INTS11 regulates hematopoiesis by promoting PRC2 function

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INTS11, the catalytic subunit of the Integrator (INT) complex, is crucial for the biogenesis of small nuclear RNAs and enhancer RNAs. However, the role of INTS11 in hematopoietic stem and progenitor cell (HSPC) biology is unknown. Here, we report that INTS11 is required for normal hematopoiesis and hematopoietic-specific genetic deletion of Ints11 leads to cell cycle arrest and impairment of fetal and adult HSPCs. We identified a novel INTS11-interacting protein complex, Polycomb repressive complex 2 (PRC2), that maintains HSPC functions. Loss of INTS11 destabilizes the PRC2 complex, decreases the level of histone H3 lysine 27 trimethylation (H3K27me3), and derepresses PRC2 target genes. Reexpression of Ints11 or PRC2 proteins in Ints11-deficient HSPCs restores the levels of PRC2 and H3K27me3 as well as HSPC functions. Collectively, our data demonstrate that INTS11 is an essential regulator of HSPC homeostasis through the INTS11-PRC2 axis.

INTRODUCTION

Hematopoietic stem and progenitor cells (HSPCs) are a rare population of hematopoietic cells, having capabilities of both self-renewal and differentiation to all lineages of hematopoietic cells, such as myeloid, erythroid, T, and B cells (1). A tight balance between self-renewal and differentiation is crucial to maintain homeostasis of the hematopoietic system. The ability of hematopoietic stem cells (HSCs) to repopulate the hematopoietic compartment is regulated by both intrinsic and extrinsic mechanisms (2). The cooperation between the stochastic and epigenetic changes fine-tunes the transcriptome profiles required for normal hematopoiesis (3, 4).

Integrator (INT) is an evolutionarily conserved complex and contains at least 14 subunits (5). INT was discovered by Baillat and colleagues in 2005 and was characterized originally as an important factor for U-rich small nuclear RNA (snRNA) 3′-end processing (6). Multiple studies demonstrate additional roles for INT in RNA polymerase II (RNAPII) transcription initiation, pause-release, elongation, and termination at protein-coding genes (7–9). In addition, INT is crucial for the biogenesis of enhancer RNA (eRNA) (10). More recently, INT has been shown to trigger premature transcription termination and attenuate gene expression (11–13). All these studies point to the essential role of INT family members in RNA biogenesis.

INT components int3 and int9 were shown to regulate snRNA processing and the differential fate of adult stem cells in Schmidtea mediterranea (14). Tao and colleagues (15) reported that int5 knockdown in zebrafish embryos leads to blockage of red blood cell differentiation because of perturbation of smad1/5 splicing. Knockdown of INTS13 with small hairpin RNAs in human myeloid HL-60 cells revealed that INTS13 is required to activate specific enhancers during myeloid differentiation (16). In a recent study, Yoshimi and colleagues (17) demonstrated that aberrant splicing of INTS3 is associated with leukemogenesis, and overexpression of INTS3 slows down leukemia progression in vivo. INTS11, the only subunit of the INT protein family with endonuclease activity (18), has been shown to play critical roles in mouse adipose differentiation and ciliogenesis in cultured human cells (19, 20). Unexpectedly, Drosophila IntS11 is not essential for neural development (21). These studies imply a tissue-specific function of INTS11. Up to date, the role of INT in adult stem cell and specifically HSC regulation remains unknown.

In the current study, we investigated the role of INTS11 in HSPCs both in vitro and in vivo using our newly generated hematopoietic-specific Ints11 knockout mouse model system. We show that INTS11 is essential for normal hematopoiesis, as Ints11 deletion led to G1-S cell cycle arrest and resulted in HSC impairment. Intriguingly, we identify a previously unknown mechanistic function for INTS11 in cooperation with the Polycomb repressive complex 2 (PRC2) to maintain histone H3 lysine 27 trimethylation (H3K27me3) at genes critical for cell cycle progression and HSPC functions. Loss of INTS11 destabilizes the PRC2 complex, decreases the H3K27me3 level, and derepresses PRC2 target genes. Reexpression of Ints11 or PRC2 proteins in Ints11-deficient HSPCs restores the levels of PRC2 and H3K27me3 as well as HSPC functions. Our study demonstrates an indispensable role of INTS11 in normal hematopoiesis regulating the expression of genes critical for HSPC functions through the newly discovered Ints11/PRC2 axis.

RESULTS

INTS11 is required for hematopoiesis

To determine the expression levels of Ints11 in wild-type (WT) hematopoietic lineages, different hematopoietic cell subpopulations were sorted and subjected to quantitative polymerase chain reaction (qPCR). Ints11 mRNA was ubiquitously expressed in all the hematopoietic lineages examined, with a higher level in the HSPCs (Fig. 1A). To investigate the function of INTS11 in hematopoiesis, we generated Ints11−/− heterozygous mice (Fig. S1A) and bred them with the Vav1-Cre mice (22). However, VavCre+;Ints11−/− animals were never observed, suggesting embryonic lethality (Fig. 1B). Subsequent kinetic dissection...
of embryos from embryonic day (E) 13.5 to 18.5 confirmed the lethality upon deletion of Ints11 at the embryonic stage (fig. S1B). VavCre−;Ints11flox/flox embryos were observed at a normal rate of Mendelian ratios up to E17.5, but a much lower frequency at E18.5 than VavCre− embryos. The successful deletion of Ints11 was confirmed in the tail and CD45+ fetal liver by PCR and qPCR, respectively (fig. S1, C and D). Western blotting demonstrated complete deletion of INTS11 protein in fetal liver cells at E13.5 (Fig. 1C). While there was no difference in the sizes between the two genotypes of the embryos, VavCre−;Ints11flox/flox embryos were pale (Fig. 1D and fig. S1E). The liver of VavCre−;Ints11flox/flox embryos was also pale, which was significantly smaller and less cellular than that of the control embryos (Fig. 1, E to G). Flow cytometric analysis of HSPC populations in VavCre−;Ints11flox/flox fetal livers revealed a severe reduction
in the cell populations of Lin−cKit+Scal− (LKS−), Lin−Scal−cKit+ (LSK), long-term (LT-) HSCs (Lin−cKit+Scal−CD34+CD135−), short-term (ST-) HSCs (Lin−cKit+Scal−CD34+CD135+), and multipotent progenitors (MPPs; Lin−cKit+Scal−CD34+CD135+) (Fig. 1, H to J, and fig. S1, F and G), indicative of impaired development of HSPCs in Ints11-deficient embryos. Consistently, colony-forming unit cell (CFU-C) assays demonstrated that fetal liver cells of VavCre+;Ints11flx/flx embryos were unable to form colonies (Fig. 1K and fig. S1H). Further phenotypic analysis showed a significant decrease in erythroid precursors in Ints11-deficient fetal livers (fig. S1, I and J). These data indicate that INTS11 is essential for sustaining fetal hematopoiesis.

