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The Satb1 protein directs hematopoietic stem cell differentiation toward lymphoid lineages

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Summary

How hematopoietic stem cells (HSCs) produce particular lineages is insufficiently understood. We searched for key factors that direct HSC to lymphopoiesis. Comparing gene expression profiles for HSCs and early lymphoid progenitors revealed that Satb1, a global chromatin regulator, was markedly induced with lymphoid lineage specification. HSCs from Satb1-deficient mice were defective in lymphopoietic activity in culture and failed to reconstitute T lymphopoiesis in wild-type recipients. Furthermore, Satb1 transduction of HSCs as well as embryonic stem cells robustly promoted their differentiation toward lymphocytes. Whereas genes that encode Ikaros, E2A, and Notch1 were unaffected, many genes involved in lineage decisions were regulated by Satb1. Satb1 expression was reduced in aged HSCs with compromised lymphopoietic potential, but forced Satb1 expression partly restored that potential. Thus, Satb1 governs the initiating process central to the replenishing of lymphoid lineages. Such activity in lymphoid cell generation may be of clinical importance and useful to overcome immunosenescence.

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Keywords

Satb1; hematopoietic stem cells; early lymphoid differentiation; aging

Introduction

To maintain the immune system, hematopoietic stem cells (HSCs) differentiate to lymphoid-primed multipotent progenitors (LMPPs) and then to lymphoid-specified progenitors in a process accompanied by the loss of erythroid-megakaryocyte and myeloid potential (Adolfsson et al., 2005; Lai and Kondo, 2008). Accumulating evidence has suggested that combinations of transcription factors coordinately and sequentially regulate lymphopoiesis. Five transcription factors, PU.1, Ikaros, E2A, EBF, and Pax5 are hierarchically involved in the early steps of B-lineage differentiation (Medina et al., 2004). Whereas EBF and Pax5 specifically act in B-lineage determined progenitors, PU.1 and Ikaros are expressed in earlier hematopoietic progenitors and involved in multiple lineage decision processes (Scott et al., 1997; Yoshida et al., 2006). E2A, an indispensable factor for B lymphopoiesis, can also affect T lymphocyte formation by regulating Notch1 expression (Ikawa et al., 2006). Furthermore, recent reports have shown that E2A proteins are expressed in primitive hematopoietic progenitors and play a critical role in early lymphoid specification (Dias et al., 2008; Yang et al., 2008; Semerad et al., 2009). However, whether the initiation of lymphoid differentiation is regulated entirely by transcription factors in a hierarchical manner remains unclear.

The immune system changes qualitatively and quantitatively with ontogeny and age (Miller and Allman, 2005; Montecino-Rodriguez and Dorshkind, 2006). Indeed, lymphocyte progenitors expand substantially in the fetal liver (FL), but their production shifts to bone marrow (BM) and becomes stable after birth. With age, replenishment of the adaptive immune system declines (Rossi et al., 2005; Sudo et al., 2000). Qualitative changes in lymphopoietic activity of HSCs are reflected in in vitro cell culture experiments. If key inducers in early lymphoid lineages can be identified, they will be useful for expanding lymphocytes in culture for clinical purposes. Additionally, manipulating the expression of relevant genes might boost the immune system of immunocompromised and elderly people.

We have developed a method to sort early lymphoid progenitors (ELPs) from Rag1-GFP reporter mice (Igarashi et al., 2002; Yokota et al., 2003a). ELPs expressing Rag1 are present in the $Sca1^{+}c\text{-}kit^{hi}$ HSC-enriched fraction; they displayed high B- and T-lymphopoietic potential, but limited myelo-erythroid potential and self-renewal ability. In contrast, $Rag1^{-}Sca1^{+}c\text{-}kit^{hi}$ HSCs effectively reconstitute and sustain the lymphohematopoietic system for long periods in lethally irradiated recipients. We conducted gene array comparisons between those 2 fractions with the goal of discovering molecules involved in the transition of HSC to lymphoid lineages.

Herein, we showed that Special AT-rich Sequence Binding 1 (Satb1), a nuclear architectural protein that organizes chromatin structure, plays an important role in lymphoid lineage specification. In parallel with or ahead of key transcription factors, the expression of Satb1 increased with early lymphoid differentiation. In functional assays, lymphopoietic activity was compromised in Satb1-deficient hematopoietic cells, but the induced expression of Satb1 strongly enhanced lymphocyte production from HSCs. Furthermore, exogenous Satb1 expression primed lymphoid potential even in embryonic stem (ES) cell-derived mesoderm cells and aged BM-derived HSCs. Global analysis of potential Satb1 target genes identified a number that may have critical roles in early lymphopoiesis. The findings demonstrate that

the earliest steps in lymphopoiesis are regulated by an epigenetic modifier and indicate how modulation of the process might be used to induce or rejuvenate the immune system.

Results

Profiling gene expression of Rag1$^+$ ELP in fetal liver

We sorted the Rag1^{lo} c-kit^{hi} Sca1$^+$ ELP fraction and the Rag1$^-$ c-kit^{hi} Sca1$^+$ HSC-enriched fraction with high purity from E14.5 FL of Rag1-GFP knock-in heterozygous embryos and performed gene arrays. We found that transcripts of *Trbv14* as well as *Ighm* genes were up-regulated even in very early lymphoid progenitors (Supplemental Table 1). Furthermore, we detected increased expression of *Il7r*, *Notch1*, and *Flt3* genes encoding cell surface receptors important for B or T lymphocyte differentiation in the ELP fraction. In addition to discovering many signal transduction kinases with unknown functions in lymphopoiesis, our search identified *Lck* and *Xlr4b* genes as being involved in lymphoid differentiation signals. Transcripts for some of these lymphoid-related genes had already been detected in the Rag1$^-$ HSC-enriched fraction (see the microarray data; accession number CBX73). These results suggest that lymphoid-lineage specification begins even before the emergence of Rag1^{lo} ELP. Additionally, the microarray data identified new candidate genes that might be important for early lymphoid development.

Expression of *Satb1* increases with early lymphoid specification and declines with age

Our major goal was to find key genes involved in the specification of lymphoid fates. Since the microarray data showed that expression of various lymphoid-related genes was activated before the ELP stage, we hypothesized the existence of a modulator that synchronously regulates multiple genes. Among the list in Supplemental Table 1, *Satb1* attracted attention because it was originally identified as a protein binding to the enhancer region of the *Igh* gene and later shown to play a critical role in T cell development (Alvarez et al., 2000; Dickinson et al., 1992). Additionally, recent studies had demonstrated that it serves as a master regulator for many genes, including cytokines, cytokine receptors and transcription factors (Cai et al., 2006; Han et al., 2008; Notani et al., 2010; Yasui et al., 2002).

To explore possible relationships between Satb1 and early lymphopoiesis, we examined its expression in primitive hematopoietic progenitors. The HSC-enriched Rag1-GFP$^-$ Flt3$^-$ lineage marker-negative (Lin$^-$) Sca1$^+$ c-kit^{hi} (LSK) fraction, the LMPP-enriched fraction, the ELP-enriched fraction, the common lymphoid progenitor (CLP)-enriched fraction, and the myeloid progenitor-enriched Lin$^-$ c-kit^{hi} Sca1$^-$ fraction were sorted from BM of 8- to 10-week-old mice. Transcripts for *Satb1* were then quantitatively evaluated with real-time RT-PCR. *Satb1* expression increased substantially when HSC differentiated into LMPP and ELP (Figure 1A). This trend matched that of other early lymphoid lineage-related genes including those that encode PU.1 (*Sfpi1*), Ikaros (*Ikzf1*), E2A (*Tcf3*), and Notch1 (Supplemental Figure 1). Importantly, in contrast to its expression in the lymphoid lineage, *Satb1* expression was shut off when HSC differentiated to committed myeloid progenitors. These results suggest that *Satb1* is potentially involved in early lymphoid differentiation.

