right panel) normalized to young *Sirt1fl/fl* control (n = 6 mice).
(G) qRT-PCR analysis 14 weeks after tamox treatment. Results are relative to *Sirt1fl/fl* (n = 6 replicates from two independent experiments).
(H) qRT-PCR analysis of young (12-week-old) and old (16-months-old) WT LSK cells. Results are relative to young LSK (n = 9 replicates from three independent experiments). All data are expressed as mean ± SEM (*p < 0.05).

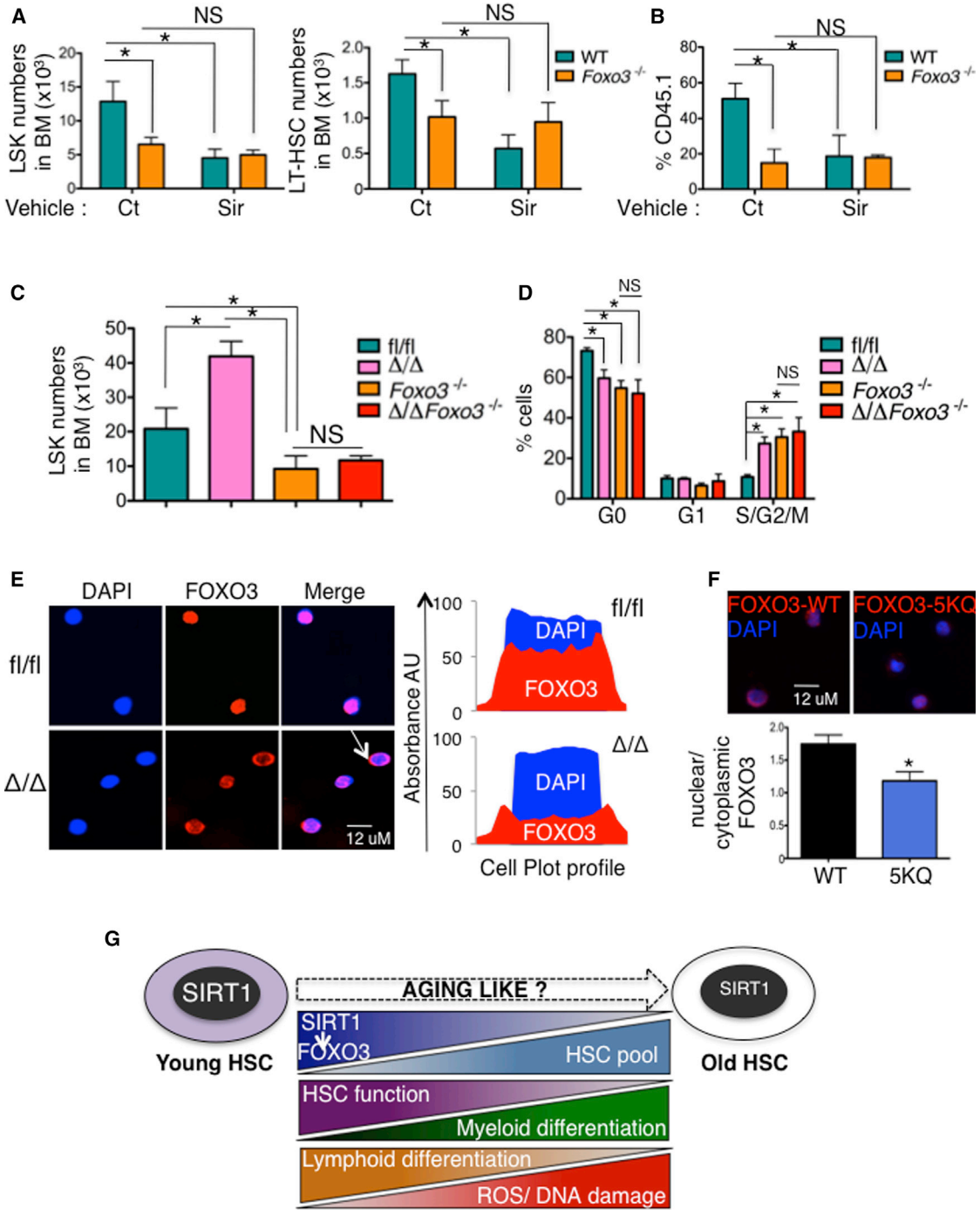

Figure 7. SIRT1 Controls Hematopoietic Homeostasis by Promoting FOXO3 Nuclear Localization and Activity in HSCs

(A) Total number of BM LSK (left panel) cells and LT-HSC (right panel) isolated from WT and *Foxo3*$^{-/-}$ mice treated with Sirtinol (Sir) or vehicle control (Ct) for 3 weeks (n = 3 mice in each group).

(B) Long-term competitive repopulation of 100 transplanted HSC (from A) as measured by the percent of CD45.1 in the PB of recipient mice 16 weeks after transplantation (n = 5 mice in each group).

(C) Total number of LSK cells isolated from BM of indicated mice (n = 3 mice in each group).