To determine whether INTS11 is required for adult hematopoiesis, we crossed Ints11flx/flx mice with Mx1-Cre mice and induced Ints11 deletion (Ints11flx/Δ) by three daily intraperitoneal injections of polyinosine-polycytidine [poly(I:C)]. Recombination of the Ints11 floxed allele occurred 72 hours after the first poly(I:C) injection (fig. S2A). There was a >90% reduction of Ints11 mRNA and the protein in the bone marrow (BM) Lin−cKit+ (LK) cells of Ints11flx/Δ mice compared with Mx1Cre+ (WT) controls (Fig. 2, A and B). Adult mice lacking Ints11 expression died within 3 weeks of poly(I:C) injection (Fig. 2C). All the mice developed pancytopenia (Fig. 2D). Flow cytometric analysis of peripheral blood (PB) showed that Ints11flx/Δ mice had a marked reduction of myeloid cells (Gr1+/Mac1+) but relatively increased percentages of T and B cells (Fig. 2, E and F, and fig. S2, B and C). BM cellularity was markedly reduced (Fig. 2G). Analysis of bone histological sections revealed a substantial reduction of BM cellularity of Ints11flx/Δ mice (Fig. 2H). In addition, flow cytometric analysis demonstrated that the percentages of myeloid (Gr1+/Mac1+), erythroid progenitor cells (CD71+/Ter119+), and lymphoid (T and B220−) cells were significantly reduced in the BM of Ints11flx/Δ mice compared with those of WT control mice (Fig. 2, I and J, and fig. S2, D to G). Despite the comparable body weights between WT and Ints11flx/Δ mice (fig. S2H), Ints11flx/Δ mice exhibited lower spleen weight and significantly decreased cellularity compared with WT controls (Fig. 2, K and L, and fig. S2I). The histological analysis of Ints11flx/Δ spleen sections showed a decreased proportion of myeloid cells (Fig. 2M). Flow cytometric analysis of the spleen showed that the percentage of myeloid cells was significantly reduced, whereas the percentages of T and B cells were relatively higher in the spleens of Ints11flx/Δ than in WT controls (fig. S2, J to M).

We next examined the effects of Ints11 deletion on BM HSPC populations 72 hours after poly(I:C) injection. We observed no difference in the cellularity of BM between Ints11flx/Δ and their littermate controls (fig. S3A). Flow cytometric analysis revealed that the numbers of LSK cells and ST-HSCs were significantly decreased in the BM of Ints11flx/Δ mice compared with WT controls (fig. S3, B and C), while the numbers of LKS− cells, LT-HSCs, MPPs, as well as cKit+ cells and LK cells were comparable (fig. S3, B to E). By day 12 after poly(I:C) injection, Ints11 deletion led to a marked reduction of HSPCs in the BM of Ints11flx/Δ mice (Fig. 3, A and B, and fig. S3, F to H). CFU-C assays confirmed that Ints11-deficient BM cells formed minimum number of colonies (Fig. 3, C and D). Together, these results indicate that INTS11 is required for the maintenance of the HSPC pool.

INTS11 function in hematopoiesis is cell autonomous

To verify the cell intrinsic effects of Ints11 deletion in HSC functions, we performed competitive BM transplantation assays by injecting equal numbers of BM cells from BoyJ mice (CD45.1+) and Mx1Cre+;Ints11flx/Δ mice (CD45.2+) or Mx1Cre+ littermates (CD45.2+) into lethally irradiated BoyJ recipient mice (CD45.1+) (fig. S3I). Ints11 deletion was induced by poly(I:C) injection upon confirmation of comparable engraftment rates of CD45.1+ versus CD45.2+ cells in the PB of the recipient mice. Ints11-deficient CD45.2+ cells were rapidly outcompeted by cotransplanted CD45.1+ WT cells (Fig. 3E). Myeloid cells were the first diminished cell population in the PB of the recipient mice (Fig. 3F) followed by a persistent reduction of T and B cell populations over 30 weeks after poly(I:C) injection (fig. S3, J and K). The CD45.2+ Ints11flx/Δ cells were almost eliminated in the BM 30 weeks after the poly(I:C) injection (Fig. 3G). These data indicate a cell-autonomous requirement for INTS11 in fetal and adult hematopoiesis.

