

Conversion of T cells to B cells by inactivation of polycomb-mediated epigenetic suppression of the B-lineage program

Tomokatsu Ikawa,^{1,2,3} Kyoko Masuda,^{2,4} Takaho A. Endo,⁵ Mitsuhiro Endo,⁶ Kyoichi Isono,⁶ Yoko Koseki,⁶ Rinako Nakagawa,⁷ Kohei Kometani,⁸ Junichiro Takano,^{1,6} Yasutoshi Agata,⁹ Yoshimoto Katsura,^{2,10} Tomohiro Kurosaki,^{7,8} Miguel Vidal,¹¹ Haruhiko Koseki,⁶ and Hiroshi Kawamoto^{2,4}

¹Laboratory for Immune Regeneration, RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan; ²Laboratory for Lymphocyte Development, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan; ³PRESTO (Precursory Research for Embryonic Science and Technology), Japan Science and Technology Agency, Saitama 332-0012, Japan; ⁴Department of Immunology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan; ⁵Laboratory for Integrative Genomics, RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan; ⁶Laboratory for Developmental Genetics, RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan; ⁷Laboratory for Lymphocyte Differentiation, Immunology Frontier Research Center, Osaka University, Suita 565-0871, Japan; ⁸Laboratory for Lymphocyte Differentiation, RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan; ⁹Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; ¹⁰Division of Cell Regeneration and Transplantation, Advanced Medical Research Center, Nihon University School of Medicine, Tokyo 173-8610, Japan; ¹¹Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain

In general, cell fate is determined primarily by transcription factors, followed by epigenetic mechanisms fixing the status. While the importance of transcription factors controlling cell fate has been well characterized, epigenetic regulation of cell fate maintenance remains to be elucidated. Here we provide an obvious fate conversion case, in which the inactivation of polycomb-mediated epigenetic regulation results in conversion of T-lineage progenitors to the B-cell fate. In T-cell-specific Ring1A/B-deficient mice, T-cell development was severely blocked at an immature stage. We found that these developmentally arrested T-cell precursors gave rise to functional B cells upon transfer to immunodeficient mice. We further demonstrated that the arrest was almost completely canceled by additional deletion of *Pax5*. These results indicate that the maintenance of T-cell fate critically requires epigenetic suppression of the B-lineage gene program.

[*Keywords:* T-cell development; epigenetics; reprogramming; B cell; lineage commitment; transcription factor]

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The process by which progenitors with multiple developmental potentials terminate some of them is termed lineage commitment. Lineage commitment generally proceeds in two steps: First, in the specification step, progenitors are biased to produce a certain lineage but still retain their other potentials. Second, in the determination step, progenitors become determined to produce that lineage but not others (Rothenberg et al. 2008; Mandel and Grosschedl 2010; Aloia et al. 2013).

In general, during the progression to the lineage determination step, transcription factors initially determine

which genes are activated or silenced, and this is followed by epigenetic mechanisms that stably maintain the activated or silenced status of the affected genes by histone modification or DNA methylation. In that sense, transcription factors primarily serve as major lineage determinants. Indeed, it is well known that cell fate can be changed by genetically modifying transcription factors. For example, deletion of PAX5 and EBF1 in early B-lineage cells leads to generation of other hematopoietic lineage

Corresponding authors: tomokatsu.ikawa@riken.jp, kawamoto@frontier.kyoto-u.ac.jp

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cells (Nutt et al. 1999; Cobaleda et al. 2007; Nechanitzky et al. 2013), and overexpression of CEBP α leads to conversion of B- or T-lineage cells into macrophages (Xie et al. 2004; Laiosa et al. 2006). Moreover, recent studies of so-called “reprogramming” have shown that somatic cells can be converted into embryonic stem (ES)-like cells or directly into other types of somatic cells when they are provided appropriate transcription factors (Takahashi and Yamanaka 2006; Ieda et al. 2010; Szabo et al. 2010).

On the other hand, in the case of epigenetic mechanisms, alteration of the epigenetic status usually does not lead to drastic cell fate conversions. Polycomb group (PcG) proteins are some of the major players in epigenetic repression of genes (Sparmann and van Lohuizen 2006; Aloia et al. 2013). In mice deficient for a polycomb component, an impairment of anterior transformation of vertebral identity as well as a defect in the development of various types of hematopoietic cells have been reported (van der Lugt et al. 1994; Iwama et al. 2004; Cales et al. 2008; Miyazaki et al. 2008; Oguro et al. 2010). However, so far, no cases of obvious cell fate conversion have been reported in mice with genetically modified polycomb components. This is also true for other epigenetic modulators, such as DNA methyltransferase (Trowbridge et al. 2009; Challen et al. 2014) or AT-rich sequence binding 1 (Satz1) (Sato et al. 2013; Will et al. 2013).

We focused on the process of lineage restriction from multipotent hematopoietic stem cells (HSCs) to T-cell progenitors. T cells are produced in the thymus from progenitors derived from fetal liver or bone marrow (BM). In the thymus, during the immature CD4⁻CD8⁻ (double-negative [DN]) stage, cells differentiate from the DN1 to DN4 stages, and TCR β chain gene rearrangement takes place at the DN3 stage. DN cells then differentiate into the CD4⁺CD8⁺ (double-positive [DP]) stage and then into the mature CD4-single-positive (SP) or CD8SP stage. It was shown previously that the restriction step from HSCs toward T-cell progenitors is initiated by the production of myelo-lymphoid progenitors (Lu et al. 2002) followed by the myeloid-T progenitors (Bell and Bhandoola 2008; Wada et al. 2008). We further showed that such myeloid-T progenitors become determined to the T-cell lineage at the DN2 stage (Masuda et al. 2007) and that this T-lineage determination step is driven by the transcription factor Bcl11b (Ikawa et al. 2010; Li et al. 2010a,b). It then became of interest to ask how the T-cell-determined status is maintained.

PcG proteins consist of two main complexes, termed Polycomb-repressive complex 1 (PRC1) and PRC2. PRC2 complexes initially induce histone H3K27 trimethylation (H3K27me3), and, subsequently, RING finger E3 ligases of PRC1 complexes catalyze histone H2AK119 monoubiquitylation, leading to a conformational change of histones to a repressive state (Aloia et al. 2013). Ring1B and its paralog, Ring1A, are components of PRC1 that mediate monoubiquitylation of H2AK119. They associate with many other proteins such as Bmi-1, Mel18, and Phc1 to form the core PRC1 complexes. Previous reports have shown that deletion of *Mel18* or *Bmi1* results in the impaired proliferation of thymocytes at the DN2 stage or

DN3 stage, respectively (Miyazaki et al. 2005, 2008). However, the deletion of each gene does not completely abrogate PRC1 function, since these molecules have other compensatory partner molecules. It has been reported that the deletion of *Ring1b* led to severe gastrulation defects and embryonic lethality (Voncken et al. 2003), whereas the inactivation of *Ring1a* in mice resulted in no overt phenotypes, suggesting the functional predominance of Ring1B over Ring1A (del Mar Lorente et al. 2000).