Lymphopoietic activity becomes compromised during aging. Accumulating evidence suggests that the earliest lymphoid progenitor pools proximal to HSC are deficient in aged BM (reviewed by Miller and Allman, 2005). Indeed, the Rag1$^+$ ELP population markedly decreases with age (data not shown). The down-regulation of genes mediating lymphoid specification and function is likely a major cause (Rossi et al., 2005). Because *Satb1* has been listed in microarray panels as a down-regulated gene in aged HSC (Chambers et al., 2007; Rossi et al., 2005), we sorted Rag1-GFP$^-$ LSK and ELP-enriched Rag1-GFP$^+$ LSK from BM of 6-week-old or 2-year-old Rag1-GFP heterozygous mice and examined their

expression. In agreement with previous studies, our real-time RT-PCR identified an approximate 50% reduction of *Satb1* transcripts in aged Rag1-GFP$^-$ LSK cells (Figure 1B, left panel). The few ELP recovered from aged mice expressed amounts of *Satb1* comparable to those in ELP from young mice. Recent purification methods for HSC with CD150, a SLAM family receptor that marks HSC even in aged BM (Yilmaz et al., 2006), identified an approximate 80% reduction in *Satb1* transcripts in aged HSC compared with ones from young mice (Figure 1B, right panel). These observations suggest that *Satb1* may be a key molecule related to immunosenescence.

Satb1 deficiency reduces the lymphopoietic activity of hematopoietic stem and progenitor cells

T cell development in the thymus is impaired in *Satb1*$^{-/-}$ mice (Alvarez et al., 2000). Although the profile of B220, IgM and IgD expression appears to be unaffected in the *Satb1*$^{-/-}$ spleen, the total number of B cells is reportedly reduced to approximately 25% of wild-type at 2 weeks of age (Alvarez et al., 2000). We have determined that the number and frequency of cells that can be recovered from lymphoid organs were reduced in E18.5–19.5 *Satb1*$^{-/-}$ fetuses. Body sizes of *Satb1*$^{-/-}$ fetuses were not different from wild-type (WT) and heterozygous littermates (Supplemental Figure 2A, 2B).

We then sorted Lin$^-$ cells from FL of *Satb1*$^{-/-}$ mice or their WT littermates and cultured them with stromal cells that support lymphopoiesis. T cell differentiation can be recapitulated in vitro with hematopoietic cells cultured with OP9 expressing the Notch ligand Delta-like 1 (OP9-DL1). Under these co-culture conditions, the differentiation patterns of WT and *Satb1*$^{-/-}$ Lin$^-$ cells differed significantly (Figure 2A, 2B). The majority of *Satb1*$^{-/-}$ cells were arrested in the CD44$^-$CD25$^+$ stage and did not differentiate into CD44$^-$CD25$^-$ cells. Reduction of IL-7 from culture media normally induces maturation of the CD4$^-$CD8$^-$ double-negative (DN) into CD4$^+$CD8$^+$ double-positive (DP) cells and subsequently into either the CD4$^+$ or the CD8$^+$ single-positive cells. However, more than half of the *Satb1*$^{-/-}$ cells were arrested in DN stages even after the IL-7 reduction, and their differentiation to the DP stage was aberrantly skewed toward CD4$^+$CD8$^-$ (Figure 2C).

Substantial differences were also observed in B-lineage cell production. In co-culture with MS5, which supports B as well as myeloid lineages in the presence of SCF, Flt3-ligand and IL7, *Satb1*$^{-/-}$ progenitors exhibited significant reductions in B-lymphopoietic potential (Figure 2D, 2E). Co-culture with OP9, which originated with M-CSF-deficient mice and supported the B lineage predominantly, also yielded reduced B/myeloid ratios with *Satb1*$^{-/-}$ progenitors (Supplemental Figure 2C). Essentially the same results were obtained when cultures were initiated with LSK Flt3$^-$, more stringently purified HSC (Supplemental Figure 2D, 2E). In addition, B cell lineage output was also reduced when *Satb1*$^{-/-}$ LMPP or CLP were cultured (Supplemental Figure 2F). In contrast, the myeloid potential was retained in *Satb1*$^{-/-}$ progenitors (Figure 2D and 2E). Indeed, the Lin$^-$ fraction of E14.5 *Satb1*$^{-/-}$ FL contained more myeloid-erythroid progenitors than that of the WT control (Figure 2F).

In transplantation experiments, we observed that CD45.2$^+$ *Satb1*$^{-/-}$ HSC sorted from 2-week-old BM did not effectively reconstitute CD3$^+$ T-lineage cells in lethally irradiated CD45.1$^+$ WT recipients (Figure 3A). Peripheral blood CD3$^+$ T-lineage recoveries from *Satb1*$^{-/-}$ HSC were decreased approximately 90% compared with that from WT HSC (Figure 3B). Conversely, we observed varied amounts of reconstitution of the B lineage and no reduction in reconstitution of the myeloid lineage resulted from Satb1 ablation (Figure 3B). Compromised T cell lineage contributions of *Satb1*$^{-/-}$ HSC were also evident in the thymus and spleen (Figure 3C, 3D). Although T lymphopoiesis in the thymus was replaced by either WT or *Satb1*$^{-/-}$ donor cells when FL HSC were transplanted, thymocytes were reduced in the *Satb1*$^{-/-}$ recipients and their differentiation was affected. Besides apparent

stagnation at the DP stage and marked reduction of the DN population (Figure 3C, upper panels), c-kit^{hi} cells in the CD44^{+}CD25^{-} DN1 stage were rare in *Satb1*$^{-/-}$ recipients (Figure 3C, lower panels). The reduced contribution of *Satb1*$^{-/-}$ cells was also evident in CD3^{+} splenic T lymphocytes. Interestingly, T cells in the spleens of *Satb1*$^{-/-}$ recipients contained substantial percentages of DP and DN cells. Such T cell lineage cells are extremely rare in normal mouse spleens (Figure 3D).

Taken together, these results demonstrate that Satb1 is indispensable for normal T lymphopoiesis but not for myelopoiesis. The factor may normally have a lesser role in B-lineage differentiation. Furthermore, our data indicate that abnormalities of lymphoid development observed in *Satb1*$^{-/-}$ mice are intrinsic to *Satb1*$^{-/-}$ hematopoietic cells.

Forced expression of Satb1 in HSC induces lymphopoiesis

Next we conducted overexpression experiments to define the role of Satb1 in lineage fate decisions of HSC. LSK Flt3^{-} cells were sorted from BM of adult WT mice and then retrovirally transduced with either a fluorescence-alone expressing control or a native Satb1 construct combined with a GFP-expressing vector. Successfully transduced cells were sorted according to GFP expression. Real-time RT-PCR and western blots revealed that Satb1-transduced cells expressed more than 10-fold *Satb1* transcripts and Satb1 proteins compared to control cells (Supplemental Figure 3A).

The sorted cells were cultured with stromal cells that supported lymphopoieisis. Results from these experiments complemented the observations with *Satb1*$^{-/-}$ cells. Satb1 transduction enhanced T cell lineage growth in OP9-DL1 co-cultures (Figure 4A and 4B). By day10 of the culture, cells had been increased more than 5-fold by Satb1-transduction, and a majority of the recovered cells had progressed to the DN2 and DN3 stages. Differentiation to the DP stage was also advanced by the Satb1-transduction (Figure 4A). The kinetics of cell differentiation and expansion in the B cell lineage showed more changes. Whereas both control and Satb1-transduced cells produced substantial numbers of B-lineage cells, the latter produced B220^{+}CD19^{+} cells more quickly and effectively (Supplemental Figure 3B). Specifically, the Satb1 transduction resulted in approximately 50~300 fold and 5-fold greater recovery of B220^{+}CD19^{+} cells on day10 in the MS5 and OP9 co-cultures, respectively (Figure 4C and 4D). Notably, Satb1 transduction negatively influenced the output of myeloid cells, particularly Mac1^{lo}Gr1^{+} granulocytes (Supplemental Figure 3C). In addition, CFU-GM formation of HSC was decreased by Satb1 transduction (Supplemental Figure 3D).