Up-regulated genes in Ints11-deficient LK cells are enriched for PRC2 targets

To identify the molecular pathways regulated by INTS11 in HSPCs, we performed RNA sequencing (RNA-seq) using Ints11flx/Δ BM LK cells. It is known that snRNAs are relatively stable, as their levels only marginally decrease over 72 hours (11, 12). We thus examined genome-wide expression profiles in WT and Ints11flx/Δ LK cells 72 hours after poly(I:C) injection. Compared with WT cells, the deletion of Ints11 resulted in an aberrant gene expression signature in LK cells consisting of 1807 up-regulated and 352 down-regulated genes [Fig. 4A and fig. S4A; fold change > 1.8 and false discovery rate (FDR) < 0.05]. Ints11 deletion decreased the Ints7 mRNA level in LK cells compared with WT cells, while the expression of other INT subunits remained at the same levels in Ints11flx/Δ BM LK cells as in WT cells (fig. S4, B and C). Gene set enrichment analysis (GSEA) showed that a subset of the down-regulated genes in Ints11-deficient cells was enriched for the metabolism of RNA (Fig. 4B), supporting the previous finding that INTS11 is required for RNA processing (6, 10). GSEA further revealed that genes associated with the regulation of G1-S cell cycle transition and apoptosis were dysregulated in Ints11flx/Δ LK cells (Fig. 4C and fig. S4, D and E). Unexpectedly, PRC2 target genes were up-regulated in Ints11flx/Δ LK cells (Fig. 4, D and E, and fig. S4F). qPCR confirmed the changes on selected PRC2 target genes, including genes associated with cell cycle, apoptosis, and HSC functions (Fig. 4F and fig. S4G).

Enrichment of cell cycle–related gene signatures led us to investigate whether deletion of Ints11 changes the cell cycle profile of HSPCs. In line with the gene expression changes, the cell cycle analysis demonstrated that the proportion of lineage-negative cells in the BM in G0-G1 was significantly increased and the S phase was significantly reduced upon Ints11 deletion, suggesting a failure of entering S phase from G0-G1 in Ints11flx/Δ cells (fig. S4, H and I). Cyclin-dependent kinase inhibitor p21Cip1 (Cdkn1a for gene name) plays a predominant role in maintaining HSC quiescence (23, 24). Consistently, Western blot analysis revealed an up-regulation of p21Cip1, which may contribute to cell cycle arrest and G1- to-S phase transition in Ints11flx/Δ HSPCs (fig. S4J). In addition, there was a mild increase in apoptotic cells [7-amino-actinomycin D–negative (7-AAD−) /annexin V+] in Ints11flx/Δ lineage–negative BM cells compared with WT controls (fig. S4K).

Recently, single-cell RNA-seq (scRNA-seq) has emerged as a powerful tool to dissect hematopoietic progenitors and other cell populations (25). Given the heterogeneity of HSPCs, we performed a scRNA-seq analysis of 21,495 BM cKit+ cells from WT and Ints11flx/Δ mice to capture the impact of Ints11 deletion on transcriptomic changes.
Fig. 2. INTS11 deletion leads to hematopoietic failure. (A) Ints11 mRNA levels in BM Lin−cKit+ (LK) cells from WT (n = 9) and Ints11Δ/Δ mice (n = 13) 72 hours after first poly(I:C) injection. (B) INTS11 protein levels in LK cells from WT and Ints11Δ/Δ mice. (C) Kaplan-Meier survival curve representing percent survival of Ints11Δ/Δ mice following poly(I:C) injection. Log-rank (Mantel-Cox) test; WT, n = 10; Ints11Δ/Δ, n = 13. (D) PB counts of WT and Ints11Δ/Δ mice (n = 10 per genotype). WBC, white blood cells; RBC, red blood cells. (E) Flow cytometric analysis of myeloid cells in PB from WT and Ints11Δ/Δ mice 12 days after first poly(I:C) injection. (F) Frequencies of Gr1+/Mac1+ cells in PB from WT and Ints11Δ/Δ mice (n = 8 per genotype). (G) BM cellularity of WT and Ints11Δ/Δ mice (n = 10 per genotype) 12 days after poly(I:C) injection. (H) Representative hematoxylin and eosin (H&E) staining of bone sections. Scale bar, 100 μm. (I) Flow cytometric analysis of myeloid cells in BM from WT and Ints11Δ/Δ mice 12 days after poly(I:C) injection. (J) Frequency of Gr1+/Mac1+ cells in BM from WT (n = 7) and Ints11Δ/Δ mice (n = 10). (K and L) Weight (K) and cellularity (L) of the spleen from WT and Ints11Δ/Δ mice (n = 9 per genotype). (M) Representative H&E staining of spleen sections. Scale bar, 10 μm. Data are means ± SEM. Unpaired Student’s t test: **P < 0.01 and ***P < 0.001.
in HSPC populations. Fifteen major clusters (subpopulations) were identified by the unsupervised clustering method after integrating WT and \(\text{Ints1}^{\Delta/\Delta}\) cells (Fig. 4G and fig. S5A). Loss of \(\text{Ints1}\) skewed the cluster distribution with increased megakaryocyte-erythroid progenitors (MEPs; 7.43 versus 2.87%) and decreased monocyte progenitors (MonoPs; 2.53 versus 9.64%) (Fig. 4G and fig. S5B). We observed that there was a slight increase of LT-HSCs and ST-HSCs in \(\text{Ints1}^{\Delta/\Delta}\) cells compared with WT controls (fig. S5, A and B).

MPP subsets emerge from HSCs, and myeloid-biased MPP2 and MPP3 work together with lymphoid-primed MPP (LMPP) to control blood production (26). After scoring individual cells with several module scores based on the expression levels of population-specific gene signatures, we found that a higher erythroid/megakaryocyte score and a lower monocyte score were significantly enriched in all \(\text{Ints1}\)-deleted HSCs and MPPs (Fig. 4H and fig. S5C). These data support the observation of increased MEP and reduced MonoP populations.

