Forced expression of the histone demethylase Fbxl10 maintains self-renewing hematopoietic stem cells

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Abstract

Objective. The methylation status of histones changes dramatically depending on cellular context and defines cell type-specific gene expression profiles. Histone demethylases have recently been implicated in this process. However, it is unknown how histone demethylases function in the maintenance of self-renewing hematopoietic stem cells (HSCs).

Materials and Methods. We profiled the expression of histone demethylase genes in mouse hematopoietic cells and listed genes preferentially expressed in HSCs. We analyzed the impact of a selected gene by transducing CD34⁺c-Kit⁺Sca-1⁺Lineage marker⁻ (CD34⁺KSL) HSCs using retroviral system followed by *in vitro* methylcellulose colony assays and *in vivo* competitive repopulation assays.

Results. We found that *F-box and leucine-rich repeat protein 10* (*Fbxl10*, also known as *Jhdm1b* or *Kdm2b*), is highly expressed in CD34⁺KSL HSCs. *Fbxl10* encodes a demethylase specific to the histone H3 mono/di-methylated at lysine 36 (H3K36me1/me2) and forms complexes with polycomb-group (PcG) proteins as essential regulators of HSCs. Forced expression of *Fbxl10* in HSCs expanded numbers of colony-forming cells with multi-lineage differentiation potential in culture and prevented exhaustion of the long-term repopulating potential of HSCs following serial transplantation. *Fbxl10* tightly repressed the expression of cyclin-dependent kinase inhibitor genes, including *Ink4a*, *Ink4b*, and *Ink4c*, through direct binding to their promoters and gene bodies and demethylation at H3K36. Increased levels of mono-ubiquitylation of H2A at target loci also suggested the collaboration of *Fbxl10* with PcG proteins.

Conclusion. Our findings implicate *Fbxl10* in the maintenance of self-renewal capacity of HSCs, thus highlight a role of histone demethylation for the first time in the epigenetic regulation of HSCs.

Introduction

Hematopoietic stem cells (HSCs) are primitive cells capable of both self-renewal and differentiating into all of the mature blood cell lineages. The capacity to self-renew is an essential feature of all stem cells, generating at least one daughter cell that has stem cell properties similar to the parental cell. Thus, the ability to self-renew is critical to maintaining the HSC pool size, and thereby keeps a supply of mature blood cells throughout the lifetime of organisms. The self-renewal process is regulated by both intrinsic and extrinsic signals [1-3]. Although great progress has been made in identifying the many signaling pathways and transcription factors involved in HSC regulation, recent findings also uncovered epigenetic machinery, such as polycomb-group (PcG) proteins, as major regulators of HSCs [4, 5].

Post-translational covalent modifications of histone N-terminal tails include methylation, acetylation, ubiquitylation, sumoylation, and phosphorylation, and are central to the epigenetic regulation of transcription, replication, and repair [6]. Histone acetylation marks are generally associated with transcriptional activation, whereas histone methylation marks contribute to transcriptional activation or repression depending upon the types and sites of modified residues and the degree of methylation. For example, tri-methylation of histone H3 at lysine 4 (H3K4me3), lysine 36 (H3K36me3), and lysine 79 (H3K79me3) is closely associated with transcriptional activation, whereas tri-methylation of H3 at lysine 9 (H3K9me3), H3 at lysine 27 (H3K27me3), and H4 at lysine 20 (H4K20me3) is linked to transcriptional repression. The role of histone methylation marks in stem cells is best known for embryonic stem (ES) cells. Comprehensive genome-wide analyses have demonstrated that ES cells establish 'bivalent domains' that contain coexisting active (H3K4me3) and repressive (H3K27me3) histone methylation marks at promoters of developmental regulator

genes to keep these genes “poised” for activation in ES cells. Upon differentiation, promoters with bivalent domains are resolved into a monovalent state, either active or repressive [7]. Recent findings have revealed that bivalent domains also exist in HSCs [8, 9]. Actually, our recent analysis showed that the B cell lineage regulator genes are transcriptionally repressed by bivalent domains before lineage commitment in HSCs [10]. Specific patterns of histone methylation marks at promoters of target genes thus play a key role in cell fate decision.

Although the methylation of histones has been traditionally considered a static modification, the identification of a large number of histone demethylases strongly suggests a dynamic nature of histone methylation. Histone demethylases are divided into two families. The first to be described was the amine oxidase family, lysine-specific demethylase 1 (LSD1), a demethylase specific to H3K4me2/me1 [11]. Thereafter, jumonji C (JmjC) domain-containing proteins were identified [12]. The majority of histone demethylases belong to the JmjC domain-containing family. Histone demethylases have been implicated in diverse cellular functions through the regulation of gene expression [13, 14]. In adults, JMJD3 (jumonji domain containing 3), a H3K27me2/3-specific demethylase, and LSD1 play a crucial role in differentiation of neural and epidermal stem cells [15, 16] and proliferation of neural stem cells [17], respectively. However, it is unknown how histone demethylases function in the maintenance of self-renewing HSCs.

F-box and leucine-rich repeat protein 10 (Fbxl10), also known as Jhdm1b or Kdm2b, belongs to the JmjC domain-containing histone demethylases, and contains an N-terminal JmjC domain, a CXXC zinc finger domain, a PHD finger, an F-box, and eight leucine-rich repeats [12, 18, 19]. Fbxl10 demethylates H3K36me2 and H3K36me1, but not H3K36me3 [12, 20, 21]. The effect of Fbxl10 in cultured cells remains controversial. Fbxl10 promotes cell proliferation and functions as a physiological inhibitor of senescence in mouse

embryonic fibroblasts (MEFs) [20-22] but overexpression of Fbxl10 inhibits the proliferation of HeLa cells [19, 23]. Furthermore, the roles of Fbxl10 in the context of whole organisms have not been characterized.