In order to completely inactivate the function of PRC1 complexes, we used a *Ring1b* conditional deletion in the setting of a *Ring1a*-deficient background. We found that the T-cell development was blocked at the immature CD4⁻CD8⁻ DN stage in the thymus of Ring1A/B-double-deficient mice. This developmental block was partially rescued by the additional deletion of *Cdkn2a*, a cell cycle inhibitor, resulting in the generation of CD4⁺CD8⁺ DP cells. We further showed that these DP cells were able to differentiate into functional IgM⁺ B cells upon transfer to immunodeficient mice. On the other hand, the severe T-cell developmental block seen in *Ring1a/b* conditional deficient mice was almost overcome by additional deletion of *Pax5*, demonstrating that *Pax5* is a major target of polycomb during early T-cell development. These results indicate that the maintenance of T-cell fate requires continuous epigenetic suppression of the B-lineage-specific gene program.

Results

Ring1a/b is essential for T-cell development

To examine the expression profiles of Ring1B in the hematopoietic system, we first analyzed Ring1B-YFP reporter mice in which YFP-coding sequences were knocked into the *Ring1B* gene locus (Isono et al. 2013). Ring1B was expressed at a higher level in DN cells than DP, CD4SP, or CD8SP cells, peaking at the DN2 stage (Supplemental Fig. S1A–D). Additionally, the expression of Ring1B in DN cells was higher than that of mature T and B cells in the spleen or myeloid and B cells in the BM (Supplemental Fig. S1E–H).

To study the function of *Ring1a/b* during T-cell differentiation, we crossed *Ring1a*^{-/-} mice and mice with transgenic expression of *Cre* recombinase driven by the *Lck* promoter and carrying loxP-flanked alleles encoding Ring1B [*LckCre-Ring1a*^{-/-}*Ring1b*^{fl/fl}: Lck double knockout] mice (Fig. 1A,B; Supplemental Fig. S2A). The T-cell-specific *Lck-Cre* gene specifically deletes the floxed *Ring1b* allele at the DN2–3 stage in T cells in the thymus. In Lck double-knockout mice, the number of thymocytes was reduced to ~5% of that in the *Ring1a*^{-/-}*Ring1b*^{fl/fl} control mice (Fig. 1B). TCR β ⁺ cells were nearly absent, and T-cell development was completely blocked at the DN3 stage in Lck double-knockout mice. While the proportion of TCR $\gamma\delta$ ⁺ cells was increased, their absolute number did not significantly change in Lck double-knockout mice (Supplemental Fig. S2C). This could be because most of the $\gamma\delta$ ⁺ T-cell precursors diverge before the DN3 stage, where the LckCre activity becomes on.

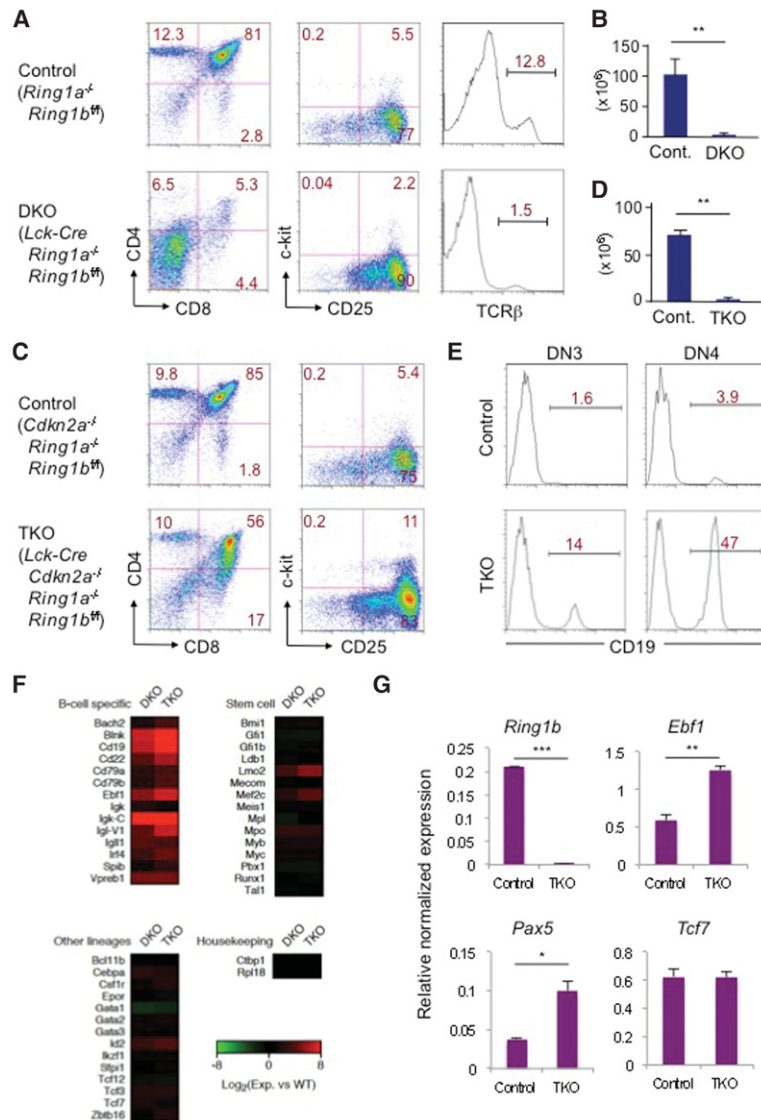


Figure 1. *Ring1a/b* is essential for T-cell development. (A) Flow cytometric analysis of cells in the thymus of *LckCre(-)* (control) or *LckCre(+)* (double knockout [DKO]) *Ring1a^{-/-}Ring1b^{fl/fl}* mice. Thymocytes were analyzed for the expression of CD4 versus CD8, c-kit versus CD25 gated on Lineage marker-negative (Lin^-) cells, and TCR β . (B) The number of thymocytes in control or double-knockout mice. (C) Flow cytometric analysis of thymic cells from *LckCre(-)* (control) or *LckCre(+)* (triple knockout [TKO]) *Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* mice. Thymocytes were analyzed for the expression of CD4 versus CD8 and c-kit versus CD25 gated on Lin^- cells. (D) The number of thymocytes in control or triple-knockout mice. (E) Expression of CD19 by DN3 and DN4 cells from control and triple-knockout mice. (F) Derepressed genes in Lin^- thymocytes from double-knockout and triple-knockout mice compared with control mice identified by microarray analysis. (G) Thymic DN3 cells from *Ert2Cre(-)* (control) or *Ert2Cre(+)* (triple knockout) *Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* mice were sorted and cultured on TSt-4/Delta-like 1 (DLL1) cells in the presence of 4-hydroxytamoxifen (4-OHT). The cultured cells were harvested at day 3, and quantitative RT-PCR (qRT-PCR) was performed for expression of the indicated genes. Data are representative of at least three independent experiments. Mean \pm SD. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$, Student's *t*-test in B, D, and G.