(D) LSK cell-cycle distribution of mice from (C) measured by KI67/DAPI staining (n = 3 mice per genotype).

(E) FOXO3 immunostaining (left panel) and quantification (right panel) measuring the cell plot profile are shown.

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HSC aging. Specifically, the expansion of HSC numbers, albeit transient, followed by depletion overtime of HSC function overlaps with aged associated HSC defects. These abnormalities combined with anemia, myeloid skewed HSC differentiation, enhanced specific production of GMPs and immune deficiency, enhanced oxidative stress, greater DNA damage, and age-related modulations of gene expression suggest that young SIRT1-deleted HSCs may exhibit a premature aging phenotype (Chambers et al., 2007; Morrison et al., 1996; Sudo et al., 2000; Yilmaz et al., 2006). A greater fraction of HSCs in old BM is cycling, although this increased cycling may only be observed in mice older than 22–24 months (Dykstra and de Haan, 2008; Morrison et al., 1996; Yilmaz et al., 2006). In agreement with an aging-like HSC phenotype, a significant fraction of SIRT1-deficient HSCs exited quiescence and entered the cell cycle (Figures 2A–2C; Figures S2I and S2J). Nonetheless, SIRT1-deleted HSCs do not phenocopy aged HSCs, as the MPP subset is increased in SIRT1-deleted HSPCs in contrast to aged HSPCs (Rossi et al., 2005, 2007a). Although the defects of SIRT1-deleted HSCs are cell autonomous, our studies do not rule out potential participation of non-cell-autonomous mechanisms. This work suggests a model in which SIRT1 wires together a combinatorial transcriptional program in HSPCs. Overall, our findings predict that a decline in SIRT1 function, as it may occur with age (Figures 6H and 7G), would result in disruption (or destabilization) of SIRT1-regulated HSPC transcriptional program.

Recent studies suggest that aging changes the clonal composition of HSC compartment rather than their intrinsic properties (Dykstra and de Haan, 2008). While our findings are in agreement with a potential SIRT1 repression of myeloid biased HSC clones that dominate with age, they also suggest that SIRT1 may have additional protective functions toward key HSC programs that become compromised with age, such as the capacity to repair damaged DNA.

The question of whether SIRT1 has any functions in normal adult HSC has long been debated. Depending on their strain, a significant proportion of germline-deleted SIRT1 mice die perinatally, resulting in only a fraction of mice surviving to adulthood. SIRT1 was dispensable for the HSC activity, perhaps due to developmental adaptation of HSCs in the surviving mice (Leko et al., 2012; Li et al., 2012; Narala et al., 2008; Yuan et al., 2012). On the other hand, using a tamoxifen-inducible conditional deletion approach, it was shown recently that SIRT1 is required for HSPC genome stability under stress (Singh et al., 2013). We demonstrated that SIRT1 is essential for HSC function and lineage specification under homeostatic conditions even when BM is not stressed (Figures 1 and S2).

SIRT1 Regulates Hematopoietic Stem Cell Lineage Decision

One of the most unexpected findings was the skewed generation of myeloid lineage at the expense of the lymphoid compartment uncovered in young SIRT1-deleted HSCs. The propensity toward myeloid differentiation associated with immune defects is one of the hallmarks of HSC aging in both mouse and human (Linton and Dorshkind, 2004; Pang et al., 2011; Rossi et al., 2005; Sudo et al., 2000). The specific increased generation of GMPs (Figures 5A and 5B) was greatly similar to the mild specific increase in GMPs produced in old BM (Rossi et al., 2005, 2007a). The shift in the lympho-myeloid cell ratio in the BM, spleen, and peripheral blood of SIRT1-deficient mice (Figures 3 and 4; Figures S3 and S5; Table S1) that became highly pronounced overtime underscores the function of SIRT1 in balancing the generation of hematopoietic lineages from HSCs.

The increased expression of GATA-1 and GATA-2 in SIRT1-deleted hematopoietic progenitors (Figures 5C and 5D) is in agreement with the function of these factors in GMPs and eosinophil production (Hirasawa et al., 2002; Iwasaki et al., 2006). The reduced expression of myeloid transcription factors C/EBPα and PU.1, specifically in multipotential progenitors (Figure 5C), was unexpected. These results may indicate that relative expression of myeloid transcription factors, as is the case for their order of expression (Iwasaki et al., 2006), may influence lineage specification.