Studies in *Drosophila* have demonstrated that dKDM2 (a homolog of the mammalian FBXL10) forms a multiprotein complex called dRAF (dRing-associated factors), which contains dRing (a homolog of the mammalian RING proteins) and Psc (a homolog of the mammalian BMI1 protein) [24]. dKDM2 enhances *homeotic* gene silencing by PcG complexes, suggesting that the dRAF and polycomb repressive complex (PRC) 1 cooperate *in vivo*. dKDM2 also stimulates mono-ubiquitylation of histone H2A (H2Aub1) by dRing and Psc in a manner independent of dKDM2 catalytic activity [24]. Thus, the dRAF complex can coordinate the removal of an active mark, H3K36me2, with addition of a repressive mark, H2Aub1, although neither of these two activities appears to depend on the other. In mammals, Fbxl10 is included in the BCOR (BCL6 corepressor) complex, which also contains Ring1A/B, Bmi1, and/or nervous system polycomb 1 (Nspc1; a paralog of Bmi1) [25, 26]. Ring1A/B and Bmi1 are core components of PRC1 and play a crucial role in the maintenance of HSCs. All these findings suggest functional crosstalk between Fbxl10 and PRC1 proteins in HSCs.

To explore the relevance of histone demethylases in HSCs, we profiled their gene expression and found that *Fbxl10* is highly expressed in HSCs. In the present study, we investigated the role of Fbxl10 in HSCs by conducting a gain-of-function analysis and demonstrated a novel function in maintaining the self-renewal capacity of HSCs.

Materials and methods

Mice

C57BL/6 (B6-CD45.2) mice were purchased from Japan SLC. C57BL/6 mice congenic for the Ly5 locus (B6-CD45.1) were purchased from Sankyo Lab Service. All mice were bred and maintained in the Animal Research Facility of the Graduate School of Medicine, Chiba University in accordance with institutional guidelines. All experiments using mice received approval from the Chiba University Administrative Panel for Animal care.

Microarray analysis

Each hematopoietic cell fraction was purified by cell sorting from bone marrow (BM) of 8-week-old C57BL/6 mice. Total RNA was extracted using an ISOGEN reagent (Nippon Gene) and used to generate Biotin-labeled cRNA. Preparation of cRNA, hybridization, and scanning of the oligonucleotide microarrays (GeneChip Mouse genome 430 2.0 arrays; Affymetrix) were performed according to the manufacturer's instructions as reported previously [27]. Microarray data were submitted to Gene Expression Omnibus under accession number GSE27787.

Quantitative real-time PCR analysis

Total RNA was isolated using TRIZOL LS solution (Invitrogen) and reverse transcribed by the ThermoScript RT-PCR system (Invitrogen) with a random hexamer primer. Real-time quantitative PCR was performed with an ABI prism 7300 Thermal Cycler (Applied Biosystems) using FastStart Universal Probe Master (Roche). *Hypoxanthine-guanosine phosphoribosyl transferase 1 (Hprt1)* expression was used to calculate relative expression levels. The combination of primer sequences and universal Probe Library (Roche) were

designed by the Universal ProbeLibrary assay design center (<https://www.roche-applied-science.com>) and are listed in Supplemental data (Supplementary Table 1).

Purification of mouse HSCs

Mouse HSCs (CD34⁺KSL cells) were purified from BM of 8-week-old mice. Mononuclear cells were isolated on Ficoll-Paque PLUS (GE Health care). The cells were stained with an antibody cocktail consisting of biotinylated anti-Gr-1, Mac-1, IL-7R α , B220, CD4, CD8, and Ter119 monoclonal antibodies. Lineage-positive cells were depleted with goat anti-rat IgG microbeads (Miltenyi Biotec) through an LS column (Miltenyi Biotec). The cells were further stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34, phycoerythrin (PE)-conjugated anti-Sca-1, and allophycocyanin (APC)-conjugated anti-c-Kit antibodies. Biotinylated antibodies were detected with APC-Cy7-conjugated streptavidin. Analysis and sorting were performed on a FACS Aria II (BD bioscience). Antibodies used are listed in Supplemental data (Supplementary Table 2). Other hematopoietic cell fractions used in microarray and quantitative real-time PCR analyses are described in Supplemental data (Supplementary Table 3).

Retroviral vectors and virus production

Full-length mouse *Fbxl10* cDNA tagged with myc at the N-terminus was subcloned into the MIGR1 retroviral expression vector, which contained long-terminal repeats (LTRs) of murine stem cell virus (MSCV) and an internal ribosomal entry site (IRES) upstream of the enhanced green fluorescent protein (GFP) as a marker gene. For chromatin immunoprecipitation (ChIP) assays, mouse *Fbxl10* cDNA tagged with a 3 $\times$ Flag in the retroviral vector pGCDNsam was used. A recombinant vesicular stomatitis virus

glycoprotein (VSV-G)-pseudotyped high-titer retrovirus was generated by a 293gpg packaging cell line [28]. The virus in supernatants of 293gpg cells was concentrated by centrifugation at 6,000g for 16 hours.