In stromal-free cultures containing SCF, Flt3-ligand and IL7, Satb1 expression strongly induced CD19^{+} cell production from the LSK fraction (Supplemental Figure 3E). When calculated on a per-cell basis, 1 LSK cell with Satb1 overexpression produced approximately 450 CD19^{+} cells, whereas only 50 cells with this B-lineage marker were produced from control progenitors. As for other hematopoietic lineages, DX5^{+}CD3e^{-} NK cells emerged when IL-15 was added to the stromal cell-free cultures. Co-expression of NK1.1 and/or CD94 confirmed the NK-lineage, and their numbers were also enhanced by Satb1 overexpression (Supplemental Figure 3F). Interestingly, the same Satb1-transduced LSKs differentiated to neither conventional nor plasmacytoid dendritic cells (Supplemental Figure 3G).

The results from in vitro bulk cultures and assessment of lymphoid lineage cell numbers might reflect enhanced survival of lymphoid progenitors rather than priming or expansion of lymphoid potential in individual clones. Notably, no obvious increase in apoptotic cells occurred in any tested cultures with *Satb1*$^{-/-}$ cells or *Satb1*$^{-/-}$ lymphopoietic organs (data not shown and Supplemental Figure 3H). Additionally, Satb1 overexpression conferred

growth advantages to hematopoietic progenitors without influencing their viability in any of the cultures we used (data not shown). To investigate further the mechanisms through which Satb1 exerts its effect on early progenitors, we performed limiting dilution assays. On average, 1 in 3.1 control cells and 1 in 2.6 Satb1-transduced cells gave rise to blood cells, indicating that both are highly potent progenitors for hematopoietic cell growth (Figure 4E, left). Nevertheless, we observed significant differences between them regarding the frequencies of progenitors with lymphopoietic potential. While 1 in 41 Satb1-transduced Flt3$^-$ LSK cells produced B cells, only 1 in 143 control cells were lymphopoietic under these conditions (Figure 4E right). In the same experiment, fresh Flt3$^-$ LSK cells without retroviral transfection produced hematopoietic cells and B cells at a frequency of 1 in 6.7 cells and 1 in 61 cells, respectively (data not shown).

These results suggest that Satb1 expression affects early lineage decisions in individual HSC and expands the growth and differentiation of lymphoid cells in vitro. To evaluate whether these findings were of practical value, we performed in vivo transplantation experiments with SATB1-transduced LSK Flt3 cells. We observed enhanced contribution of the SATB1-transduced cells to both T and B lineages in short-term engraftment (Figure 4F). To assess whether the overexpression of SATB1 induces tumors, we evaluated long-term and short-term lymphohematopoiesis after transplantation. In 8 transplanted mice, SATB1-overexpressing cells did not induce tumors, at least during 3 months of observation.

Satb1 regulates lymphoid lineage-related genes in HSC

During early lymphocyte differentiation, several transcription factors have been shown to play roles in a hierarchical manner. To identify the target genes of Satb1, we first examined whether the exogenous expression of Satb1 influences the expression of lineage-relevant transcription factors in LSK Flt3$^-$ cells. Although high Satb1 expression was achieved, no significant up-regulation was observed in the expression of *Sfpi1*, *Ikzf1*, *Tcf3*, or *Notch1* (data not shown). The expression of *Cebpa*, which is important for myeloid differentiation, was also not significantly affected (data not shown).

Next, to find candidate genes involved in the Satb1 induction of lymphopoiesis, we performed a microarray comparing gene expression between Satb1- and control-transduced LSK Flt3$^-$ cells (Supplemental Table 2). In accordance with the results described above, the data showed no significant changes in the expression of *Sfpi1*, *Ikzf1*, *Tcf3*, *Notch1* or *Cebpa*. However, several transcription factors involved in lymphoid differentiation, *Sp4*, *Maf*, *Fos*, and *Id3*, were up-regulated in Satb1-transduced cells (Figure 5A). Cytokines such as *Il7* and *Kitl*, which are critical for lymphocyte differentiation and generally believed to be stromal cell products, were induced in hematopoietic progenitor cells themselves by ectopic expression of Satb1 (Figure 5B). While receptors for IL4 or IL17 were induced, *Csf3r*, encoding the G-CSF receptor was down-regulated. Interestingly, among lymphoid-related genes, *Rag1*, which is indispensable for both T and B cell differentiation, was strongly induced by Satb1 (Figure 5C). Expression of the CD86 gene that correlates with lymphoid competency (Shimazu et al., 2012) was also significantly elevated.

As a complementary experiment, we performed a set of microarray analyses comparing gene expression signatures between WT and *Satb1*$^{-/-}$ cells (Supplemental Table 3). We again observed no direct correlations between Satb1 expression and *Ikzf1*, *Tcf3*, or *Notch1*, but confirmed that the expression of numerous lineage-related genes was influenced. The expression of *Il7* and *Kitl* was detectable in WT hematopoietic progenitors, and their levels were significantly lower in the *Satb1*$^{-/-}$ progenitors. Of note, *Satb2*, which is a homolog of *Satb1*, as well as *Bright*, which codes a B cell-specific AT-rich sequence binding protein (Herrscher et al., 1995), were up-regulated in *Satb1*$^{-/-}$ HSC. In addition, the *Satb1*$^{-/-}$ HSC aberrantly expressed *Rag1* and *Pax5*, whose levels decreased with differentiation to LMPP.

These results indicate that Satb1 expression globally influences many genes involved in lineage fate decisions during the specification of HSC toward lymphoid lineages.

Satb1 induces lymphopoiesis in ES cells

Next, we examined whether the exogenous expression of Satb1 is sufficient to promote lymphopoiesis in ES cells. In the OP9 co-culture system (Nakano et al., 1994), ES cells can produce mesoderm cells in 4.5 days, which have potential to become hematopoietic and endothelial cells. After a short period of retroviral transduction with the control-GFP or the Satb1-GFP vector, ES-derived mesoderm cells were cultured with OP9 in the presence of SCF, Flt3-ligand and IL7. As shown in Figure 6A, although both control- and Satb1-transfected cells contained substantial numbers of GFP^+ cells, the latter produced $CD45^+$ hematopoietic cells effectively. Further phenotype revealed that most of the $CD45^+$ GFP^+ cells produced from the Satb1-transfected cells expressed B220 and CD19 (Figure 6A right panels). Notably, those cells were also positive for AA4.1, CD11b and CD5, suggesting that they were likely B1-B-lineage cells (Figure 6B).

Next, we established ES cell clones, which can be induced to express Satb1-GFP on removal of tetracycline (Tet) from the culture medium. Eight days after Tet deprivation (day 12.5; Figure 6C), approximately 15% of the recovered cells were GFP^+ (data not shown). Thirty-five percent of these cells expressed CD45 and included substantial numbers of $AA4.1^+$ $CD19^+$ B-lineage cells (Figure 6D, right panels). Conversely, in the presence of Tet, the proportions of $AA4.1^+$ and $CD19^+$ cells among the $CD45^+$ faction were very low (Figure 6D left panels). A majority of the $CD19^+$ cells among the Satb1-GFP^+ ES-derived cells were positive for Mac1 or CD5, again indicating a preference for the B1-B lineage (Figure 6E). In cytospin preparations, many of the ES-derived cells cultured with Tet showed myelo-monocytic morphology whereas Satb1/GFP^+ cells exhibited lymphocyte-like morphology (Figure 6F). Finally, a PCR-based *Igh* rearrangement assay confirmed D_H-J_H recombination in the Satb1-GFP^+ ES-derived cells (Figure 6G).

To test T-lineage potential, we transduced the control-GFP or the Satb1-GFP vector to ES-derived mesoderm cells and cultured them with OP9-DL1 cells. The Satb1-transduced cells effectively produced $CD4^+$ $CD8^+$ DP cells with rapid kinetics (Figure 6H, 6I). Substantial numbers of ES-derived T-lineage cells expressed TCR-γδ or -β, and Satb1-transduced cells were advanced in this regard (data not shown). Taking these results together, we conclude that Satb1 expression directs even ES-derived cells toward lymphoid lineages.