We next performed GSEA analysis to assess the transcription signatures associated with specific HSPC populations. Consistent with the findings observed in bulk RNA-seq, \(\text{Ints1}^{\Delta/\Delta}\) cells showed a significant increase in the expression of PRC2 targets as well as the genes controlling cell cycle arrest and apoptosis in almost all HSC and MPP populations (Fig. 4, I and J). Unexpectedly, genes up-regulated in HSC signatures were enriched in all the HSCs and MPPs of \(\text{Ints1}^{\Delta/\Delta}\) mice (Fig. 4J). Moreover, we observed higher scores of stemness and apoptosis, but modest changes of proliferation score in \(\text{Ints1}^{\Delta/\Delta}\) mice.
Fig. 4. Loss of Ints11 derepresses PRC2 target genes in HSPCs. (A) Heatmap depicting significantly dysregulated (1807 up-regulated and 352 down-regulated) genes in Ints11^−/− LK cells compared with WT controls (FDR < 0.05 and |fold change| > 1.8). (B to E) GSEA shows that genes involved in the regulation of RNA metabolism (B) and cell cycle transition (C) are down-regulated, and PRC2 targets (D and E) are up-regulated, in Ints11^−/− LK cells. The normalized enrichment score (NES), P value, and FDR are shown. (F) Relative mRNA expression of genes associated with cell cycle, apoptosis, and PRC2 targets in LK cells of WT (n = 6) and Ints11^−/− mice (n = 8) 72 hours after first poly(I:C) injection. Data are means ± SEM. Unpaired Student's t test: **P < 0.01 and ***P < 0.001. (G) Uniform manifold approximation and projection (UMAP) visualization of hematopoietic cell clusters identified from cKit+ cells of WT and Ints11^−/− mice. Each dot represents one cell, and cluster identity is color-coded (Seurat). LMPP, lymphoid-primed MPP; NeuP, neutrophil/granulocyte progenitor (proNeu, early specifying; preNeu, late committed; and immNeu, immature neutrophil); BaP/Mast, basophil progenitor and mast cells; EoP, eosinophil progenitor; DCP, dendritic cell–committed progenitor; NKP, natural killer cell–committed progenitor; MΦ P, macrophage-restricted progenitor. (H) Violin plot of monocyte transcription signature in indicated populations. The score was calculated on the basis of the expression values for genes in a given gene set. Mann-Whitney U test. (I and J) GSEA with NES and FDR values for gene sets of PRC2 (I) and HSC, apoptosis, and cell cycle (J) associated with HSPC populations in Ints11^−/− cells. The colors reflect scaled NES, representing the degree of expression change. The size of the circle represents the FDR value. (K) Violin plot of stemness transcription signature in indicated populations. Mann-Whitney U test.
INTS11 interacts with the PRC2 complex

PRC2 consists of four core components: enhancer of zeste homolog 1/2 (EZH2), suppressor of zeste 12 homolog (SUZ12), embryonic ectoderm development (EED), and retinoblastoma-binding protein 4 and 7 (RBBP4/7) (27, 28). To determine whether INTS11 colocalizes with PRC2 genome-wide in the hematopoietic cells, we first investigated the chromatin targets of INTS11 in hematopoietic cells by analyzing the published INTS11 chromatin immunoprecipitation sequencing (ChIP-seq) data by Barbieri and colleagues (16). Genomic distribution analysis of the INTS11 peaks showed that 77.91% of INTS11-associated regions were localized at promoter regions (≤1 kb of transcription start sites; fig. S6A). Further comparison with the SUZ12 ChIP-seq dataset for hematopoietic cells (29) revealed that the peaks of INTS11 highly overlapped with the peaks of SUZ12 (55.4%; Fig. 5A). Gene ontology analysis demonstrated that the overlapping target genes among INTS11 and SUZ12 were enriched in RNA processing, cell cycle, apoptosis, hematopoiesis, and transcription regulation (Fig. 5, B and C, and fig. S6B).

The largely overlapping targets among INTS11 and SUZ12 prompted us to probe for interaction between INTS11 and PRC2 complex proteins. To test this hypothesis, we generated a lentiviral vector expressing FLAG-INTS11 (fig. S6C) and performed IP assays. All IPs were us to probe for interaction between INTS11 and PRC2 complex proteins. To test this hypothesis, we generated a lentiviral vector expressing FLAG-INTS11 (fig. S6C) and performed IP assays. All IPs were performed in the presence of benzonase to ensure that the protein-protein interactions were DNA independent. We used an anti-FLAG antibody to pull down INTS11 in the nuclear extracts from 293T cells expressing FLAG-INTS11, followed by Western blot using antibodies against EZH2, SUZ12, and EED, the core subunits of the PRC2 complex. We found that INTS11 interacts with endogenous EZH2, SUZ12, and EED (Fig. 5D). Reciprocal IP of either EZH2 or SUZ12 led to the co-IP of INTS11 (Fig. 5E and fig. S6D). To determine whether PRC2 could interact with endogenous INTS11 in vivo, we transfected plasmids expressing hemagglutinin (HA)–EZH2 or HA-SUZ12 individually into 293T cells. IP analysis of nuclear extracts using the anti-HA antibody recognizing endogenous INTS11 confirmed that INTS11 forms a complex with PRC2 (Fig. 5F).

We then assessed whether endogenous INTS11 formed a complex with PRC2 members in hematopoietic cells. We performed IPs for INTS11 or EZH2 and analyzed for INTS11, EZH2, and SUZ12 in nuclear extracts of human K562 cells. Both EZH2 and SUZ12 coimmunoprecipitated with INTS11, whereas INTS11 and SUZ12 coimmunoprecipitated with EZH2 (Fig. 6B). These results demonstrate that INTS11 interacts with PRC2 in hematopoietic cells.

To assay whether INTS11 directly interacts with PRC2, we next perform a glutathione S-transferase (GST) pull-down assay. GST-INTS11 fusion proteins were captured with glutathione beads (fig. S6E) and were incubated with recombinant PRC2 protein complex (EZH2/SUZ12/EED). The results showed that GST-INTS11, but not GST or empty glutathione beads, precipitated the PRC2 core subunits EZH2, SUZ12, and EED (Fig. 6C). To further determine which subunit of PRC2 interacts with INTS11, we incubated GST-INTS11 fusion protein with recombinant protein of FLAG-tagged EZH2, SUZ12, or EED, respectively. Compared with GST alone control, the GST-INTS11 fusion protein was able to pull down EZH2, but not SUZ12 or EED (Fig. 6D and fig. S6F). These findings indicate that INTS11 physically interacts with PRC2.