Transduction of CD34⁻KSL HSCs

CD34⁻KSL HSCs were transduced with the indicated retrovirus as previously described with minor modifications [29]. CD34⁻KSL HSCs were sorted into 96-well micro-titer plates coated with the recombinant human fibronectin fragment CH-296 (RetroNectin; Takara Shuzo) at 100 cells per well and incubated in α -Minimal essential medium (α -MEM) supplemented with 1% fetal bovine serum (FBS), 1% L-glutamine, penicillin, streptomycin solution (GPS; Sigma), 50 μ M 2-mercaptoethanol (2-ME), 100 ng/ml mouse stem cell factor (SCF; PeproTech), and 100 ng/ml human thrombopoietin (TPO; PeproTech) for 24 hours. Then, cells were transduced with the indicated retrovirus at a multiplicity of infection (MOI) of 1,500 in the presence of 1 μ g/ml RetroNectin and 10 μ g/ml protamine sulfate (Sigma) for 24 hours. After transduction, cells were further incubated in S-Clone SF-O3 (Sanko Junyaku) supplemented with 0.1% bovine serum albumin (BSA), 50 μ M 2-ME, 1% GPS, 50 ng/ml SCF, and 50 ng/ml TPO and subjected to assays. CD34⁺KSL progenitor cells were transduced and cultured similarly with the exception that 100 ng/ml chimeric protein of soluble IL-6 receptor α and IL-6 (FP6; kindly provided by Kyowa Hakko Kirin) was added.

Colony-forming assay

Colony assays were performed in methylcellulose-containing Iscove's modified Dulbecco's medium (Methocult M3234; Stem Cell Technologies) supplemented with 20 ng/ml mouse SCF, 20 ng/ml mouse interleukin-3 (IL-3), 20 ng/ml human TPO, and 2 units/ml human

erythropoietin (EPO), and incubated at 37°C in a 5% CO₂ atmosphere, as previously described [29-31]. The number of high proliferative potential (HPP)- and low proliferative (LPP)- colony-forming cells (CFCs), which generate a colony with a diameter greater than and less than 1mm, respectively, were evaluated by counting colonies at day 14 of culture. Because colonies with multi-lineage differentiation potential [colony-forming units-nmEM (CFU-nmEM)] are mainly derived from HPP-CFCs [32], GFP⁺ colonies derived from HPP-CFCs were individually collected, cytopun onto glass slides, and subjected to May-Grünwald Giemsa staining for morphological examination.

Western blot analysis for histone tail methylation

GFP⁺ Lineage marker⁻ (Lin⁻) c-Kit⁺ stem/progenitor cells were lysed in 100 mM Tris (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 0.65% NP-40, and protease inhibitor cocktail (PIC; Roche). Nuclei pelleted from the lysate were extracted with 0.2 N HCl, and acid-soluble histones were precipitated with 20% trichloroacetic acid. Histones were separated by SDS-PAGE and detected by Western blotting using the antibodies listed in Supplemental data (Supplementary Table 4).

ChIP assay

GFP⁺ Lin⁻ c-Kit⁺ stem/progenitor cells purified from transduced CD34⁺KSL HSCs at day 14 of culture were crosslinked with 1% formaldehyde for 15 minutes at room temperature, washed 3 times with PBS, and resuspended with swelling buffer [20 mM Hepes (pH 7.8), 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.1% NP-40, and PIC] for 10 minutes on ice. Nuclei were then lysed with RIPA buffer [10 mM Tris (pH 8.0), 140 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.1% Sodium deoxycholate (DOC), and PIC] and sonicated for 20 minutes using a Bioruptor (Cosmo Bio). The average chromatin fragment

size after sonication was approximately 500 bp. After centrifugation, the soluble chromatin fraction was recovered and precleared for 1 hour at 4°C with a mixture of Protein A and G-conjugated Dynabeads (Invitrogen) blocked with BSA and salmon sperm DNA. Chromatin was immunoprecipitated overnight at 4°C with antibody-conjugated Dynabeads. The immunoprecipitates were extensively washed with the following combination of wash buffers; RIPA buffer, high salt RIPA buffer [10 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.1% DOC, and PIC], LiCl wash buffer [10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% NP40, and 0.5% DOC], and TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. Bound chromatin was eluted and, together with input DNA, placed in elution buffer [10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 300 mM NaCl, and 0.5% SDS] to reverse crosslinking. Immunoprecipitated DNA and input DNA were treated with RNase A (Sigma) and proteinase K (Roche), and extracted with phenol:chloroform. Quantitative PCR was performed with an ABI prism 7300 Thermal Cycler using SYBR Premix Ex Taq II (Takara Bio). The primer sequences and antibodies used are listed in Supplemental data (Supplementary Table 4,5). For ChIP analysis of H2Aub1, anti-H2Aub1 antibody, anti-mouse IgM, and Protein G-conjugated Dynabeads were incubated to make anti-H2Aub1-conjugated Dynabeads. Anti-mouse IgG antibody was used as an isotype control.

Competitive repopulation assay

CD34⁺KSL HSCs (CD45.2) transduced with the indicated retrovirus were transplanted after a 10-day-culture intravenously into 8-week-old B6-CD45.1 mice irradiated at a dose of 9.5 Gy together with 2×10^5 BM competitor cells from 8-week-old B6-CD45.1 mice. For secondary transplantation, BM cells were collected from all recipient mice between 12 and 16 weeks after primary transplantation and pooled together. Then, 2×10^6 BM cells were

transplanted into 8-week-old B6-CD45.1 mice irradiated at a dose of 9.5 Gy without competitor cells. Tertiary transplantation was similarly performed using 5×10^6 BM cells. Peripheral blood cells of the recipient mice were analyzed with a mixture of antibodies that included PE-Cy7-conjugated anti-CD45.1, Pacific Blue-conjugated anti-CD45.2, PE-conjugated anti-Mac-1 and anti-Gr-1, APC-conjugated anti-B220, and APC-Cy7-conjugated anti-CD4 and anti-CD8 antibodies. Cells were analyzed on a FACS Canto II (BD bioscience). Percent donor chimerism was calculated as (percent donor cells) $\times 100 / (\text{percentage donor cells} + \text{percent recipient cells})$.