We also generated double-knockout mice using *Cd4-Cre* whose *Cre* expression was controlled by the *Cd4* promoter and found that the frequency of T cells in the thymus and spleen was similar to that of the control mice (Supplemental Fig. S3A,B), indicating that *Ring1a/b* is dispensable at later stages of T-cell development.

Cdkn2a, a cyclin-dependent kinase (CDK) inhibitor, is known to be an important target of polycomb-mediated repression in hematopoietic lineages. Inactivation of *Cdkn2a* has been shown to restore the defective self-renewal capacity of HSCs and thymocyte proliferation observed in *Bmi1*-deficient mice (Miyazaki et al. 2008; Oguro et al. 2010). To study the role of *Cdkn2a* on the *Lck* double-knockout phenotype, we generated *Lck* double-knockout mice on a *Cdkn2a^{-/-}* background (*Lck* triple-knockout mice). We found that in the *Lck* triple-knockout mice, the number of thymocytes was not restored; however, some DP cells were generated (Fig. 1C, D; Supplemental Fig. S2B,D). Such minimal rescue in

this setting may indicate the existence of other essential target genes whose derepression hampers T-cell development. We then noticed that CD19 is expressed by DN3 and DN4 cells in the *Lck* triple-knockout mice (Fig. 1E). Global gene expression profiles of Lineage marker-negative (Lin^-) cells in the thymus revealed that several B-cell lineage-specific genes but not other lineage markers or stem cell-related genes were substantially up-regulated in the *Lck* double-knockout and *Lck* triple-knockout T-cell progenitors (Fig. 1F). To further characterize the DN3 and DN4 cells in the thymus of *Lck* double-knockout mice, expression of B-lineage-associated genes was analyzed by quantitative RT-PCR (qRT-PCR). Expression of *Ebf1* and *Pax5* in DN3 cells of *Lck* double-knockout mice was substantially up-regulated, although the level was still low compared with that of CD19⁺ cells in normal BM (Supplemental Fig. S4A,C). Of note, the expression of *Ebf1*, *Pax5*, *Cd79a*, *Cd79b*, and *Vpreb1* was increased dramatically in DN4 cells in *Lck* double-knockout mice,

indicating that the derepression of B-lineage-associated genes started at the DN3 stage but became prominent at the DN4 stage (Supplemental Fig. S4B,C). This is consistent with a previous report that showed the premature derepression of B-lineage genes in HSCs from *Bmi1*-deficient mice (Oguro et al. 2010).

To confirm that derepression of B-lineage genes really occurs at the DN3 stage, we generated triple-knockout mice with an *Ert2-Cre* transgene encoding a hormone-inducible Cre-estrogen receptor fusion protein [*Ert2Cre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* [ERT2 triple-knockout] mice], in which triple-knockout status can be induced by exposure to 4-hydroxytamoxifen (4-OHT). DN3 cells from ERT2 triple-knockout mice were cultured on TSt-4/Delta-like 1 (DLL1) cells, which are stromal cells that support the proliferation and differentiation of T-lineage cells, in the presence of 4-OHT. After 72 h of induction, cells were harvested, and mRNA expression was analyzed by qRT-PCR. Down-regulation of *Ring1b* and up-regulation of B-lineage genes such as *Ebf1* and *Pax5* were seen (Fig. 1G). These data indicate that a subset of B-lineage-associated genes is directly repressed by PRC1 in T-cell pro-

genitors. As shown in Supplemental Figure S3A, CD19 expression in CD4SP and CD8SP cells in the thymi of *Cd4Cre-Ring1a^{-/-}Ring1b^{fl/fl}* mice was barely detectable, indicating that suppression of B-cell genes by *Ring1a/b* persists until the DP stage, whereas *Ring1a/b* is dispensable at later stages of T-cell development.

Conversion of thymic DP cells from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* (triple-knockout) mice into B cells in vivo

To determine the developmental plasticity of the affected T-cell progenitors, DP cells from the thymi of *Lck* triple-knockout and control mice were transferred to sublethally irradiated immunodeficient mice (Fig. 2A). Whereas DP cells from control mice gave rise to CD4⁺SP and CD8⁺SP mature T cells in the spleen, those from triple-knockout mice failed to generate T cells. Surprisingly, instead, the triple-knockout DP cells gave rise to CD19⁺IgM⁺ B cells in the spleens and BM of reconstituted mice (Fig. 2B,C). Similarly, upon transferring to immunodeficient mice, the CD19⁻DN3 cells from *Lck* triple-