Decreased H3K27me3 enrichment upon Ints11 deletion contributes to the up-regulation of PRC2 targets

To determine whether the loss of Ints11 affects PRC2 activities and functions, we evaluated the mRNA and protein levels of PRC2 subunits in BM LK cells of WT and Ints11/LK mice 72 hours after poly(I:C) injection. The protein levels of EZH2, SUZ12, and RBBP4/7 were greatly reduced in Ints11/LK cells compared with WT controls (Fig. 7A), whereas the levels of CTFC and HDAC1 were comparable between Ints11/LK and WT cells (fig. S7A). Unexpectedly, there was no change in mRNA levels of Gata2, Sux12, Eed, and Rbbp4/7 in Ints11/LK cells (fig. S7B). The PRC2 protein complex is known to catalyze methylation on lysine 27 of histone H3 (H3K27) (27). We next performed Western blot analysis to compare the levels of H3K27me3 between WT and Ints11/LK cells. The results showed that Ints11/LK cells exhibited a decreased level of global H3K27me3 compared with WT LK cells, while the level of H3K27me2 was identical between Ints11/LK and WT cells (Fig. 7B). Further analysis using fetal liver cells pooled from VavCre−/Ints11/LK or VavCre−/Ints11/LK embryos revealed that the levels of H3K27me3, EZH2, SUZ12, and EED were also decreased in VavCre−;Ints11/LK or compared with those in VavCre− controls (fig. S7C). These data suggest that loss of INTS11 destabilizes the PRC2 complex proteins, thus decreasing the enzymatic activity of the PRC2 complex on H3K27 trimethylation.

To evaluate whether the dysregulated gene expression following Ints11 deletion was associated with the changes of histone modifications, we performed ChIP-seq for H3K27me3 and H3K4me3 using WT and Ints11/LK LK cells. Normalized global read density and locus-level enrichment revealed a significant reduction in genome-wide H3K27me3 occupancy in Ints11/LK cells compared with WT cells (P < 2.2 × 10−16) (Fig. 7, C and D, and fig. S7D). However, there were no significant changes of H3K4me3 peaks globally between WT and Ints11/LK LK cells (fig. S7E), suggesting a limited impact of Ints11 on H3K4me3 in HSPCs.

To determine the impact of decreased H3K27me3 on gene expression in Ints11/LK LK cells, we integrated the RNA-seq and ChIP-seq data. We found that 718 (39.7%) up-regulated genes were associated with reduced H3K27me3 peaks (Fig. 7E). Gene ontology analysis revealed that the up-regulated genes were enriched in hematopoiesis,
**Fig. 5.** INTS11 forms a complex with PRC2. (A) Venn diagram showing the overlap among binding sites (peaks) of INTS11 (GSE106359) and SUZ12 (GSE59090) in hematopoietic cells. (B) Gene ontology analysis of the genes that were co-occupied by INTS11 and SUZ12. (C) Representative ChIP-seq tracks show that INTS11 and SUZ12 co-occupy PRC2 target gene promoters in hematopoietic cells. (D) Nuclear extracts of 293T cells transfected with FLAG-tagged INTS11 or empty vector control (EV) were immunoprecipitated (IP) with anti-FLAG and probed for PRC2 proteins. Arrowhead indicates the FLAG-fusion protein. (E) Reciprocal SUZ12 IP from nuclear extracts of 293T cells transfected with FLAG-tagged INTS11 or EV, and representative immunoblot analysis. (F) Nuclear extracts of 293T cells transfected with HA-tagged EZH2 (top) or SUZ12 (bottom) were IP with anti-HA or mouse IgG, and Western blotting was performed. (G) Western blot analysis of the samples above (F) using antibodies against INT subunits INTS1, INTS3, and INTS4. (H) Soluble nuclear and chromatin-bound fractions from 293T cells transfected with HA-tagged SUZ12 were subjected to IP with anti-HA antibody followed by Western blot analysis with indicated antibodies.
stem cell development, cell cycle, and apoptosis (Fig. 7F). We also examined the occupancies of H3K27me3 on genes associated with cell cycle, apoptosis, and HSC function, respectively. Up-regulated genes, including Cdkn1a, Gata2, Perp, and Trib1, in Ints11Δ/Δ LK cells, were correlated with a lower intensity of H3K27me3 (Fig. 7G and fig. S7F). ChIP-qPCR confirmed significant decreases in H3K27me3 at the promoters of selected genes that were activated in Ints11Δ/Δ LK cells, in contrast to minimal changes in H3K4me3 occupancies (Fig. 7H and fig. S7G). These data suggest that the key up-regulated genes are directly targeted by PRC2 in Ints11-deficient HSPCs. The association of PRC2 with INTS11 is required for transcriptional repression in HSPCs.