Results

Expression profiles of histone demethylase genes in hematopoietic cells

Histone methylation status is dynamically regulated by histone methyltransferases and histone demethylases. Histone methyltransferases, including MLL and EZH2, have been studied extensively on their role in HSCs [33,34] but little is known about histone demethylases. To understand the role of histone demethylases in HSCs, we first compared the gene expression profiles of 26 histone demethylases in various hematopoietic cell fractions of BM by microarray analysis. We focused on CD34⁺KSL HSCs which comprise only 0.004 % of mouse BM mononuclear cells [35] in comparison with terminally differentiated blood cell lineages. The microarray analysis identified 3 unique genes (*Fbxl10*, *Jmjd1b*, and *LSD1*) that were preferentially expressed in primitive hematopoietic populations, including CD34⁺KSL HSCs, CD34⁺KSL multipotent progenitors (MPPs), and Lin⁻ cells (Fig. 1A). Among these genes, *Fbxl10* showed the greatest expression in CD34⁺KSL HSCs, but was markedly downregulated in terminally differentiated cells. We then examined basal expression levels of *Fbxl10* in various BM hematopoietic cell populations by quantitative real-time PCR (Fig. 1B). Consistent with the microarray data, the level of *Fbxl10* expression was high in CD34⁺KSL HSCs and CD34⁺KSL MPPs. Among myeloid progenitors, the expression of *Fbxl10* was downregulated during the differentiation of common myeloid progenitors (CMPs) into granulocyte–macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs). Levels of *Fbxl10* expression were very low in mature myeloid cells and erythroblasts compared to in CD34⁺KSL HSCs. These results demonstrate that *Fbxl10* is preferentially expressed in primitive hematopoietic cells and downregulated along with differentiation. The expression profile suggested a role for *Fbxl10* in the epigenetic regulation of HSCs.

Forced expression of *Fbxl10* in CD34⁺KSL HSCs expands colony-forming cells *ex vivo*

To clarify whether *Fbxl10* has an effect on HSCs, we next overexpressed *Fbxl10* in CD34⁺KSL HSCs using a retrovirus system (Fig. 2A). CD34⁺KSL HSCs were transduced with *empty control* or *Fbxl10* and then incubated for 13 days (14 days' culture in total) in the presence of SCF and TPO, the cytokine combination which supports the proliferation of HSCs and progenitors rather than their differentiation. In all experiments, transduction efficiency was determined by flow cytometry using GFP as a marker antigen and ranged from 80% to 90 % in the *control* and 60% to 75% in the *Fbxl10*-transduced cells (Supplementary Fig. 1). Forced expression of *Fbxl10* gave no apparent growth advantage to cells and no morphological change was observed under the microscope compared to the *control* (Fig. 2B, 2C). Nonetheless, flow cytometric analyses of GFP⁺ transduced cells at day14 of culture revealed a 2-fold increase in the proportion of KSL cells in the *Fbxl10* culture compared to the *control* culture (*Control* $0.98 \pm 0.10\%$ vs. *Fbxl10* $1.93 \pm 0.53\%$, $P < 0.05$), although an increase in the proportion of Lin⁺ cells in the *Fbxl10* culture was not statistically significant (*Control* $20.46 \pm 5.16\%$ vs. *Fbxl10* $27.52 \pm 4.32\%$, $P = 0.11$) (Fig. 2D). In addition, the proportion of myeloid progenitors (c-Kit⁺Sca-1⁺Lin⁺) within GFP⁺ cells was also significantly higher in *Fbxl10*-transduced cells (*Control* $1.99 \pm 1.15\%$ vs. *Fbxl10* $6.41 \pm 2.28\%$, $P < 0.01$).

Next, the expansion of primitive progenitor cells was evaluated by colony assay. The total number of CFCs derived from *Fbxl10*-transduced CD34⁺KSL HSCs was slightly increased in the 3.5-day culture compared to the *control* ($P = 0.07$), but increased up to 2-fold in the 10-day culture ($P < 0.01$) (Fig. 3A). Among CFCs, the number of HPP-CFCs, which generate a colony with a diameter greater than 1mm, was markedly increased with *Fbxl10* compared to the *control* ($P < 0.01$). Morphological evaluation of the colonies

derived from HPP-CFCs revealed that the *control* and *Fbxl10* cultures retained comparable numbers of colony-forming units-nmEM (CFU-nmEM) with the potential for differentiation into multiple myeloid lineages at day 3.5 of culture ($P = 0.07$ at χ^2 test) while after 10-day culture, the *Fbxl10* culture retained more CFU-nmEM than the *control* culture ($P < 0.01$ at χ^2 test) (Fig. 3B). Given that 60 % of freshly isolated CD34⁻KSL HSCs can be defined as CFU-nmEM as previously described [29], the net expansion of CFU-nmEM numbers in the *Fbxl10* cultures was 50.7 to 65.9-fold at day 10 of culture, whereas that in the *control* cultures was 2.2 to 9.9-fold (Fig. 3C), indicating a significant expansion of CFU-nmEM on forced expression of *Fbxl10*. We further asked whether Fbxl10 could augment the colony-forming capacity of CD34⁺KSL MPPs. We transduced *Fbxl10* into CD34⁺KSL MPPs and carried out colony-forming assays. Unexpectedly, *Fbxl10* did not increase the number of CFCs or CFU-nmEM in the 3.5-day culture (Supplementary Fig. 2) and neither *control* nor *Fbxl10*-transduced CD34⁺KSL MPPs gave rise to HPP-CFCs in the 10-day culture (data not shown). These results indicated that forced expression of Fbxl10 acts mainly in self-renewing CD34⁻KSL HSCs. Collectively, increased number of CFCs with multi-lineage differentiation potential together with the higher proportion of KSL cells suggested that forced expression of Fbxl10 better maintains primitive hematopoietic cells.