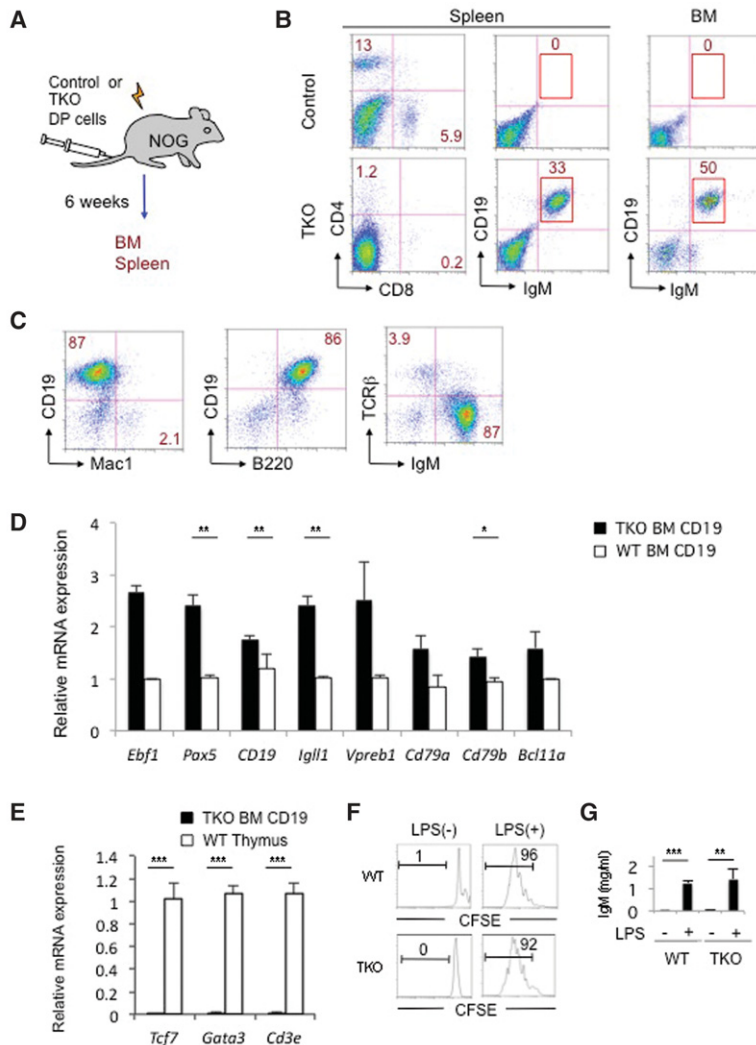


Figure 2. Conversion of thymic DP cells from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* (triple-knockout [TKO]) mice into B cells in vivo. (A) Schematic representation for the developmental potential of DP cells from control or triple-knockout mice. (B) Flow cytometric analysis of cells in the spleens and BM of NOD/Shi-*scid*, IL2R γ^{null} (NOG) mice transplanted with DP cells from control or triple-knockout mice. Expression of CD4 versus CD8 and CD19 versus IgM is shown. (C) Flow cytometric analysis of cells in the BM of NOG mice transplanted with DP cells of triple-knockout mice at 6 wk after injection. Cells were analyzed for the expression of CD19 versus Mac1, CD19 versus B220, and TCR β versus IgM gated on CD45.2. (D,E) qRT-PCR analysis of converted B cells purified from the BM for the expression of B-lineage-associated (D) or T-lineage-associated (E) genes. (F,G) In vitro stimulation of converted B cells for investigating proliferation and IgM secretion. Converted and wild-type splenic B cells were sorted and labeled with CFSE. The labeled cells were stimulated with LPS and IL-4 for 4 d. Flow cytometric analysis (F) and IgM secretion measured by ELISA (G) of converted (triple-knockout) and wild-type B cells after stimulation are shown. Data are representative of at least three independent experiments. Mean \pm SD. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$, Student's *t*-test in D, E, and G.

knockout mice also gave rise to CD19⁺IgM⁺ cells in the spleens and BM, whereas the DN3 cells and DP cells of Lck double-knockout mice failed to generate B-lineage cells (Supplemental Fig. S5). These data indicate that Lck triple-knockout T-cell progenitors retain the potential to differentiate into B cells even after they reach the DP stage. Of note, we were not able to detect intermediate B-cell progenitors in the BM and spleen (Fig. 2B,C; Supplemental Fig. S5). Moreover, we failed to detect the transdifferentiation in vitro (data not shown), suggesting that the T-cell progenitors of Lck triple-knockout mice directly converted to CD19⁺IgM⁺ cells.

The CD19⁺IgM⁺ cells derived from Lck triple-knockout DP cells were found to have a complete B-cell lineage identity because they express a set of B-cell lineage-associated genes at levels comparable with wild-type B cells, while T-cell lineage-associated genes such as *Tcf7*, *Gata3*, or *Cd3e* were almost undetectable (Fig. 2D,E). To determine the function of the converted B cells, the CD19⁺IgM⁺ cells derived from Lck triple-knockout DP cells were labeled with CFSE and cultured with LPS and IL-4 for 4 d. The B cells converted from LCK triple-knockout DP cells had a normal proliferative response and IgM secretion upon LPS and IL-4 stimulation, indicating that the converted B cells were functional (Fig. 2F,G).

Rearrangements of immunoglobulin heavy chain (*Igh*) and *Tcrβ* chain genes in B cells derived from DP cells in the thymi of Lck triple-knockout mice

Consistent with the surface expression of IgM, PCR analysis revealed D_H-J_H and V_H-D_H rearrangements on the *Igh* gene locus as well as V_κ-J_κ and V_λ-J_λ rearrangements of the immunoglobulin light chain (*Igl*) locus in CD19⁺IgM⁺

cells in the spleens of mice reconstituted with Lck triple-knockout DP cells, although they seemed to be oligoclonal. The converted B cells derived from Lck triple-knockout DP cells also carried V-DJ rearranged *Tcrβ* chain genes, confirming the T-cell origin of the cells (Fig. 3A).

To further confirm the conversion of T-lineage cells to B cells, we examined the T-cell- and B-cell-specific V-DJ rearrangements at the single-cell level by using the seminested PCR technique in individual B cells from the spleens of mice reconstituted with Lck triple-knockout DP cells (Nechanitzky et al. 2013). We detected both V_β and V_H rearrangements in 14%–40% of the individual converted B cells (Fig. 3B; Table 1). These data demonstrate that the conversion event stably takes place at a certain frequency. Whereas one could argue that such oligoclonality is reminiscent of preleukemic clones, this is unlikely because the converted cells never developed into leukemia in recipient mice even several months after transplantation. Furthermore, the low rearrangement frequency of both V_β and V_H loci was also shown previously in the case of the conversion of B cells to T-lineage cells of *Pax5* knockout and *Ebf1* knockout mice (Cobaleda et al. 2007; Nechanitzky et al. 2013).

Association of *Ring1B* with the promoter regions of B-lineage-associated genes in thymocytes

Previous studies have suggested that the PcG proteins regulate differentiation and maintenance of HSCs by controlling the expression of various target genes (Oguro et al. 2010; Aloia et al. 2013; Xie et al. 2014). Of note, the PRC1 component Bmi-1 is implicated in the repression of transcription factors essential for the generation of B-lineage cells, such as *Ebf1* and *Pax5* (Oguro et al. 2010).