Ectopic Satb1 expression in aged HSC restores lymphopoietic potency

As shown in Figure 1B, the *Satb1* expression in HSC declines with age. This decline might be correlated with the age-dependent impairment of lymphopoiesis. Therefore, we examined whether Satb1 expression restores the lymphopoietic activity of progenitors from aged mice. Rag1-GFP^- LSK cells of 2-year-old mice were transduced with control or Satb1-DsRed vectors. After 72 h of transduction, $DsRed^+$ cells were sorted and cultured on OP9 in the presence of SCF, Flt3-ligand, and IL7. The Satb1-transduced cells produced a percentage of Rag1-GFP^+ $B220^+$ cells that was significantly higher than that of control cells (Figure 7A). Indeed, most of the aged Rag1-GFP^- LSK cells were prone to differentiate into Rag1-GFP^+ cells as a result of exogenous Satb1 expression. With respect to the recovered B-lineage cell counts, approximately 3-fold more $B220^+$ Rag1-GFP^+ $Mac1^-$ cells were obtained through Satb1 overexpression (Figure 7B).

Conversely, fewer B-lineage cells were generated from aged ELP than from young ELP despite their similar expression of *Satb1* (Figure 1B, Supplemental Figure 4A). B-lineage differentiation of aged ELP also showed decreased Rag1 expression (Supplemental Figure

4B). Nonetheless, aged ELP showed substantial lymphopoietic activity in MS5 co-cultures, in which aged HSC scarcely produced B-lineage cells (Supplemental Figure 4A). These results suggest that the down-regulation of Satb1 expression is involved in the compromised lymphopoietic potential of aged HSC and that ectopic induction of Satb1 can at least partially restore the activity.

Discussion

Despite accumulating evidence that multiple transcription factors support lymphocyte differentiation, ones which specifically direct HSC to the lymphoid lineage have remained elusive. One aim of this study was to describe molecular signatures of early stages of lymphopoiesis by comparing gene expression patterns between HSC and ELP. While we observed that many genes specific for the lymphoid lineage including *Tcr*, *Igh* and *Il7r* were highly induced at the ELP stage, some lymphoid genes were already expressed at low levels in the HSC-enriched fraction. Among them, we were particularly interested in chromatin modifiers because of their ability to control spatial and temporal expression of essential genes. Our screen identified Satb1 whose expression was previously linked to T lymphocyte differentiation (Alvarez et al., 2000). We show that Satb1 plays a critical role in directing HSC to lymphoid lineages.

Satb1 was originally identified as a protein which binds specifically to genomic DNA in a specialized DNA context with high base-unpairing potential (termed base-unpairing regions; BURs) (Dickinson et al., 1992). Satb1 is predominantly expressed in the thymus and subsequent studies revealed critical roles in thymocyte development (Alvarez et al., 2000), T cell activation (Cai et al., 2006), and Th2 differentiation (Notani et al., 2010). In thymocyte nuclei Satb1 has a cage-like distribution and tethers BURs onto its regulatory network, thus organizing 3-dimensional chromatin architecture (Cai et al., 2003). By recruiting chromatin modifying/remodeling factors, Satb1 establishes region-specific epigenetic status at its target gene loci and regulates a large number of genes (Yasui et al., 2002; Cai et al., 2003). Increased Satb1 expression in hematopoietic progenitors compared with HSC has been observed by others (Forsberg et al., 2005; Ng et al., 2009); however, no study has been conducted concerning the role of Satb1 in differentiation of HSC to either lymphoid or myeloid progenitors. Our results clearly show a tight association of Satb1 expression with lymphoid lineages even at the earliest stages. In addition, *Satb1*$^{-/-}$ HSC are hampered to produce lymphocytes in vitro and in vivo that is consistent with the phenotypes originally described in *Satb1*$^{-/-}$ mice, suggesting an indispensable role of Satb1 in physiological lymphopoiesis.

Although we have previously identified molecules regulating early lymphoid differentiation, information about ones that initiate the process has been elusive (Oritani et al., 2000; Yokota et al., 2003b, 2008). The present study demonstrates that ectopic expression of Satb1 strongly induces differentiation toward lymphoid lineages and promotes lymphocyte growth from primitive progenitors, even when they are derived from aged BM or ES cells. We believe that these findings are important because they reveal that the earliest step of lymphopoiesis is affected by a global chromatin organizer. In addition, our results suggest that Satb1 expression could be a useful biomarker of aging and be manipulated to reverse immunosenescence.

Lymphoid fate decisions are not necessarily determined by a few transcription factors or cytokines that positively regulate the differentiation in a hierarchical manner. The process should involve “closed windows” as well as “open opportunities”. Gene array studies comparing HSC and ELP have shown that various lymphoid-related genes appear to be synchronously upregulated in ELP, whereas stem cell-related or myeloid-related ones are

downregulated. From these observations, we speculated that a master regulator is present and involved in the synchronicity along with the hierarchical factors; further, we focused on the function of SATB1 in this process. Our results show that once Satb1 is substantially expressed in HSC, it regulates hundreds of genes, including *Rag1*, *Il7*, *kitl* and *Csf3r*, which together determine the lymphoid lineage fate. Satb1 itself has the determinant role in regulating a set of genes to exhibit the phenotype that we observed in vitro and in vivo experiments.

Increasing Satb1 beyond physiologic levels in HSC and ES cells strongly augmented B lymphopoiesis, while depleting Satb1 from HSC dominantly impaired T lymphopoiesis in vivo. Satb1 overexpression in HSC by itself induces an expression profile that favors B-cell production. Conversely, Satb1 deficiency might have disrupted the delicate balance of Satb1 and other BUR-binding proteins such as Satb2 or Bright. We detected minimum levels of *Satb2* and *Bright* expression in WT HSC, and their expression levels significantly increase with B-lineage differentiation (unpublished observations). Interestingly, both genes were aberrantly induced in Satb1-deficient HSC (Supplemental Table 2). Satb2 has a binding specificity similar to that of Satb1, and its expression is more predominant in the B lineage than in the T lineage (Dobreva et al. 2003). In ES cells, Satb2 function is antagonistic to Satb1 in regulating some target genes (Savarese et al., 2009). Whether these BUR-binding proteins are antagonistic or sometimes function synergistically, depending on cell differentiation or lineage remains unknown. Further studies of their functional correlation could yield important information about gene regulation in T and B lymphopoiesis.

While our data provide evidence of a lymphocyte inductive role of Satb1, an important question remains; that is, “what regulates Satb1 expression?” Depletion of long-lived mature B cells rejuvenates B-lymphopoiesis in old mice, suggesting that age-associated accumulation of aged B cells seems to be sensed by HSC or early progenitors in BM (Keren et al., 2011). It will be interesting to learn if such environmental cues influence Satb1 expression in HSC. New strategies for boosting lymphocyte regeneration or protecting this capability during aging might emerge from studies of Satb1-related molecular mechanisms.

Experimental Procedures

Animals

Rag1-GFP knock-in mice were previously described (Kuwata et al., 1999). *Satb1*$^{-/-}$ mice were also previously established (Alvarez et al., 2000). WT C57BL/6 mice and the congenic C57BL/6SJL strain (CD45.1 alloantigen) were obtained from Japan Clea (Shizuoka, Japan) and The Jackson Labs (Bar Harbor, ME), respectively. To obtain mouse fetuses, the morning of the day of vaginal plug observation was considered as embryonic day 0.5 (E0.5).

Flow cytometry and cell sorting

Cells were stained with Abs indicated in each experiment, and analyzed with FACScanto or FACSaria (BD Bioscience). Adult BM cells from Rag1-GFP heterozygotes were used to isolate Lin$^-$ c-kit^{hi} Sca-1$^+$ Flt3$^-$ Rag1-GFP$^-$ IL7Rα$^-$ (HSC-enriched), Lin$^-$ IL7Rα$^-$ c-kit^{hi} Sca-1$^+$ Flt3$^+$ Rag1-GFP$^-$ (LMPP-enriched), Lin$^-$ IL7Rα$^-$ c-kit^{hi} Sca-1$^+$ Flt3$^+$ Rag1-GFP$^+$ (ELP-enriched), Lin$^-$ c-kit^{lo} Sca-1^{lo} Flt3$^+$ Rag1-GFP$^+$ IL7Rα$^+$ (CLP-enriched), and Lin$^-$ c-kit^{hi} Sca-1$^-$ IL7Rα$^-$ myeloid progenitors (Adolfsson et al., 2005; Igarashi et al., 2002; Kondo et al., 1997). For culture experiments, we also sorted a HSC-enriched fraction from WT C57BL/6 or *Satb1*$^{-/-}$ mice according to the cell surface phenotype of Lin$^-$ c-kit^{hi} Sca-1$^+$ Flt3$^-$.