Transcriptional repression of cyclin-dependent kinase inhibitor genes by Fbxl10

A number of cyclin-dependent kinase inhibitors (CKIs) have been implicated in modulating the self-renewal potential of HSCs [36-38]. Fbxl10 regulates the proliferation and senescence of MEFs through repression of *p15^{Ink4b}*, *p16^{Ink4a}*, and *p19^{Arf}* [20, 21]. Moreover, purification of the Fbxl10 complex from murine erythroleukemia (MEL) cells revealed that Fbxl10 coexists with components of PRC1 [25], Ring1b and Bmi1, which maintain HSC self-renewal through repression of *p16^{Ink4a}* and *p19^{Arf}* [29, 30, 39]. To determine whether

forced expression of *Fbxl10* also affects expression of the CKI genes in HSCs, we performed a quantitative real-time PCR analysis on GFP⁺KSL cells purified from 14-day cultures of CD34⁺KSL HSCs. The level of *Fbxl10* expression was 8.0-fold higher in the *Fbxl10*-overexpressing cells than the *control* (Fig. 4A). As expected from the findings in MEFs, forced expression of *Fbxl10* significantly repressed the expression of *p15^{Ink4b}*, *p16^{Ink4a}*, *p18^{Ink4c}*, and *p57^{Kip2}* (Fig. 4B). We observed a mild but significant increase in *p21^{Cip1/Waf1}* expression on forced expression of *Fbxl10*, but the expression of *p27^{Kip1}* was not altered. These results demonstrated that Fbxl10 is a potent negative regulator of the expression of CKI genes, such as *Ink4* (*p15^{Ink4b}*, *p16^{Ink4a}*, and *p18^{Ink4c}*) and *Cip/Kip* (*p57^{Kip2}*) in HSCs.

Recruitment of Fbxl10 to the *Ink4b-Arf-Ink4a* and *Ink4c* locus

Fbxl10 is a demethylase specific to H3K36me2. H3K36 methylation is associated with active regions of chromatin and is also targeted not only to the gene promoters but gene bodies through the RNA polymerase II elongation. We thus analyzed the global levels of H3K36me2 in GFP⁺Lin⁻c-Kit⁺ stem/progenitor cells purified from cultured CD34⁺KSL HSCs transduced with *Fbxl10*. To obtain enough cells, we cultured transduced cells for 14 days in the presence of SCF and TPO and then purified GFP⁺Lin⁻c-Kit⁺ stem/progenitor cells by cell sorting. As expected, the level of global H3K36me2 was decreased by 60% in *Fbxl10*-overexpressing stem/progenitor cells while levels of H3K4me3 and H3K27me3 were not significantly altered (Fig. 5A), indicating that Fbxl10 regulates the transcription of CKI genes through demethylation at H3K36me2. To confirm whether Fbxl10 directly regulates these CKI genes, we carried out ChIP assays on the binding of Fbxl10 to their promoter and gene body regions in GFP⁺Lin⁻c-Kit⁺ stem/progenitor cells purified from cultured CD34⁺KSL HSCs transduced with *Fbxl10* (Fig. 5B). Because none of the available

anti-Fbxl10 antibodies worked in ChIP assays in our hands, we retrovirally expressed 3×Flag-Fbxl10 in CD34⁺KSL HSCs. As expected, ChIP assays using an anti-Flag antibody revealed the direct binding of Fbxl10 to the *p15^{Ink4b}*, *p19^{Arf}*, *p16^{Ink4a}*, and *p18^{Ink4c}* promoters and gene bodies, but not to the *p57^{Kip2}* promoter (Fig. 5C), and a moderate but significant decrease in H3K36me2 levels at the CKI gene promoters and gene bodies on forced expression of *Fbxl10* (Fig. 5D). Corresponding to the report on *Drosophila* KDM2 [24], ChIP assays also detected a moderate but significant increase in levels of H2Aub1 at CKI gene promoters and gene bodies on forced expression of *Fbxl10* (Fig. 5E). These results defined *p16^{Ink4a}*, *p15^{Ink4b}*, and *p18^{Ink4c}* as direct targets of Fbxl10 and suggested that Fbxl10 regulates the expression of these genes through demethylation of H3K36 and mono-ubiquitylation of H2A.

Forced expression of *Fbxl10* prevents exhaustion of the long-term repopulating potential of HSCs following serial transplantation

We next asked whether Fbxl10 has a positive effect on HSCs *in vivo*. Because the effect of forced expression of *Fbxl10* on CFCs was evident after day 10 in culture, we performed competitive repopulation assays using 10-day cultured cells corresponding to 30 initial CD34⁺KSL HSCs from CD45.2 mice and 2×10⁵ BM competitor cells from CD45.1 mice (Fig. 6A). Unexpectedly given the results *in vitro*, the flow cytometric analysis of peripheral blood revealed no obvious difference in the chimerism of donor-derived cells between the *control* and *Fbxl10*-transduced cells at 12 weeks after primary transplantation (Fig. 6B). The *Fbxl10*-transduced cells displayed repopulation kinetics and lineage contribution similar to those of the *control* (Supplementary Fig. 3). Because the 10-day *ex vivo* culture included quite a lot of hematopoietic progenitors, which could contribute to the short-term but multi-lineage repopulation in primary recipients, we evaluated the long-term

repopulating ability, which is essential for evaluation of the self-renewal potential of HSCs, by serial transplantation (Fig. 6A). At 12 weeks after the secondary transplantation, the percentage of donor-derived cells was slightly higher in recipients that received *Fbxl10*-transduced HSCs compared with those that received *control* HSCs, although the difference in total chimerism was not significant ($p = 0.21$). Finally, in the tertiary transplantation, *Fbxl10*-transduced HSCs still retained high repopulating capacity with the multi-lineage differentiation potential, while the *control* HSCs barely repopulated hematopoiesis and 5 recipients received *control* cells died before the analysis probably due to infection because of the poor engraftment (Fig. 6B-D), clearly indicating that forced expression of *Fbxl10* in CD34⁺KSL HSCs prevents exhaustion of the long-term repopulating potential of HSCs following serial transplantation.