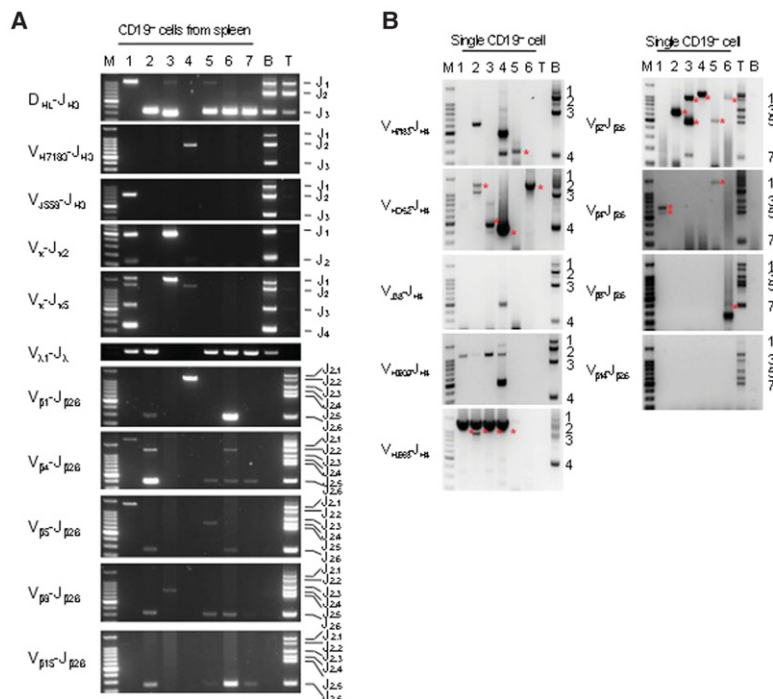


Figure 3. Rearrangements of *Igh* and *Tcrβ* chain genes in B cells derived from DP cells in the thymi of Lck triple-knockout mice. (A) Analysis of *Igh* V(D)J, *Igl* V_κ-J_κ and V_λ-J_λ, and *Tcrβ* V(D)J gene rearrangement in converted B cells derived from thymic DP cells of triple-knockout mice. BM CD19⁺ cells and DP thymocytes from normal B6 mice were used as controls. (B) Seminested single-cell PCR analysis to detect T-cell- and B-cell-specific rearrangements of *Igh* and *Tcrβ* loci in the individual converted B cells in the spleen. Asterisks indicate rearrangements detected. Data are shown from three independent experiments.

Table 1. Quantification of V(D)J rearrangement of *Igh* and *Tcrb* loci in individual B cells converted from DP cells in the thymi of *Cdkn2a Ring1a/b* triple-knockout mice

	V _H (D)J _H rearrangement	V _β (D)J _β rearrangement	Both rearrangements
Experiment 1	75.0%	30.2%	24.0%
Experiment 2	96.9%	41.7%	40.6%
Experiment 3	85.4%	17.7%	14.6%

Single-cell PCR analysis of *Igh* and *Tcrβ* gene rearrangement. The percentages of V_H(D)J_H and V_β(D)J_β rearrangements detected in individual CD3⁺CD19⁺IgM⁺ cells in the spleens of mice transplanted with CD4⁺CD8⁺ cells in the thymi of *Cdkn2a Ring1a/b* triple-knockout mice are shown. Data are shown as three independent experiments.

To examine Ring1B occupancy of B-lineage gene loci in normal T-lineage cells, chromatin immunoprecipitation (ChIP)-on-chip analysis of thymocytes was performed. The results revealed the binding of Ring1B to B-lineage-associated genes such as *Pax5*, *Ebf1*, *Irf4*, and *Irf8* as well as H3K27me₃, a hallmark of polycomb-repressive activity, in the same regions (Fig. 4A). The binding of Ring1B and H3K27me₃ mark was not observed in the promoter regions of *Gata3* or *Bcl11b*. We confirmed that binding of Ring1B and H3K27me₃ marks was at the promoter regions of *Pax5* and *Ebf1* but not *Bcl11b* loci by ChIP analysis in normal T cells (Fig. 4B). To further confirm the Ring1B occupancy at the promoter regions of B-lineage loci in T cells, we performed ChIP-PCR analysis in DN3 and DP cells sorted from normal thymocytes. The substantial binding of Ring1B at the promoter regions of *Pax5* and *Ebf1* loci was observed in DN3 cells, whereas the binding of Ring1B was dramatically reduced in DP cells (Fig. 4C). These results indicate that Ring1A/B binds directly to B-lineage-associated gene loci to repress ectopic expression of these genes during T-cell development.

Deletion of PAX5 restores T-cell development in *Ring1A/B*-deficient mice

To determine whether the main role of Ring1A/B is to repress *Pax5* and the related B-lineage-associated gene programs, we investigated whether deletion of *Pax5* would restore the development of T cells in the thymi of *Ring1A/B*-deficient mice. We bred *LckCre-Ring1a^{-/-}Ring1b^{fl/fl}* mice with *Pax5^{fl/fl}* mice to generate T-cell-specific *Ring1a/b Pax5* triple-knockout mice. Notably, CD4⁺CD8⁺DP, CD4SP, and CD8SP cells were normally generated in the thymi of PAX5 triple-knockout mice. The proportions of TCRβ⁺ and TCRγδ⁺ cells were restored by the deletion of *Pax5*. The total number of thymocytes in PAX5 triple-knockout mice was also recovered to control levels (Fig. 5A,B; Supplemental Fig. S6). Similarly, The CD4⁺ T cells and CD8⁺ T cells were also normally generated in the spleens of PAX5 triple-knockout mice (Fig. 5C). These data indicated that deletion of *Pax5* restored the differentiation potential of *Ring1a/b*-deficient T-cell progenitors. Thus, PAX5 is one of the critical targets of PcG proteins during early T-cell development.

Discussion

In this study, we dissected the role of PcG proteins in the maintenance of T-cell fate by using the T-cell-specific *Ring1a/b*-deficient mice. We demonstrated that the *Pax5* locus is one of the main targets of PcG proteins to suppress the B-cell potential during early T-cell development. The maintenance of T-cell fate by PcG proteins continued throughout the thymocyte development. However, the deletion of *Ring1a/b* driven by the *Cre* transgene under the control of the *Cd4* promoter did not show any