Stromal cell co-culture

Murine stromal cell lines MS5 and OP9 were generous gifts from Dr. Mori (Niigata University) and Dr. Hayashi (Tottori University), respectively. Freshly isolated or transduced cells were co-cultured with stromal cells in α-MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL) and rm IL7 (1ng/mL). The cultures were fed twice a week and maintained for the indicated periods in each experiment. OP9-DL1 cells originated by Dr. Kawamoto (Riken, Japan) were obtained from Riken Cell Bank (Tsukuba, Japan) and used to produce T-lineage cells. In this case, cells were cultured in the presence of rm Flt3-ligand (5 ng/mL) and rm IL7 (1ng/mL) for 14 days, and rm Flt3-ligand (5 ng/mL) alone thereafter. At the end of culture, cells were counted and analyzed by flow cytometry.

Competitive repopulation assay

The CD45.1/CD45.2 system was adapted to a competitive repopulation assay. One thousand $Flt3^-$ LSK cells sorted from FL or BM of WT, Satb1 hetero-deficient, or Satb1 homozygous-deficient mice (CD45.2) were mixed with 4×10^5 unfractionated adult BM cells obtained from WT C57BL/6-Ly5.1 (CD45.1) mice, and were transplanted into C57BL/6-Ly5.1 mice lethally irradiated at a dose of 920 rad. At 8 weeks after transplantation, engraftment of CD45.2 cells was evaluated by flow cytometry.

Retrovirus transfection

Murine Satb1 expression vector was purchased from OriGene (Rockville, MD). A retrovirus expression vector for Satb1 was generated by subcloning into the pMYs-IRES-GFP or DsRed vector (a gift from Dr. Kitamura, University of Tokoyo). Conditioned medium containing high tighter retrovirus particles was prepared as reported previously (Satoh et al., 2008). Sorted HSC were cultured in D-MEM containing 10% FBS, rm SCF (100 ng/ml), rm TPO (100 ng/ml) and rm Flt3-ligand (100ng/ml) for 24 h. Then, the cells were seeded into the culture plates coated with Retronectin (Takara Bio, Shiga, Japan) and cultured with conditioned medium containing retrovirus. After 24 h, cells were washed and performed second transfection by the same condition. After 48 h from the second transfection, GFP or DsRed-positive cells were sorted by FACSaria.

Limiting dilution assays

The frequencies of lympho-hematopoietic progenitors were determined by plating cells in limiting dilution assays using 96-well flat bottom plates. Pre-established MS5 layers were plated with 1, 2, 4, 8, or 16 cells each using the Automated Cell Deposition Unit of the FACSaria. Cells were cultured in α-MEM supplemented with 10% FCS, rm SCF (10 ng/mL), rm Flt3-ligand (20 ng/mL) and rm IL7 (1ng/mL). At 10 days of culture, wells were inspected for the presence of hematopoietic clones. Positive wells were harvested and analyzed by flow cytometry for the presence of $CD45^+$ hematopoietic cells and CD45R/$B220^+$ $CD19^+$ $Mac1^-$ B-lineage cells. The frequencies of progenitors were calculated by linear regression analysis on the basis of Poisson distribution as the reciprocal of the concentration of test cells that gave 37% negative cultures.

Lymphocyte development from murine ES cells

To induce differentiation toward hematopoietic cells, E14tg2a ES cells were deprived of leukemia inhibitory factor and seeded onto OP9 cells in 6-well plates at a density of 10^4 cells/well in α-MEM supplemented with 20% FBS (Nakano et al., 1994). After 4.5 days, the cells were harvested and whole cell suspensions were transferred into a new 10-cm dish and incubated in 37 °C for 30 min to remove adherent OP9 cells. The collected floating cells were infected with the retroviral supernatant in Retronectin-coated plates by 2 h

spinoculation (1100 *g*) (Kitajima et al., 2006). Subsequently, the cells were cultured on OP9 or OP9-DL1.

Tetracycline (Tet)-regulated inducible expression of Satb1 in ES cells

To inducibly express Satb1 in ES cells, we utilized a Tet-off system as reported previously (Era and Witte, 2000), in which transcription of the target gene is initiated by the removal of Tet from the culture medium. Briefly, we initially introduced pCAG20-1-tTA and pUHD10-3-puro by electroporation and selected one clone designated E14 by culture with 1 μg/ml of Puro and/or 1 μg/ml of Tet. We further transfected pUHD10–3-Satb1-GFP, which can inducibly express Satb1 and GFP as a single mRNA through the internal ribosome entry site in response to the Tet removal, together with the neomycin-resistant plasmid pcDNA3.1-neo. After the culture with G418, we selected clones that can inducibly express GFP in response to the Tet deprivation.

DNA PCR assays for *Igh* rearrangement

DNA PCR assays were performed as reported previously (Schlissel et al., 1991). PCR was performed using genomic DNA extracted from splenocytes or ES-derived cells as a template. D_H-J_H recombination was detected as amplified fragments of 1033 bp, 716 bp and 333 bp using a primer $D_HL(5')$ and J3(3′). Germline alleles were detected as an amplified fragment of 1259 bp using a primer Mu0(5′) and J3(3′). The sequence of primers are as follows: $D_HL(5')$, GGAATTCG(AorC)TTTTTGT(CorG)AAGGGATCTACTACTGTG; Mu0(5′), CCGCATGCCAAGGCTAGCCTGAAAGATTACC; and J3(3′), GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG.

Statistical analyses

Unpaired, 2-tailed t-test analyses were used for intergroup comparisons, and p-values were considered significant if they were less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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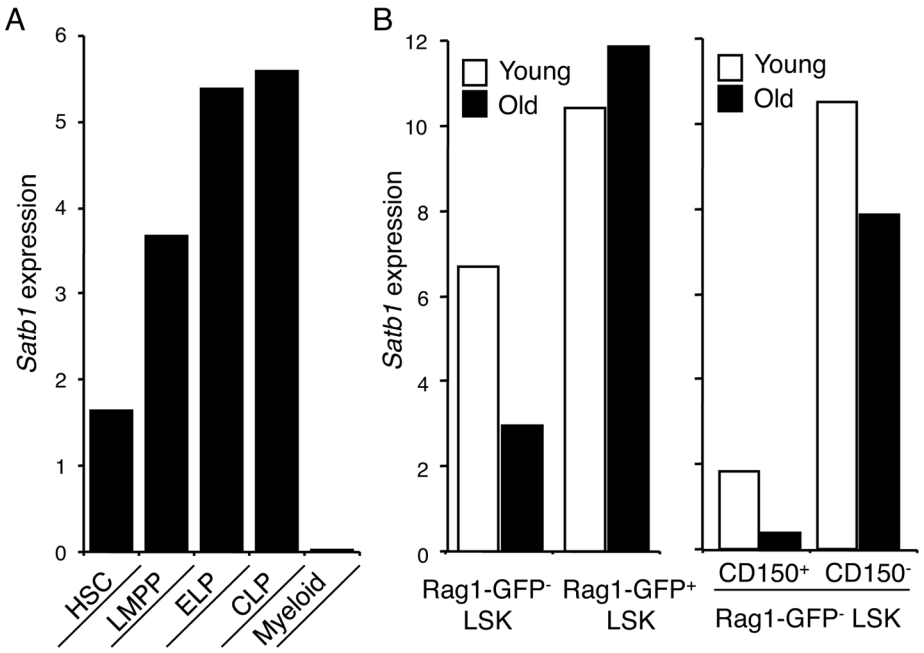

Figure 1. *Satb1* expression levels change with differentiation and aging of HSC

HSC, LMPP, ELP, CLP and the myeloid progenitor-enriched fractions were sorted from BM of 8- to 10-week-old Rag1-GFP knock-in or WT mice according to cell surface markers and GFP expression (see Experimental Procedures), and transcripts for *Satb1* were quantitatively evaluated with real-time RT-PCR. (B) The LSK Rag1-GFP^{-} and LSK Rag1-GFP^{+} fractions (left panel), or the CD150^{+} LSK Rag1-GFP^{-} and CD150^{-} LSK Rag1-GFP^{-} fractions (right panel) were sorted from 6-week-old or 2-year-old Rag1-GFP knock-in mice, respectively. Then *Satb1* expression was evaluated with real-time RT-PCR. The *Satb1* expression values were normalized by *Gapdh* expression and shown in each panel. Each data represents 2 independent examinations that showed essentially the same results. (Figure 1, see also Figure S1 and Table S1.)