To understand the role of *Fbxl10* in the transcriptional repression of the CKI genes during transplantation, we collected donor-derived KSL cells from the primary recipients at 12 weeks after transplantation. Quantitative real-time PCR analyses revealed that forced expression of *Fbxl10* repressed the transcriptional activation of *p15^{Ink4b}* and *p16^{Ink4a}* while both genes were upregulated in the *control* as in 14-day cultures of CD34⁺KSL HSCs (Fig. 6E).

Discussion

Emerging evidence supports that epigenetic regulation is critical to the establishment of gene expression profiles specific to stem cells. Among histone-modifying enzymes, however, histone demethylases are relatively newly identified. Thus, no demethylases have been implicated in the regulation of HSC function. In this paper, we provided the first evidence that a histone demethylase is involved in maintaining the self-renewal capacity of HSCs.

Fbxl10 was preferentially expressed in hematopoietic stem/progenitor cells. Forced expression of *Fbxl10* in HSCs expanded multipotent stem/progenitor cells *ex vivo* and maintained the long-term repopulating capacity of HSCs during serial transplantation. Of note, the effects of overexpressed *Fbxl10* were reminiscent to those of PcG genes such as *Ezh2* and *Bmi1*. Overexpression of *Ezh2*, which encodes a core component of PRC2, reportedly prevents exhaustion of the long-term repopulating potential of HSCs during serial transplantation [31]. In addition, we previously demonstrated that forced expression of *Bmi1* in HSCs expands multipotent stem/progenitor cells during culture *ex vivo* [29]. These findings strongly suggest crosstalk between *Fbxl10* and the PcG proteins in HSCs.

As described above, studies of the *Fbxl10* complexes in both *Drosophila* and mammalian cells have revealed that *Fbxl10* coexists with *Ring1b* and *Bmi1*, core components of PRC1 [24-26]. Although the complexes containing *Fbxl10* have been characterized as distinct from PRC1, forced expression of *Fbxl10* enhanced mono-ubiquitylation of H2A at its target gene loci. These findings suggest that *Fbxl10* regulates transcriptional repression through H3K36 demethylation as well as H2A ubiquitylation by sharing core components with PRC1 or by collaborating with PRC1 itself. Moreover, in both mouse and human fibroblasts, *Fbxl10* up-regulates the mRNA expression of *Ezh2* [21]. In fact, the expression of *Ezh2* was

upregulated on forced expression of *Fbxl10* in donor-derived KSL cells from the primary recipients. Nonetheless, ChIP assays revealed that H3K27me3 status, which is mediated by Ezh2, was not changed on *Fbxl10* overexpression at the *Ink4b-Arf-Ink4a* locus as compared with the control, indicating that the effects of *Fbxl10* on this locus are largely independent of PRC2 (Supplementary Fig. 4).

The self-renewal capacity of HSCs is maintained by close regulation of the cell cycle machinery. In particular, CKIs play a key role in controlling the self-renewal of HSCs [36-38] and the PcG complexes transcriptionally repress the *Ink4b-Arf-Ink4a* locus. In *Bmi1*-deficient mice, expression of *Ink4a* and *Arf* was markedly increased in hematopoietic cells and enforced expression of *Ink4a* and *Arf* in HSCs caused cell cycle arrest and p53-dependent apoptosis, respectively [29, 39]. Conversely, the self-renewal capacity of *Bmi1*^{-/-} HSCs was substantially restored by the deletion of both *Ink4a* and *Arf* [30]. Furthermore, an increasing level of *p16*^{*Ink4a*} was associated with aging of HSCs and loss of *p16*^{*Ink4a*} ameliorated the physiological impact of aging on HSCs [40, 41]. These findings define the *Ink4a-Arf* locus as a critical regulator for the maintenance of HSC self-renewal. In this study, the *Ink4b-Arf-Ink4a* genes appeared to be direct downstream targets of *Fbxl10* in hematopoietic cells as part of them were noted in MEFs [20, 21], confirming again functional crosstalk between *Fbxl10* and PcG proteins. Moreover, *p18*^{*Ink4c*} was also demonstrated to be a direct target of *Fbxl10* in hematopoietic cells by ChIP assays, suggesting that *Fbxl10* targets a broad range of *Ink4* family genes which could have similar impacts on HSC self-renewal. Taken together, the *Ink4* family genes might be the major targets of *Fbxl10* to prevent HSC exhaustion during *ex vivo* culture and serial transplantation. CKI genes exhibit different expression profiles between HSCs and hematopoietic progenitors [42]. It might be important to address the contribution of *Fbxl10* to the cell type-specific regulation of CKI gene transcription. Nevertheless,

additional targets other than *Ink4* family genes might exist for Fbxl10 in HSCs. We will have to await comprehensive analyses of target genes to obtain an overall picture of its function.