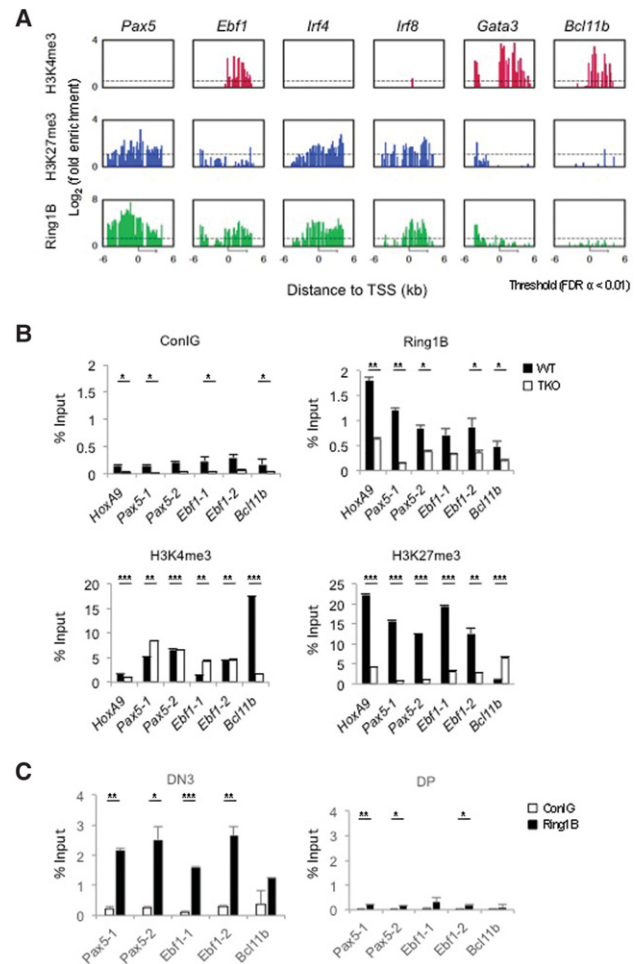


Figure 4. Association of Ring1B with the promoter regions of B-lineage-associated genes in thymocytes. (A) ChIP-on-chip analysis of total thymocytes from wild-type mice showing the H3K4me₃, H3K27me₃, and RING1B binding to the promoter regions (from -6 kb to +6 kb relative to the transcriptional start sites [TSS]) of the indicated B-lineage- or T-lineage-associated genes. (B) ChIP analysis showing binding of H3K4me₃, H3K27me₃, and Ring1B at the promoter regions of *Pax5*, *Ebf1*, and *Bcl11b* and the PcG target *Hoxa9* gene in T-lineage cells in the thymi of wild-type (WT) and Lck triple-knockout (TKO) mice. (C) ChIP analysis showing binding of Ring1B at the promoter regions of *Pax5*, *Ebf1*, and *Bcl11b* in DN3 and DP cells in the thymi of wild-type mice. Data are representative of at least three independent experiments in B and C. Mean \pm SD. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$, Student's *t*-test.

apparent defects of T cells in the spleen, indicating the critical role of PcG proteins in preventing the T-cell progenitors from choosing B-cell fate in the thymus.

The scenario of T- and B-cell lineage determination and maintenance is proposed as follows (Fig. 6). At the level of uncommitted progenitors, PcG proteins repress expression of critical B-cell lineage transcription factors such as PAX5. During the B-cell lineage determination process, *Pax5* is up-regulated by being released from PcG-mediated repression. On the other hand, even after the T-lineage determination step, which is initiated at the DN1 stage by Notch signaling and completed during the DN2 stage by *Bcl11b* up-regulation (Ikawa et al. 2010; Li et al. 2010a, b), PcG-mediated repression of *Pax5* is indispensable for maintaining the T-cell lineage. When this repression is abolished, *Pax5* is up-regulated in T-cell progenitors, and such cells can eventually be converted into B cells. Since constitutive activation of *Pax5* under control of the *Ikaros* locus was not able to induce conversion of T cells to B cells (Souabni et al. 2002), sustained epigenetic suppression of B-lineage-associated genes other than *Pax5*, such

as *Ebf1* and *Blnk*, is also required for the normal T-lineage program. The transcription factor *Bcl11b* is shown to be essential for T-cell versus NK cell fate determination (Ikawa et al. 2010; Li et al. 2010a,b). Of note, the expression of *Bcl11b* did not change in *Ring1a/b* knockout T-cell progenitors (Fig. 1F), suggesting that the PcG proteins do not directly regulate the expression of *Bcl11b* in the maintenance of T-cell fate. This may explain why *Ring1a/b*-deficient T-cell progenitors did not take the NK cell fate. During this transition phase, the myeloid lineage program is not activated, probably because both Notch signal and PAX5 activity suppress the myeloid program (Nutt et al. 1999; Busslinger 2004; Rothenberg et al. 2008).

Previous studies indicated that Ring1B is critical for the maintenance and differentiation of HSCs. Conditional inactivation of *Ring1b* in hematopoietic cells promotes myeloid lineage development at the expense of B-cell differentiation (Cales et al. 2008). The additional deletion of the *p16^{Ink4a}* locus rescued the defective proliferation of B-cell progenitors in the BM of *Ring1b*-deficient mice, suggesting an important role of *Ring1b* in repressing the expression of *p16^{Ink4a}* for the control of proliferation and differentiation of the hematopoietic progenitors. In the present study, we observed severe reduction of T cells in the thymi of *LckCre-Ring1a/b* double-knockout mice. However, the T-cell differentiation was not recovered by the additional inactivation of *Cdkn2a*, which encodes *p16^{Ink4a}* and *p19^{arf}*. Moreover, the T-cell progenitors of *Ring1a/b Cdkn2a* triple-knockout mice reprogrammed to B-lineage cells in vivo. This might reflect the functional redundancy between Ring1A and Ring1B, although it is reported that the inactivation of *Ring1a*, a paralog of *Ring1b* in mice, results in no overt phenotypes (del Mar Lorente et al. 2000). This could also be due to the functional differences of Ring1B among the cell types and the differentiation stages of the cells. Indeed, the *Ring1b*-deficient myeloid cells exhibit higher proliferative potential than normal myeloid cells (Cales et al. 2008). Of note, proteomic analysis has recently identified the functionally distinct PRC1 complexes (Gao et al. 2012). All PRC1 complexes contain Ring1A/B but can be classified into six groups based on which PcG RING fingers (PCGFs) are involved. Biochemical and genomic analysis revealed the functional difference among the PRC1 complexes. PCGFs may play a key role in controlling the cell fate decision in differentiating cells. Further analysis will be needed to determine the exact role of *Ring1a* and *Ring1b* among PRC1 complexes in regulating the determination of cell fates.