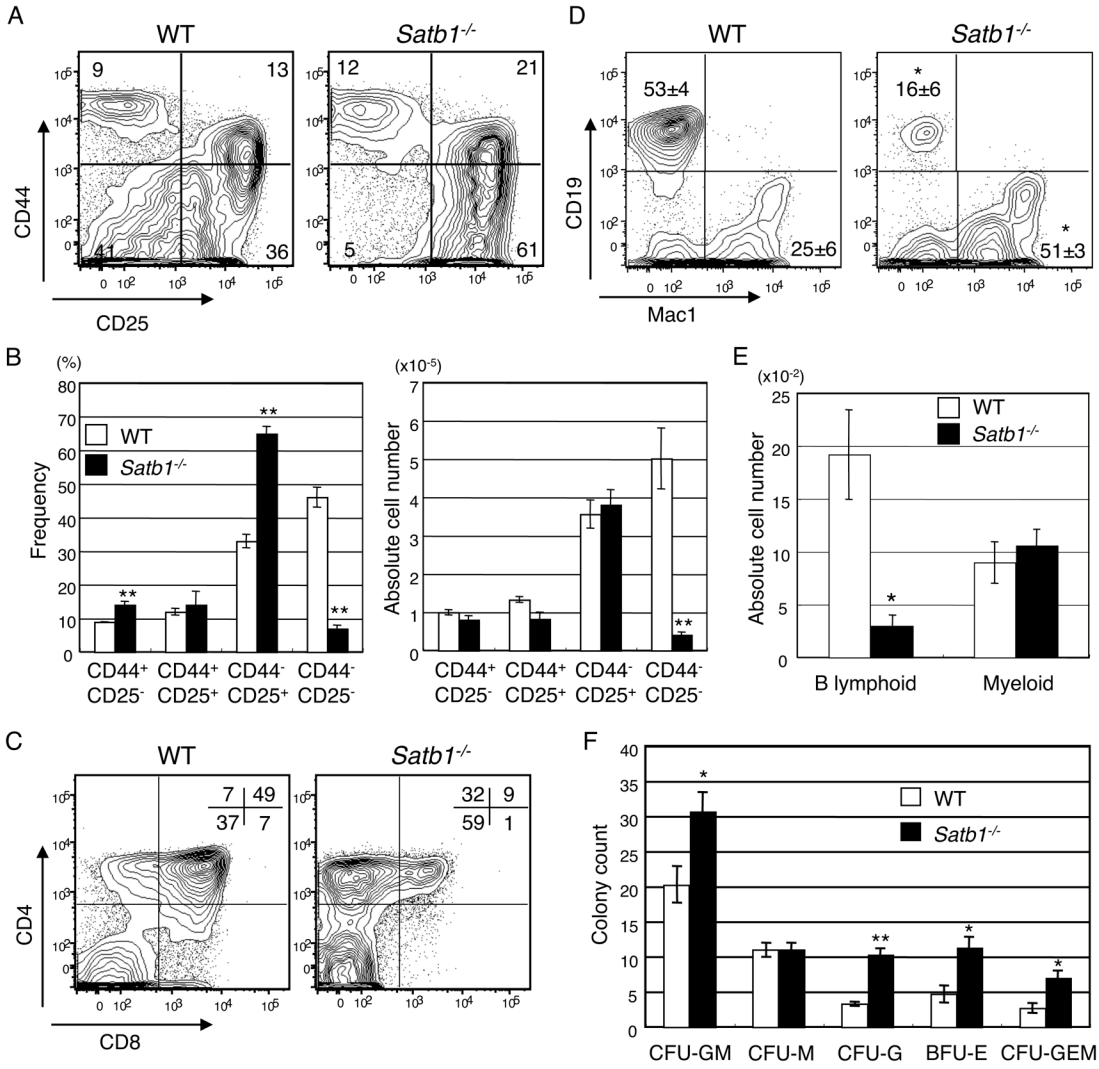

Figure 2. Satb1 deficiency alters lymphoid and myeloid activities of hematopoietic stem/progenitor cells in culture

Lin$^-$ cells were isolated from FL of E14.5 *Satb1*$^{-/-}$ embryos or their WT littermates. (A–C) Cells were co-cultured with OP9-DL1 stromal cells for evaluation of T-lineage differentiation. (A) Flow cytometry results are shown for cells recovered on day 14 and stained for CD44 and CD25/IL2Rα. (B) Frequencies and absolute numbers of each phenotype were calculated (C) A similar analysis was performed for CD4 and CD8α bearing cells recovered cells on day 18. (D,E) The same cell suspensions were co-cultured with MS5 stromal cells to assess B and myeloid lineage potentials and representative data are shown for day 7 of culture. (F) In parallel, the Lin$^-$ cells were evaluated with methylcellulose colony assays. Each dish contained 1000 sorted cells and colony counts were performed on day 10. The bars indicate numbers of CFU-GM, CFU-M, CFU-G, BFU-E, or CFU-GEM scored per dish. The results are shown as mean ± SE. Statistically significant differences between WT and *Satb1*$^{-/-}$ cells are marked with asterisks (* $p<0.05$, ** $p<0.01$). (Figure 2, see also Figure S2.)