**Reexpression of INTS11 increases the PRC2 activity and rescues the Ints11-deficient phenotypes**

To determine whether altered PRC2 activity is responsible for the defective Ints11Δ/Δ HSPCs, we harvested BM cKit+ cells from Ints11Δ/Δ mice 72 hours after poly(I:C) injection and then transduced FLAG-INTS11 into cells and performed CFU-C assays. Reexpression of INTS11 increased the protein levels of PRC2 subunits and H3K27me3 (Fig. 8, A and B, and fig. S8A) and reduced the expression of PRC2 targets, including Cdkn1a and Perp (Fig. 8C). Further analysis by ChIP-qPCR revealed normalized H3K27me3 occupancies on these PRC2 target genes (fig. S8B). Accordingly, reexpression of INTS11 significantly increased the colony-forming capacity of Ints11Δ/Δ...
Fig. 7. Decreased H3K27me3 enrichment contributes to the up-regulation of gene expression in Ints11-deficient LK cells. (A) Western blot analysis of indicated members of the PRC2 complex in LK cells from WT and Ints11^−/−^ mice 72 hours after poly(I:C) injection. (B) Western blot showing the levels of H3K27me2 and H3K27me3 in Ints11^−/−^ LK cells. H3 was used as a loading control. (C) Global levels of H3K27me3 at peaks and 5-kb regions surrounding the peak midpoint. The coverages were normalized by the sequencing depth and averaged in two biological replicates. *P value was calculated by paired Student’s t test. (D) Heatmaps of normalized H3K27me3 ChIP-seq read densities centered on the midpoints of 5985 H3K27me3-decreased regions. Each row represents a single region. (E) Venn diagram showing the overlap between genes with reduced H3K27me3 and up-regulated genes in Ints11^−/−^ LK cells. The number of genes in each section of the diagram is shown. (F) Functional enrichment analysis for 718 overlapping genes in (E). Representative significantly enriched pathways were displayed (FDR < 0.05). (G) Normalized H3K27me3 signals on the Cdkn1a and Gata2 gene loci. (H) ChIP-qPCR verified the reduction of H3K27me3 occupancies at the promoter regions of the genes in Fig. 4F (n = 4 per genotype). Data are means ± SEM. Unpaired Student’s t test: *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 8. Reexpression of INTS11 increases the PRC2 activity and rescues the Ints11-deficient phenotypes. (A and B) Western blot showing the levels of PRC2 subunits (A) and H3K27me3 (B) in Ints11Δ/Δ cells transduced with FLAG-INTS11. (C) qPCR showing the expression levels of up-regulated genes (Fig. 4F) in Ints11Δ/Δ cells transduced with FLAG-INTS11. Each dot represents an individual mouse (n = 6 to 7 per group). (D) Reexpression of INTS11 increased the colony-forming capacity of Ints11Δ/Δ cKit+ cells. Each dot represents an individual mouse (n = 8 to 9 per group). (E) Representative images of colony formation for Ints11-deficient cKit+ cells transduced with FLAG-INTS11 and controls. The images were taken on the seventh day of the assay. (F) Kaplan-Meier survival analysis of recipients receiving Ints11Δ/Δ cells expressing FLAG-INTS11. Log-rank (Mantel-Cox) test; FLAG-INTS11, n = 6; EV controls, n = 4. (G) Enhanced expression of PRC2 partially restored the colony-forming capacity of Ints11Δ/Δ cKit+ cells. Each dot represents an individual mouse (n = 5 to 6 per group). (H) Western blot showing the levels of PRC2 subunits and H3K27me3 in Ints11Δ/Δ cells transduced with EZH2 and/or SUZ12. (I) Kaplan-Meier survival analysis of recipients. Log-rank (Mantel-Cox) test; EZH2 + SUZ12, n = 5; EV controls, n = 4. Data are means ± SEM. One-way analysis of variance with Tukey’s multiple comparisons test: ***P < 0.001 and ****P < 0.0001.
cells (Fig. 8, D and E). To assess the effect of reexpression of INTS11 in Ints11Δ/Δ cells on HSPC functions in vivo, we performed phenotypic analysis following the transplantation of Mx1Cre+/Ints11Δlox/Δox BM cells (CD45.2+) expressing INTS11 or empty vector (EV) control into lethally irradiated Boyl (CD45.1+) recipient mice (fig. S8C). Ints11 deletion was induced upon confirmation of engraftment. Similar to the primary Ints11Δ/Δ mice, the recipients receiving the EV cells died within 3 weeks of poly(I:C) injection (Fig. 8F). In contrast, the reexpression of INTS11 rescued the survival of the recipient mice transplanted with Ints11Δ/Δ BM cells expressing INTS11 (Fig. 8F). Flow cytometric analysis revealed that reexpression of INTS11 markedly increased the frequencies of LKS- and LSK cell populations in the BM (fig. S8, D and E).

Deletion of Ints11 reduced PRC2 protein levels along with upregulation of PRC2 target genes in Lk cells. We then asked whether enhanced expression of EZH2 or SUZ12 in Ints11Δ/Δ cKit+ cells could restore the colony-forming activity of Ints11Δ/Δ cells. Ints11Δ/Δ cKit+ cells transduced with EZH2 and/or SUZ12 significantly increased the frequencies of CFU-C compared with the cells transduced with EV control (Fig. 8G and fig. S8F). Accordingly, enhanced expression of EZH2 and/or SUZ12 increased the H3K27me3 levels and repressed the PRC2 target gene in Ints11Δ/Δ cells (Fig. 8H and fig. S8, G to I). Enhanced expression of EZH2 and SUZ12 in Ints11Δ/Δ cells extended the survival of the recipients (Fig. 8I). In summary, these data demonstrate that alteration of PRC2 function is at least partially responsible for the effects of Ints11 on normal hematopoiesis.

**DISCUSSION**

In the current study, we identify an essential role for Ints11 as a key regulator of HSC homeostasis in vivo. We show that deletion of Ints11 in the hematopoietic system results in cell cycle arrest of G1- to S phase transition, leading to HSC dysfunction. We characterize a previously unidentified function of Ints11 in gene regulation and demonstrate that Ints11 interacts with PRC2 to regulate gene expression through the histone mark H3K27me3. We found that loss of Ints11 destabilized the PRC2 complex and decreased the levels of H3K27me3 in HSPCs. The up-regulated genes in Ints11Δ/Δ cells, which are key for cell cycle, HSC function, and apoptosis, were associated with the reduced H3K27me3 levels. Reexpression of Ints11 or EZH2/SUZ12 in Ints11Δ/Δ HSPCs increased the levels of PRC2 and H3K27me3 along with the restoration of the HSC function.