Our present results provide the first example that the inactivation of a single epigenetic machinery results in a drastic cell fate conversion, providing new insight into the mechanisms of lineage determination. We propose that the determined status is very asymmetric between T lineage and B lineage with regard to its dependency on transcriptional networks versus epigenetic regulation. The B-cell lineage is primarily maintained by B-lineage transcription factors, while maintenance of the T lineage requires epigenetic suppression of B-lineage transcription

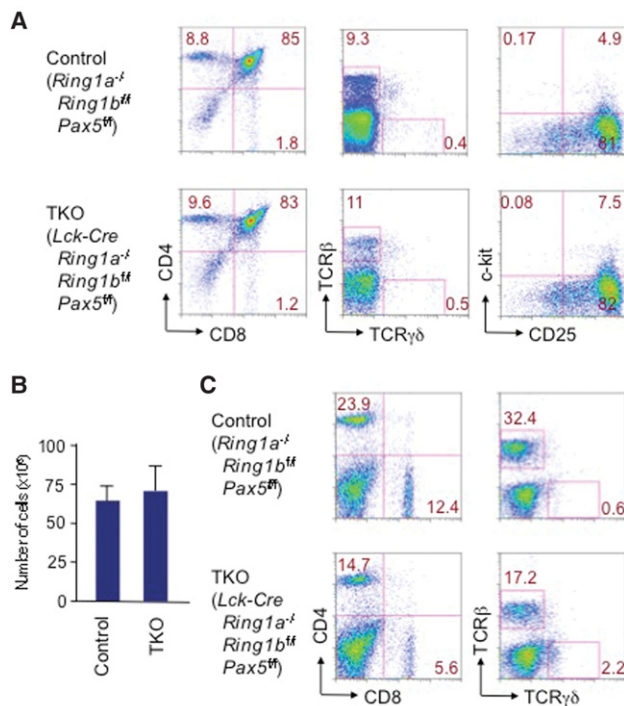


Figure 5. Deletion of *Pax5* restores T-cell development in the thymi and spleens of Ring1A/B double-knockout mice. (A) Flow cytometric analysis of cells from the thymi of *LckCre(-)* (control) or *LckCre(+)* (triple-knockout [TKO]) *Ring1A^{-/-} Ring1B^{fl/fl} Pax5^{fl/fl}* mice. Thymocytes gated on *Lin⁻* cells were analyzed for the expression of CD4 versus CD8, TCRβ versus TCRγδ, and c-kit versus CD25. (B) The number of thymocytes in control or triple-knockout mice. (C) Flow cytometric analysis of cells from the spleens of *LckCre(-)* (control) or *LckCre(+)* (triple-knockout) *Ring1A^{-/-} Ring1B^{fl/fl} Pax5^{fl/fl}* mice. Splenocytes were analyzed for the expression of CD4 versus CD8 and TCRβ versus TCRγδ. Data are representative of at least three independent experiments in A–C. Mean ± SD in B.

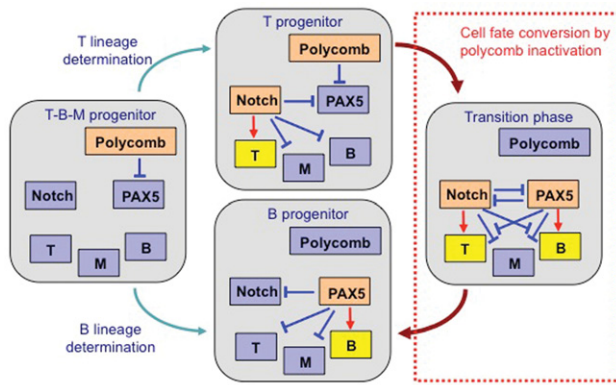


Figure 6. Model of T or B lymphoid lineage commitment regulated by PcG proteins. The predicted function of PcG proteins during T- and B-cell development from T/B/M multipotent progenitors is shown. The orange and blue boxes reflect the activity of each genetic program. For example, orange represents the activated gene program, whereas blue represents the repressed gene program in each progenitor state. Yellow represents the activated cell status in T-cell progenitors, B-cell progenitors, and a transition phase.

factors. Based on such findings, it appears that the B-cell lineage and not the T-cell lineage is the default pathway in lymphocyte development. Thus, one can conceive that the T-cell lineage was established during evolution by suppressing a pre-existing B-cell program. Alternatively, it can also be speculated that the B-cell lineage was introduced rather later during evolution by recruiting very strong transcription factors and that the T-cell lineage had to suppress them to protect its own developmental program. Although so far it is difficult to tell which is the case, the present findings may provide some clue as to how cell lineages were established during evolution.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from CLEA Japan, Inc. *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}*, *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}Pax5^{fl/fl}*, *Ert2Cre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}*, *Cd4Cre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}*, and Ring1B-YFP mice were generated and maintained in our animal facility. Mice were backcrossed to B6 mice at least six times. NOD/Shi-*scid*, IL2Ry^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals. Six-week-old to 8-wk-old female mice were used for the transfer experiments.

Antibodies

The following antibodies were purchased from BD Biosciences: fluorescein isothiocyanate (FITC)-conjugated erythroid lineage cells (TER119; 561032), MAC1 (553310), GR1 (553127), CD11C (557400), B220 (553088), THY1.2 (553004), CD8A (553031), CD4 (553651), NK1.1 (553164), CD3ε (553062), CD19 (553785), TCRγδ (553177), phycoerythrin (PE)-conjugated SCA-1 (553336), CD4 (553653), CD19 (553786), GR-1 (553128), NK1.1 (553165), TCRβ (553172), allophycocyanin (APC)-conjugated LY5.1

(558701), LY5.2 (558702), C-KIT (553556), and CD19 (550992). FITC-IGM (11-5790-81) was purchased from eBioscience.

Growth factors

Recombinant murine (rm) SCF, IL-7, and Flt3-ligand (L) were purchased from R&D.

Isolation of thymic Lin⁻ cells

Single-cell suspensions of thymocytes were prepared from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* and control mice. Cells were then incubated with mAbs specific for Lin markers (TER119, Mac-1, Gr-1, B220, CD3, CD4, and CD8) for 20 min on ice. Lin⁺ cells were depleted with Dynabeads sheep anti-rat IgG (Invitrogen) according to the manufacturer's protocol. The Lin⁻ cells were used for subsequent experiments.

In vitro deletion of Ring1b in sorted DN3 cells

Single-cell suspensions of the thymi from *Ert2Cre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* mice were prepared. Lin⁻ cells were purified using Dynabeads sheep anti-rat IgG as described above. DN3 cells were sorted as Lin⁻ ckit⁻ CD25⁺ cells after staining with FITC-Lin, PE-CD25, and APC-ckit antibodies. The purified DN3 cells were subsequently incubated on TSt-4/DLL1 cells for 72 h in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mg/mL sodium bicarbonate, 0.1 mM nonessential amino acid solution (GIBCO-BRL), 5 × 10⁻⁵ M 2-ME, 100 mg/mL streptomycin, 100 U/mL penicillin, 1 μM 4-OHT, 10 ng/mL SCF, 10 ng/mL IL-7, and 10 ng/mL Flt3-L. The cultured cells were stained with anti-CD45 antibody, and CD45⁺ cells were sorted. RNA was extracted from the sorted cells, and the RNA levels were measured by qRT-PCR.