SIRT1 Maintains HSC Homeostasis by Promoting FOXO3 Nuclear Localization and Activation

We identified FOXO3 as a SIRT1 substrate in HSCs that mediates SIRT1's effects on HSCs. FOXO3 like SIRT1 is implicated in the regulation of mouse ESC pluripotency (Han et al., 2008; Zhang et al., 2011b). Similarly, FOXO3 is required for the maintenance of HSC pool (Miyamoto et al., 2007, 2008; Yalcin et al., 2008). FOXO3 mutant myeloproliferation and decreased B and red blood cells (Hinman et al., 2009; Marinkovic et al., 2007; Miyamoto et al., 2007; Yalcin et al., 2008, 2010) (data not shown) are similarly observed in SIRT1-deficient mice. Moreover, loss of FOXO3 like (Figure S7E) SIRT1 (Figure 5B) leads

(F) The mean fluorescence of nuclear cytoplasmic ratio (lower panel) of 50 GFP-sorted mouse bone marrow mononuclear cells retrovirally expressing Flag-FOXO3-WT or Flag-FOXO3-5KQ, immunostained with anti-Flag antibody (upper panel).
(G) Model of SIRT1/FOXO3 regulation of HSC maintenance and lineage specification. The model raises the question as to whether reduced SIRT1 function contributes to the HSC aging. All data are expressed as mean ± SEM (*p < 0.05, ns, not significant).

specifically to enhanced generation of GMPs, altogether indicating that SIRT1 regulation of FOXO3 might be implicated in myeloid and B cell lineage determination. The impact of SIRT1 (Figures 3A and 3C–3F; Figures S3, S5, and S6; Table S1) and FOXO3 on the T cell compartment however seems distinct (Hedrick et al., 2012). Interestingly, SIRT1 like (Li et al., 2012; Yuan et al., 2012) FOXO3 (Ghaffari et al., 2003; Naka et al., 2010; Sykes et al., 2011) is implicated in leukemogenesis and required for the maintenance of leukemic stem cells.

Our studies combined with recent findings regarding SIRT3 (Brown et al., 2013) further implicate sirtuins in protecting HSCs from aging. Future investigations in the potential involvement of SIRT1/FOXO3 (Mouchiroud et al., 2013) in stem cell programs other than HSCs, including neural stem cells (Renault et al., 2009), should clarify whether and to what extent stem cells rely on this network for their maintenance over a lifetime.

EXPERIMENTAL PROCEDURES

Mice

The generation and genotyping of mice were performed as previously described (Price et al., 2012). SIRT1Δex4 (C57BL6, CD45.2) were crossed to Cre-ERT2 mice to generate SIRT1Δex4-ERT2 mice. The Cre induction was performed by delivering tamoxifen intraperitoneally (1 mg/mouse/day) (Sigma T5648) for 5 consecutive days to SIRT1Δex4-ERT2 mice (designated Δ/Δ). This regimen resulted in deletion of SIRT1 exon 4 and a truncated SIRT1 protein that lacked its catalytic domain. Control mice, including animals with a wild-type *Sirt1* allele and *Ert2* (*Sirt1WT/WT*), and flox-SIRT1Δex4 mice lacking *Ert2* (*Sirt1fl/fl*) were used. The efficient deletion after tamoxifen treatment was confirmed by PCR analysis of genomic tail DNA. *Foxo3*$^{-/-}$ mice, a gift of Dr. Ron Depinho (MD Anderson Cancer Center), were backcrossed ten generations onto C57BL6 (CD45.1) background (Yalcin et al., 2008, 2010). Because of the proximity of *Foxo3* and *Sirt1* genes on mouse chromosome 10, the following strategy was devised to generate *Sirt1Δ/ΔFoxo3*$^{-/-}$ mice (designated Δ/ΔFoxo3$^{-/-}$): *Sirt1fl/fl* cre^{+} *Foxo3WT* were crossed with *Sirt1WT/WT* cre^{-} *Foxo3*$^{-/-}$ mice. The F1 population *Sirt1fl/WT* cre^{+} Foxo3$^{+/-}$ were intercrossed to obtain *Sirt1fl/fl* cre^{+} *Foxo3*$^{+/-}$ mice. The F2 population *Sirt1fl/fl* cre^{+} *Foxo3*$^{+/-}$ mice were intercrossed to obtain *Sirt1fl/fl* cre^{+} *Foxo3*$^{-/-}$ mice. Young mice in all experiments were 10–12 weeks old. Mice were used in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Long-Term Repopulation Assay

Lethally irradiated (12 Gy as a split dose, 6.5 and 5.5 Gy, 4–5 hr apart) congenic C57BL6-CD45.1 mice (from the National Cancer Institute) were reconstituted with intravenous injections of 100 donor LSK CD48^{-}CD150^{+} cells from *Sirt1WT/WT* (Cre^{+}), *Sirt1fl/fl* (Cre^{-}), or SIRT1Δ/Δ mice (Cre^{+}) (all CD45.2) 4 weeks after tamoxifen treatment along with 2 × 10^{5} competitor bone marrow cells (CD45.1). For secondary transplantations, an equal number (2 × 10^{6}) of total bone marrow cells from primary recipients or three times more (6 × 10^{6}) from SIRT1Δ/Δ LSK CD48^{-}CD150^{+} were pooled and transplanted into lethally irradiated CD45.1 secondary recipients. A long-term repopulation assay of *Foxo3*$^{-/-}$ (CD45.1) mice was performed by transplantation into lethally irradiated CD45.2 recipients.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.04.015.

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