The mechanisms that maintain the balance between self-renewal and differentiation in HSCs are critical for preventing exhaustion of the stem cell pool or the development of hematopoietic malignancies (30). Posttranslational modifications (PTMs) on histone tails can promote either transcriptional silencing or activation. PRC2 is responsible for the methylation of lysine-27 on histone H3. The H3K27me3 signature is highly correlated with silent loci and is important for regulating developmental and oncopgenic genes (31). Recent studies showed that transcript cleavage by Ints11 may not be the main event to trigger transcription termination after pausing (32). Our work demonstrates that Ints11 interacts with the PRC2 complex directly and regulates the expression of genes through H3K27me3. Previous reports using loss-of-function approaches targeting individual PRC2 subunits have suggested that PRC2 proteins are essential regulators for normal hematopoiesis (33–36). The catalytic subunit EZH2 is essential for fetal hematopoiesis, but is dispensable for adult BM HSC function, due to compensation by EZH1 (33). In contrast, the core subunits of PRC2, SUZ12, and EED are required for both fetal and adult hematopoiesis. The hematopoietic specific knockout of either Suz12 or Eed in mice results in HSC exhaustion at the fetal or adult stage (34–36). Our data indicate that Ints11 and PRC2 share the molecular mechanisms in the regulation of HSPC function. However, we cannot exclude that the observed interactions could result from nonspecific hydrophobic or electrostatic interactions because of spatial proximity. Future studies are warranted to determine the specific domain of Ints11 mediating the interaction with PRC2 and evaluate the function of Ints11 upon modulation of PRC2 proteins in HSPCs.

A growing body of evidence suggests that individual INT subunits might have a function beyond their roles in the 14-subunit complex (37). For example, Ints3 and Ints6 have been shown to stably interact with human single-stranded DNA binding protein 1 and play crucial roles in the DNA damage response and homologous recombination-dependent repair of DNA double-strand breaks (38–42). In addition, Barbieri and colleagues (16) showed that Ints13 functions as an independent submodule to mediate activation of monocytic enhancers through EGR1 and NAB2. Other INT subunits, including Ints11, Ints1, and Ints6, were absent at the Ints13-specific sites (16). Our results suggest that Ints11 functions through the PRC2 complex and several INT subunits may be involved in the process. Increasing evidence suggests that PTMs, including methylation, phosphorylation, and acetylation on the PRC2 components, can affect PRC2 stability, assembly, and catalytic activity (43). However, the specific enzymes catalyzing most of these PTMs remain to be identified, and their biological significance is largely unknown. Further studies are needed to investigate how Ints11 stabilizes the PRC2 and whether other subunits’ loss influences the function of PRC2 in hematopoietic cells.

Our study shows the complex consequences of Ints11 loss for hematopoiesis and demonstrates its essential roles in HSC self-renewal and differentiation. Our findings reveal a novel noncanonical Ints11 function via a PRC2 complex in gene regulation in HSPCs.

**MATERIALS AND METHODS**

**Experimental design**

The objective of the study was to investigate the functions of Ints11 in HSPCs. A novel Ints11 conditional knockout mouse model was generated and used to determine the effects of Ints11 deletion in the hematopoietic system.

**Ints11 conditional knockout mouse model**

This mouse model was provided by R. Shiekhattar (University of Miami). Targeted Ints11 knockout mice were generated by replacing exon 2 sequences of the Ints11 gene with LoxP, neomycin, and FRT-containing cassette (fig. S1A). Ints11-targeted embryonic stem cell clones were expanded and injected into blastocystcs. The generated mice were then bred to Flp recombinase transgenic mice to create Ints11lox/+ mice. These mice were back-crossed to WT C57BL/6 mice for six rounds of breeding. Mx1-Cre, Vav1-Cre, and Boy1 (CD45.1+) mice were purchased from The Jackson Laboratory. Mx1Cre-induced gene deletion was done by intraperitoneal injection of poly(I:C) (5 mg/kg; InvivoGen) three times daily for three consecutive days. The genotyping PCR primers are listed in table S1. All animal studies were performed with approval from the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use.
Committee and conducted following institutional and national regulatory standards.

**Phenotypic analyses of mice**

PB was collected by tail vein bleeding and subjected to an automated blood count (Hemavet System 950FS, Drew Scientific). For histopathology analyses, sternums were fixed for >24 hours in 10% neutral buffered formalin at room temperature and demineralized in 12% EDTA for 2 weeks. The specimens were dehydrated using ethanol and cleared in xylene. The specimens were then embedded in melted paraffin and allowed to harden. Thin sections (4 to 5 μm) were cut and floated onto microscope slides. The slides were stained with hematoxylin and eosin (H&E) and visualized under a Keyence BZ-X810 microscope.

**Flow cytometry, cell sorting, and colony assays**

Single-cell suspensions from PB, BM, spleen, or fetal livers were stained with panels of fluorochrome-conjugated antibodies (table S2). Flow cytometric analysis of HSPCs was performed as previously described (44). The analyses were performed using a BD FACSCelesta or LSRFortessa flow cytometer (BD Biosciences). Apoptosis was assessed using the PE Annexin V Apoptosis Detection Kit (BD Biosciences). Briefly, freshly isolated BM cells were stained with lineage antibodies and phycoerythrin (PE)–annexin V/7-AAD followed by fluorescence-activated cell sorting (FACS) analysis within an hour of staining. For cell cycle analysis, BM cells were labeled with 5-bromo-2′-deoxyuridine (BrdU) for an hour in vitro, and BrdU incorporation was detected using fluorescein isothiocyanate (FITC)–conjugated anti-BrdU antibody followed by 7-AAD staining per the manufacturer’s protocol (BD Biosciences). All data were analyzed with FlowJo v10 software (Ashland, OR). For LK cell selection, magnetic-activated cell sorting was applied. BM cells were first isolated using the Direct Lineage Cell Depletion Kit (Miltenyi Biotech), and then the lineage-negative cells were sorted with cKit (CD117) MicroBeads. The purity of selected cells was routinely over 95%. For CFU-C assays, BM or fetal liver cells were plated in triplicate in methylcellulose medium (MethoCult M3134, STEMCELL Technologies) supplemented with mouse stem cell factor (mSCF; 100 ng/ml), interleukin-3 (mIL-3; 10 ng/ml), thrombopoietin (mTPO; 50 ng/ml), granulocyte-macrophage colony-stimulating factor (mGM-CSF; 10 ng/ml), human erythropoietin (hEPO; 4 U/ml), and interleukin-6 (hIL-6; 50 ng/ml, Peprotech). The colonies were then scored on day 7 of the cultures in an incubator at 37°C and 5% CO₂.