In vitro B-cell culture

CD19⁺ B cells were sorted from the splenocytes of wild-type and NOG mice transplanted with DP cells from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* mice. Collected cells were labeled with 5 μM CFSE (Nacalai Tesque) for 20 min at 37°C. Labeled cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mg/mL sodium bicarbonate, 0.1 mM nonessential amino acid solution (GIBCO-BRL), 5 × 10⁻⁵ M 2-ME, 100 mg/mL streptomycin, and 100 U/mL penicillin. LPS (Sigma-Aldrich) and IL-4 (R&D Systems) were also added as indicated. After 96 h of culture, cell division was measured by FACS, and IgM secretion was measured by ELISA as described previously (Kometsani et al. 2011).

ELISA

Ninety-six-well flat-bottomed plates were coated with 2 μg/mL anti-mouse IgM for 1 h at room temperature followed by blocking with 0.5% BSA in PBS for 1 h at room temperature. Serially diluted samples were incubated for 1 h at room temperature. After washing with PBS/0.5% Tween-20, horseradish peroxidase-conjugated anti-mouse IgM antibodies (SouthernBiotech) were used as the substrate, and absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories).

Adoptive transfer of DN3 or DP cells

DN3 or DP cells (1×10^6 cells) from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* mice were intravenously injected into the tail veins of sublethally irradiated (240 rad) NOG mice. Mice were analyzed 4–6 wk after reconstitution for donor chimerism in the BM and spleen.

PCR analysis of Igh and Tcrβ gene rearrangement

The analysis of *Igh* and *Tcrβ* gene rearrangement was performed as described previously (Ikawa et al. 2004). In brief, genomic DNA was prepared from B cells derived from the spleens and BM in mice transplanted with thymic DN3 or DP cells from *LckCre-Cdkn2a^{-/-}Ring1a^{-/-}Ring1b^{fl/fl}* and control mice using the DNeasy tissue kit (Qiagen). The reaction volume was 20 μ L, containing 2 μ L of genomic DNA (approximately equivalent to 10^4 cells), 2 μ L of 10 \times PCR buffer, 0.16 μ L of 25 mM dNTP, 4 pmol of each primer, and 0.6 U of Taq polymerase (GE healthcare). The PCR reactions were performed as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and finally 10 min at 72°C. Amplified DNA products were analyzed on an agarose gel followed by ethidium bromide staining.

Single-cell-based PCR analysis of Igh and Tcrβ gene rearrangement

The analysis was performed as described previously (Nechanitzky et al. 2013). Briefly, single converted B cells in the spleens of NOG mice derived from DP cells in the thymi of *Lck* triple-knockout mice were directly sorted into one well of a 96-well plate containing 20 μ L of PCR buffer (Takara) supplemented with 250 μ g/mL proteinase K. Single cells were digested by incubation for 60 min at 56°C followed by inactivation of proteinase K for 15 min at 95°C. The first round of PCR was done by the addition of 30 μ L of PCR mix containing dNTPs, PCR buffer, and rTaq (all from Takara) as well as primers for detecting V(D)J recombination of *IgH* and *TCRβ* gene loci (Supplemental Table S3). An aliquot of 1 μ L of the product of the first round of PCR and the nested primers (JH4 and J β 2.7) in combination with one of the V_H or V_β primers was used for the second round of PCR. Amplified DNA products were analyzed on an agarose gel followed by ethidium bromide staining.

RNA extraction and qRT-PCR

Total RNA was isolated using an RNeasy kit (Qiagen). cDNA synthesis was performed using a SuperScript VILO cDNA synthesis kit (Invitrogen) following the manufacturer's protocol. Real-time PCR was performed using SYBR Premix EX Taq (Takara) and analyzed by StepOnePlus (Applied Biosystems). The reactions were performed in duplicate for 10 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 30 sec at 55°C. The primer sequences used are in Supplemental Table S1.

Microarray analysis

RNA extraction was performed as described above. The expression profiles were analyzed using a Quant-iT RiboGreen RNA assay kit (Invitrogen). The quality of the RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent). The fluorescence intensities were detected using the Scan-Array Lite scanner (Perkin-Elmer). The PMT levels were adjusted to achieve 0.1%–0.5% pixel saturation. Each TIFF image was analyzed using Gene Pix

Pro version 6.0 software (Molecular Devices). The data were filtered to remove low-confidence measurements and globally normalized per array such that the median of the signal intensity was adjusted to 50 after normalization (accession no. GSE53650).

ChIP analysis

ChIP assays were performed as described (Ikawa et al. 2006; Endoh et al. 2008). Immunoprecipitated and input DNA were quantified by real-time PCR with the primers in Supplemental Table S2.

ChIP-on-chip analysis

ChIP-on-chip analysis was carried out using the mouse promoter ChIP-on-chip microarray set (Agilent Technologies, G4490A) as described previously (Endoh et al. 2008). Thymocytes of normal B6 mice were subjected to ChIP assay using anti-H3K4me3, anti-H3K27me3, and anti-Ring1B antibodies. Purified immunoprecipitated and input DNA were subjected to blunt ligation with linker oligo DNA, linker-mediated PCR (LM-PCR), labeling, hybridization, and washing following the Agilent mammalian ChIP-on-chip protocol. Scanned images were quantified with Agilent Feature Extraction software under standard conditions.

Assignment of regions bound by H3K4me3, H3K27me3, and Ring1B around transcription start sites was carried out using direct sequence alignment on the mouse genome database (NCBI version 36). The location of bound regions of each protein was compared with a set of transcripts derived from the Mouse Genome Informatics database. We assigned bound regions that were within –6 kb to +6 kb of the transcription start site. Alignments on the mouse genome and transcription start sites of genes were retrieved from Ensembl (<http://www.ensembl.org>).

The measured intensity ratios (immunoprecipitate/input: fold enrichment) were calculated, and the maximum value of the ratios in each promoter region (–6 kb to +6 kb around the transcriptional start site) of a gene was used to represent the fold enrichment of the gene. Fold enrichment was calculated only for probes whose signals from both immunoprecipitated and input DNAs were significant ($P < 10^{-3}$) (accession no. GSE53650).

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