In this study, forced expression of *Fbxl10* in CD34⁻KSL HSCs markedly expanded CFC numbers during a 10-day culture *ex vivo*. Nonetheless, the expression of *Fbxl10* in CD34⁺KSL MPPs did not enhance their colony-forming capacity, although *Fbxl10* was highly expressed not only in CD34⁻KSL HSCs but also in CD34⁺KSL MPPs. These findings strongly indicate that Fbxl10 functions in an HSC-specific manner and promotes the production of highly potent stem/progenitor cells. These findings are in line with those for *p15^{Ink4b}*-deficient mice. Loss of *p15^{Ink4b}* resulted in an increase in not only CMPs but also KSL cells that was not accompanied by the expansion of long-term reconstituting HSCs [43]. Thus, the increase in CFCs caused by Fbxl10 might be partially due to silencing of *p15^{Ink4b}*. Although *Fbxl10*-transduced HSCs did not show any advantages in repopulating capacity compared with the *control* in the primary transplantation, they retained a markedly greater repopulating capacity during serial transplantation. Thus, forced expression of *Fbxl10* maintains the self-renewal capacity of HSCs probably by antagonizing various stresses that limit this capacity. This notion was supported by the finding that Fbxl10 repressed many of the CKI genes, which are often upregulated upon various stresses, to very low levels during transplantation.

In a study using retroviral-insertional mutagenesis, *Fbxl10* was identified as one of the common targets of provirus integration in molony murine leukemia virus (MoMuLV)-induced rat T cell lymphomas [22]. In fact, *FBXL10* expression is upregulated in various human hematological malignancies, including acute myeloid and lymphoblastic leukemia, as well as in seminoma [21]. Furthermore, the *INK4B-ARF-INK4A* locus, a direct target of Fbxl10, is commonly inactivated by homozygous deletion or hypermethylation of

the promoter in various human hematological malignancies [44]. Although we did not observe the development of leukemia or lymphoma in recipient mice repopulated with the *Fbxl10*-overexpressing cells during serial transplantation, it would be of importance to investigate whether the upregulation of *FBXL10* contributes to the development and maintenance of self-renewing leukemic stem cells. It will be also intriguing to analyze the collaboration of FBXL10 and PcG proteins in the context of hematological malignancies.

In conclusion, our study identified Fbxl10, a demethylase acting on H3K36, as a novel epigenetic regulator of HSCs, which plays an important role in maintaining the self-renewal capacity and/or multipotency of HSCs. Together with accumulating evidence on the role of histone modifiers in HSCs, our findings underscore the dynamic regulation of histone modifications as a new field of HSC research.

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Figure legends

Figure 1. Expression profiles of histone demethylase genes in hematopoietic cells

(A) Heat map of the expression of 26 histone demethylase genes in a microarray analysis of various bone marrow hematopoietic cell fractions (Red, increased expression; Green, decreased expression). Cells analyzed were CD34⁻KSL HSCs, CD34⁺KSL MPPs, KSL cells, Lineage marker⁻ cells, B220⁺ B lymphocytes, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, NK1.1⁺ NK cells, Ter119⁺ erythroblasts, Gr-1⁺ neutrophils, and Mac-1⁺ monocytes/macrophages. *NKT cells were defined as CD3ε⁺NK1.1⁺ cells. (B) Quantitative RT-PCR analysis of *Fbxl10* in the bone marrow hematopoietic cell fractions. Cells analyzed were CD34⁻KSL HSCs, CD34⁺KSL MPPs, Lineage marker⁻ cells, CMPs, GMPs, MEPs, B220⁺ B lymphocytes, Ter119⁺ erythroblasts, Gr-1⁺ neutrophils, and Mac-1⁺ monocytes/macrophages. Each value was normalized to *Hprt1* expression and presented as the fold-increase compared to the levels in CD34⁻KSL HSCs. Data are shown as the mean ± standard deviation (SD) for triplicate analyses. *, statistically significant relative to the CD34⁻KSL HSCs ($p < 0.05$).

Figure 2. Forced expression of *Fbxl10* maintains HSCs in an undifferentiated state *in vitro*

(A) Schematic diagram of the experimental process. CD34⁻KSL HSCs were transduced with the *Control* or *Fbxl10* retrovirus, cultured in SF-O3 medium with 0.1% BSA, SCF, and TPO, and subjected to analyses at the indicated time points. (B) Growth of CD34⁻KSL HSCs transduced with the *Fbxl10* retrovirus. CD34⁻KSL HSCs transduced with the indicated retroviruses were cultured in the presence of SCF and TPO, and the results are shown as the mean ± SD for triplicate cultures. (C) Morphology of CD34⁻KSL HSCs at day

14 of culture. CD34⁻KSL HSCs transduced with the indicated retroviruses were observed under an inverted microscope at day 14 of culture. (D) Flow cytometric analysis of CD34⁻KSL HSCs at day14 of culture. Representative flow cytometric profiles of KSL cells in cultures of CD34⁻KSL HSCs transduced with the indicated retroviruses are depicted (upper panels) and the percentages of Lin⁻ (lower panel) and KSL cells (upper and lower panels) among GFP⁺ cells are indicated as the mean $\pm$ SD (n = 5). *p* values are results of Student's t-test for the frequency of KSL or Lineage marker⁻ cells.