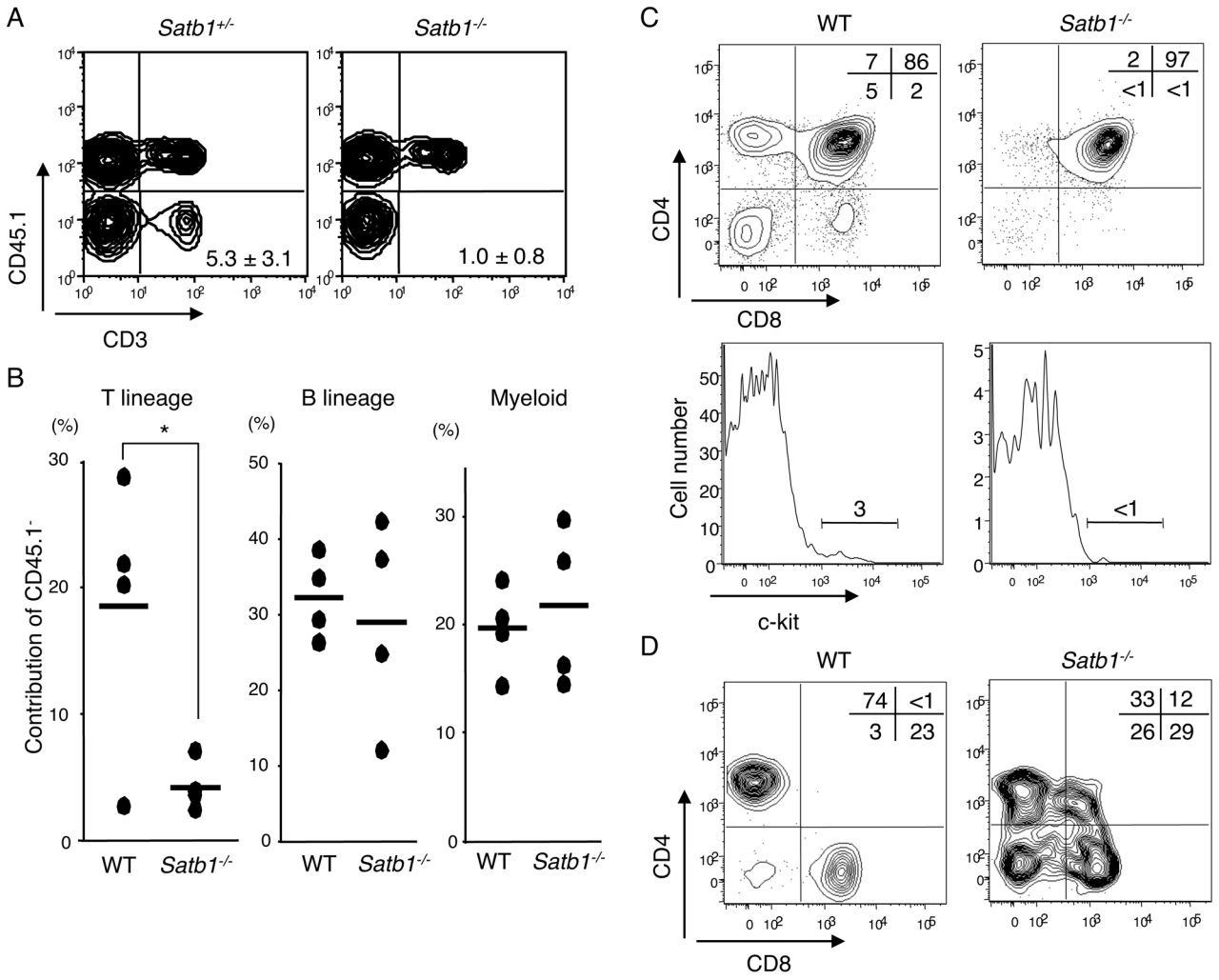

Figure 3. Defective T lymphopoiesis from transplanted *Satb1*$^{-/-}$ HSC

(A, B) One thousand stem cell enriched Flt3^{-} LSK cells were sorted from BM of 2-week-old Satb1 deficient or littermate mice (CD45.2). They were then mixed with 4 × 10^{5} adult BM cells obtained from WT (CD45.1) mice, and were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, peripheral blood cells of the recipients were identified with anti-CD45.1 and anti-CD3. Numbers in each panel of (A) represent percentages of CD3^{+} CD45.1^{-} cells among the total leukocytes and are shown as averages with SD (n=4 in each). Chimerisms of CD45.1^{-} cells in the CD3^{+} T-lineage, the CD45R/B220^{+} B lineage, or the Gr1^{+} myeloid lineage were determined. Statistical significance: *p<0.05. (C,D) One thousand Flt3^{-} LSK cells sorted from E14.5 FL of Satb1 homo-deficient or their WT littermates (CD45.2) were transplanted into lethally irradiated WT CD45.1 mice. At 8 weeks after transplantation, T-lineage reconstitution in the thymus and the spleen was analyzed. The CD4 and CD8 profiles of CD45.2^{+} thymocytes (C, upper panels) and the c-kit expression of CD45.2^{+} CD3^{-} CD4^{-} CD8^{-} CD44^{+} CD25^{-} thymocytes (C; lower panels) are shown. (D) Representative CD4 and CD8 profiles are shown for CD45.2^{+} CD3^{+} cells in recipient spleens.

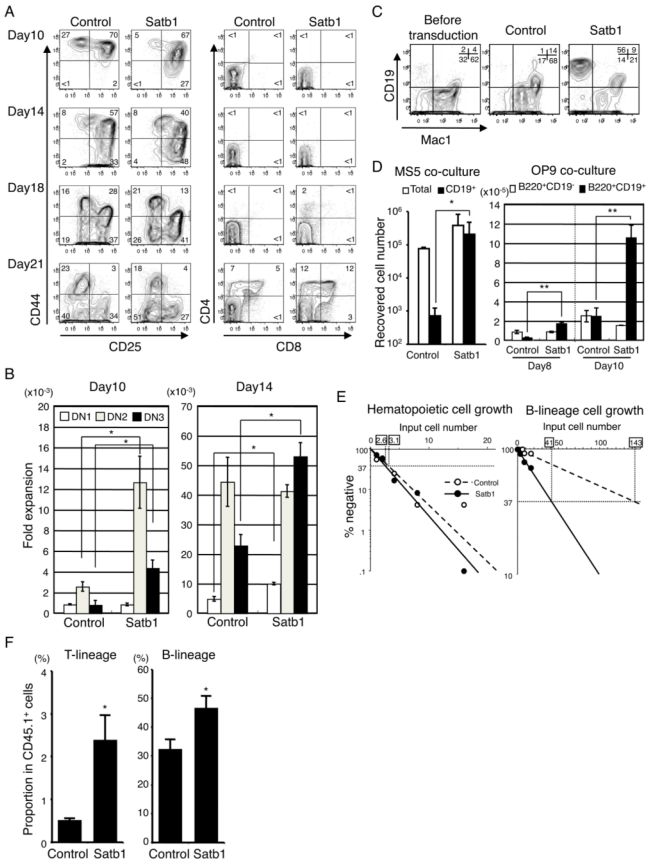

Figure 4. Satb1 overexpression promotes lymphopoiesis

LSK Flt3$^-$ cells obtained from WT BM were retrovirally transduced with either a fluorescence-alone expressing control or a native Satb1 combined with GFP expressing vector. Successfully transduced cells were cultured, and their differentiation and proliferation were analyzed at the indicated period. (A and B) Time course analyses were performed for T-lineage cell generation in the OP9-DL1 co-culture. Absolute numbers of recovered cells were divided by the numbers of transduced LSK Flt3$^-$ cells used to initiate the cultures to obtain the fold expansion values. Data are shown as mean ± SE. (C) CD19 and Mac1 profiles are shown for cells recovered from MS5 co-cultures on day 10. The left panel shows data obtained from fresh LSK Flt3$^-$ cells that did not undergo the retroviral infection. (D, left panel) The absolute numbers of total recovered cells and B-lymphoid cells in the MS5 co-culture. (D, right panel) The output of B220$^+$ CD19$^-$ or B220$^+$ CD19$^+$ B-lineage cells was evaluated in the OP9 co-culture. Cultures were established in triplicate. Data are shown as mean ± SE. Statistical significance: * $p<0.05$, **$p<0.01$. (E) Limiting-dilution analyses were performed to determine the frequencies of hematopoietic progenitors that could give rise to CD19$^+$ B-lineage cells. Input cell numbers corresponding to each 37% negative value are shown in rectangles. (F) One thousand LSK Flt3$^-$ cells (CD45.1) transduced with either Satb1-expressing or control vectors were transplanted to lethally irradiated WT mice (CD45.2) with 1×10^5 adult BM cells (CD45.2). Two weeks after transplantation, peripheral blood was collected to determine the proportion of CD4/CD8$^+$ T lineage and CD19$^+$ B lineage in CD45.1$^+$ cells. Data are shown as mean ± SE. Statistical significance: *$p<0.05$. (n=5 in each group). (Figure 4, see also Figure S3.)

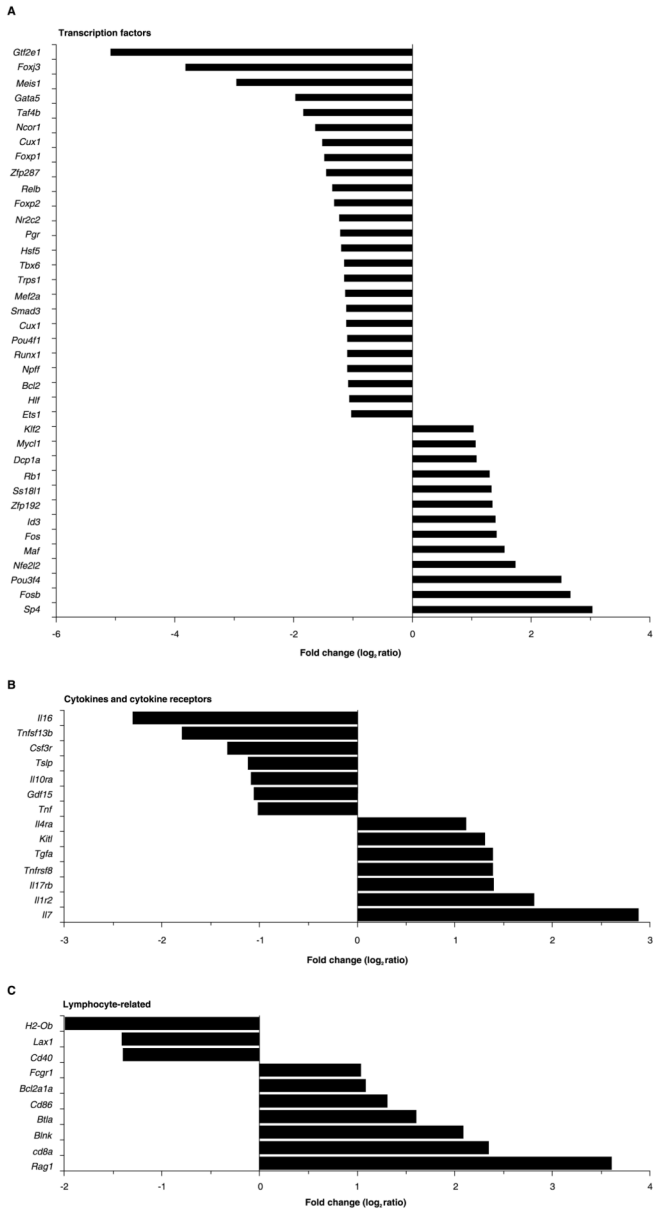

Figure 5. Genes affected by Satb1 expression

A microarray experiment was performed to compare gene expression in Satb1 and control-transduced LSK Flt3^{-} cells. Up-regulation in Satb1-transduced cells is shown as positive in each figure. (A) Transcription factors, (B) cytokine and cytokine receptors, and (C) other lymphoid lineage-related genes are summarized. (Figure 5, see also Table S2 and Table S3.)

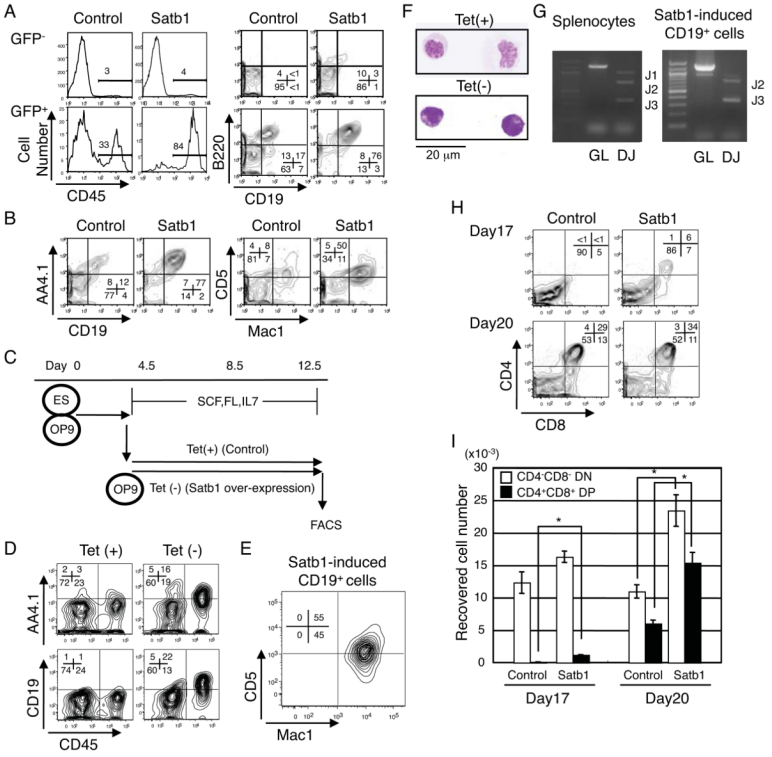

Figure 6. Satb1 promotes lymphoid differentiation from ES-derived cells

E14tg2a ES cells were deprived of leukemia inhibitory factor and seeded onto OP9 cells. After 4.5 days, the differentiated mesoderm cells were infected with retroviral supernatants containing control-GFP or Satb1-GFP expressing vectors. Subsequently, the cells were cultured on OP9 for 8 days. At the end of culture, all cells were harvested and stained with the antibodies indicated in each panel. (A, left panels) Total recovered cells were divided according to GFP expression. The percentages of $CD45^+$ cells in GFP^- (upper panels) and GFP^+ populations (lower panels) are shown. (A, right panels) CD45R/B220 and CD19 profiles of the $CD45^+$ cells corresponding to the left panels. (B) Representative AA4.1 and CD19 or Mac1 and CD5 profiles of the GFP^+ $CD45^+$ cells recovered from control or Satb1-transduced culture. (C) The experimental design used with a Tet-off system (upper panel). ES cells, which inducibly express Satb1 by Tet deprivation, were established. After 4.5 days of culture without leukemia inhibitory factor in the presence of Tet, the differentiated cells were reseeded onto new OP9 stromal cells with or without Tet. Subsequently, FACS analysis was performed after 8 days of culture (day12.5). Tet (+) indicates profiles of GFP^- cells cultured with Tet (D, left panels). Tet (−) panels show profiles of Satb1/GFP^+ cells cultured without Tet (D, right panels). (E) Mac1 and CD5 expression on the Satb1/GFP^+ $CD19^+$ cells grown without Tet. (F) Morphology of ES-derived hematopoietic cells on day 12.5. (G) DNA PCR assays of germline (GL) or D_H-J_H rearranged *Igh* chain (DJ) genes were performed with the Satb1/GFP^+ $CD19^+$ cells recovered without Tet (right panel). Splenocytes were used as a positive control for the D_H-J_H recombination (left panel). On each gel, a size marker was loaded in the left lane. (H, I) E14tg2a ES cells were differentiated to mesoderm cells for 4.5 days and then infected with the retroviral supernatant containing control-GFP or Satb1-GFP expressing vectors for 3 days. Subsequently the cells were cultured on OP9-DL1 and T-lineage output was evaluated on the indicated days. Data are shown as mean ± SE. Statistical significance: * $p<0.05$.

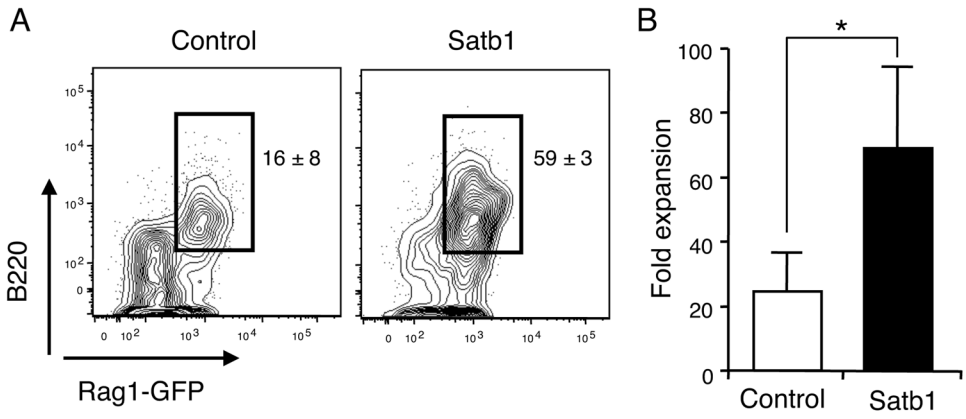

Figure 7. Satb1 overexpression restores lymphopoietic activity of aged HSC

(A) Rag1/GFP^{-} LSK cells were sorted from 2-year-old mice and retrovirally transduced with control or Satb1-DsRed vectors. Successfully transfected cells were cultured on OP9 cells. Cultures were established in triplicate. Numbers in each panel indicate the frequency of Rag1/GFP^{+} CD45R/B220^{+} cells. (B) Yields of CD45R/B220^{+} Rag1/GFP^{+} Mac1^{-} B-lineage cells per 1 input control- or Satb1-transduced Rag1/GFP- LSK cell were calculated and given as averages with SD bars. Statistical significance: *$p<0.05$. (Figure 7, see also Figure S4.)