Figure 3. Forced expression of *Fbxl10* in CD34⁻KSL HSCs expands CFCs with multi-lineage differentiation potential *ex vivo*

(A) *Fbxl10* expands CFC numbers *ex vivo*. Colony assays were performed to evaluate the numbers of CFCs in cultures of CD34⁻KSL HSCs transduced with an *Fbxl10* retrovirus. Absolute numbers of LPP and HPP-CFCs which gave rise to colonies with a diameter less and greater than 1mm, respectively are shown as the mean $\pm$ SD for triplicate cultures. *p* values were calculated by Student's t-test for the total number of colonies. (B) *Ex vivo* expansion of CFU-nmEM on forced expression of *Fbxl10*. Proportions of colony types were defined by the composition of colonies. The GFP⁺ HPP colonies were recovered and examined by microscopy to determine colony types. Proportions of colony types are depicted: n, neutrophils; m, macrophages; E, erythroblasts; M, megakaryocytes. *p* values were calculated by the χ^2 test for the distribution of colony types. (C) Net expansion of CFU-nmEM during the indicated time periods in cultures *ex vivo*. The results are shown as the mean $\pm$ SD for triplicate cultures. *, statistically significant relative to the *control* (*p* < 0.01).

Figure 4. Repression of cyclin-dependent kinase inhibitor genes by *Fbxl10* in HSCs

(A and B) Quantitative RT-PCR analysis of the expression of *Fbxl10* (A) and cyclin-dependent kinase inhibitor genes (B) in *Fbxl10*-overexpressing KSL cells. GFP⁺ KSL cells were purified by cell sorting from cultured CD34⁺ KSL HSCs transduced with *Control* or *Fbxl10* at day 14 of culture. Each value was normalized to *Hprt1* expression and the expression level of each gene in control cells was arbitrarily set to 1. Data are shown as the mean $\pm$ SD for triplicate analyses. * $p < 0.05$ ** $p < 0.01$. N.D. indicates not detected.

Figure 5. Regulation of histone modification status of the *Ink4b-Arf-Ink4a* and *Ink4c* loci by *Fbxl10*

(A) Levels of dimethylation at histone H3K36me2 in *Fbxl10*-transduced cells. Acid-extracted histones were purified from GFP⁺ Lineage marker⁻c-Kit⁺ stem/progenitor cells in cultures of CD34⁺ KSL HSCs transduced with *Control* or *Fbxl10*, and analyzed by Western blotting using the indicated methylation state-specific histone antibodies. Levels of histone modifications were normalized to the amount of histone H3 and are indicated relative to control values. (B) Schematic diagram of the *Ink4b-Arf-Ink4a* and *Ink4c* loci indicating the genomic structure. Exons are demarcated by black boxes. Regions amplified from the precipitated DNA by site-specific quantitative PCR are indicated by arrows. (C-E) ChIP analyses of GFP⁺ Lineage marker⁻c-Kit⁺ stem/progenitor cells purified from cultured CD34⁺ KSL HSCs transduced with *Control* (black bars) or *Fbxl10* (white bars) at day 14 of culture. Antibodies specific to the Flag tag (C), H3K36me2 (D), or H2Aub1 (E) were used. There were no detectable or very low levels of background signals with IgG isotype controls at all amplified regions. Percentages of input DNA are shown as the mean $\pm$ SD for triplicate analyses. *, statistically significant relative to the *control* ($p < 0.01$). Data shown are representative of at least three independent experiments.

Figure 6. Maintenance of the long-term repopulating activity of HSCs following serial transplantation through forced expression of *Fbxl10*

(A) Schematic diagram of the experimental strategies for competitive repopulation assays. Cells at day 10 of culture *ex vivo* corresponding to 30 initial CD34⁺KSL HSCs (CD45.2) transduced with the indicated retroviruses were mixed with 2×10^5 BM competitor cells (CD45.1) and transplanted intravenously into a CD45.1 mouse irradiated with a dose of 9.5 Gy. At 12 weeks after the primary transplantation, 2×10^6 BM cells from the recipients were transplanted into secondary recipients without competitor cells. At 12 weeks after the secondary transplantation, 5×10^6 BM cells from the secondary recipients were sequentially transplanted into tertiary recipients without competitor cells. Data are representative of two independent experiments. (B) Percent chimerism of donor cells in peripheral blood. Each dot shows the percent chimerism of donor cells in the peripheral blood of recipients 12 weeks after transplantation. Bars indicate the mean (n=12, 5 recipients received *control* cells died in 3rd BMT before the analysis). (C) Lineage contribution of donor cells at 12 weeks after transplantation in (B). The proportion of donor-derived cells in each cell lineage is shown as the mean $\pm$ SD. *, statistically significant relative to the *control* ($P < 0.01$). All WBCs, Myeloid, T-lymphoid, and B-lymphoid represent all white blood cells, Gr-1⁺ and/or Mac-1⁺ myeloid cells, CD4⁺ or CD8⁺ T lymphocytes, and B220⁺ B lymphocytes, respectively. (D) Representative flow cytometric analysis of peripheral blood donor-derived (CD45.2⁺) multi-lineage engraftment at 12 weeks after tertiary transplantation. The percentage of cells in each quadrant is indicated. (E) Quantitative RT-PCR analysis of *Ink4* genes in donor-derived KSL cells from BM of recipient mice at 12 weeks after primary transplantation. Each value was normalized to *Hprt1* expression. The 14-day cultures of CD34⁺KSL HSCs in the presence of SCF and TPO were used as reference cells and their expression was arbitrarily set to 1. Data are shown as the mean $\pm$ S.D. for triplicate analyses.

N.D. indicates not detected.

A

B

Figure1

Figure 2

A

B

C

Fold expansion of CFU-nmEM after *ex vivo* culture

		Day 3.5	Day 10
Exp.1	Control	1.0 ± 0.2	2.2 ± 0.4
	Fbx110	2.4 ± 0.1 *	50.7 ± 0.8 *
Exp.2	Control	0.5 ± 0.1	9.9 ± 1.5
	Fbx110	1.2 ± 0.2 *	65.9 ± 2.0 *

Figure 3

Figure 4

Figure 5

Figure 6