**Transplantation assay**

Competitive repopulation assay was performed by transplanting a mixture of 1 × 10⁵ 4-week-old BoyJ (CD45.1⁺) BM competitor cells and 1 × 10⁶ of either 4-week-old Mx1Cre⁺ or Mx1Cre⁺;Ints11flex/flex (CD45.2⁺) BM cells into lethally irradiated (9.5 Gy) BoyJ recipients through the tail vein injection. One month after the transplantation, poly(I:C) (10 mg/kg) was injected in the recipient mice to induce Ints11 deletion. Flow cytometric analyses were performed to monitor the chimerism by examining the percentages of CD45.1⁺ and CD45.2⁺ over 30 weeks after the poly(I:C) injection. In vivo rescue assay, BM cells were collected from 5-fluorouracil (5-FU; Selleckchem)–treated Mx1Cre⁺;Ints11flex/flex (CD45.2⁺) mice. Mice were given a single dose of 5-FU (150 mg/kg) by intraperitoneal injections and were sacrificed 48 hours after injection. Mononuclear cells were transduced with the packaged viruses in the culture medium supplemented with mSCF (100 ng/ml) and hIL-6 (50 ng/ml) and then transplanted into lethally irradiated BoyJ recipient mice (CD45.1⁺) through the tail vein injection. Four weeks after the transplantation, recipients were injected with a total of five doses of poly(I:C) (10 mg/kg, every other day) to induce Ints11 deletion.

**Quantitative PCR analysis**

Total RNA was extracted with TRIzol reagent (Invitrogen), and complementary DNA (cDNA) was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. qPCR was performed in triplicate using an Applied Biosystems QuantStudio 3 system with the Fast SYBR Green Master Mix (Applied Biosystems). The expression of gene of interest was calculated using the 2⁻ΔΔCt method by normalization to the housekeeping gene β-actin. All qPCR primers used are listed in table S1.

**Plasmid constructs, cell culture, and lentiviral transduction**

The full-length cDNAs of mouse Ints11, Ezh2, or Suz12 were tagged with 3×FLAG or HA and independently cloned into a lentiviral vector, as previously described (45). The human hematopoietic cell line K562 was obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. 293TN cells (System Biosciences) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and transfected with the plasmid of Ints11 full-length (FLAG-INTS11), an EV (FLAG-EV), HA-EZH2, or HA-SUZ12 using Lipofectamine 3000 (Thermo Fisher Scientific). The viral supernatant was harvested 48 hours after transfection. BM cKit⁺ cells purified from Ints11ΔΔ mice 72 hours after poly(I:C) injection were transduced with the packaged viruses, and the positive cells were then sorted for further experiments using a BD FACSAr IA machine.

**Western blot, IP, and GST pull-down assays**

Lysates from BM LK cells were prepared and then resolved on NuPAGE 4 to 12% bis-tris gels (Invitrogen). Immunoblotting was performed with the following antibodies (table S2): rabbit polyclonal anti-INTS11 (1:1000), rabbit polyclonal anti-H3K27me3 (1:1000), rabbit polyclonal anti-H3K27me2 (1:1000), rabbit polyclonal anti-H3K27me1 (1:1000), rabbit monoclonal anti-HA (1:1000), rabbit monoclonal anti-EZH2 (1:1000), rabbit monoclonal anti-SUZ12 (1:1000), rabbit monoclonal anti-EED (1:1000), rabbit monoclonal anti-EZH2 (1:1000), rabbit monoclonal anti-RBBP4 (1:800), rabbit monoclonal anti-p21Cipl (1:1000), rabbit monoclonal anti-mouse CTCF (1:1000), rabbit polyclonal anti-mouse HDAC1 (1:1000), mouse monoclonal anti-β-actin (1:4000), mouse monoclonal anti-mouse FLAG (1:1000), and mouse monoclonal anti-mouse HA (1:1000). After incubation with anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG antibodies conjugated with horseradish peroxidase (GE Healthcare), signals were detected using the Prometheus ProSignal ECL reagents (Genesee Scientific). Images were taken on a KwikQuant Imager system (Kindle Biosciences).

For the IP assays, nuclear extracts were prepared as previously described with the presence of benzazene (46). Lysates were incubated overnight with the indicated antibodies. After washing of the beads four times with IP buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 1% Triton X-100, and protease inhibitor cocktail], the associated proteins were collected and subjected to Western blot analysis. The chromatin-bound fraction and the soluble nuclear fraction were prepared using the Subcellular Protein Fractionation Kit (Thermo Fisher Scientific).
The full-length human INTS11 was cloned into the pET expression vector downstream of the GST tag. Escherichia coli strain BL21 (DE3) was transformed with pET-hINTS11 or the EV expressing GST alone. GST fusion proteins were purified on Pierce Glutathione Agarose beads (Thermo Fisher Scientific). An equal amount of GST protein was incubated with PRC2 recombinant proteins (table S2) in binding buffer [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 10 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors]. After washing the beads, the bounded proteins were analyzed by Western blotting.

**Size exclusion chromatography**

Chromatography was performed using 0.5 ml of nuclear extracts obtained from 293T cells expressing FLAG-INTS11 by fractionation on a Superose 6 Increase 10/300 GL column (Cytiva) in a buffer composed of 50 mM tris-HCl (pH 8.0) and 150 mM NaCl with a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected across the entire protein elution profile and analyzed by Western blotting.

**Bulk RNA-seq and data analysis**

BM LK cells from Ints11<sup>L/A</sup> and WT littermates were purified for 72 hours after first poly(I:C) injection (same time point applied in scRNA-seq and ChIP-seq). Total RNA from individual mice was isolated with TRIzol reagent (Invitrogen). The RNA libraries were prepared using the Illumina TrueSeq strand-specific mRNA sample preparation system followed by sequencing with a read length of 2 × 75 base pairs (bp) using Illumina NextSeq 500.

After being trimmed by Trimomatic (v0.38) (47), more than 135 million reads per sample were aligned to the mouse genome (GRCm38/mm10) using STAR (v2.7.0e) (48). The read raw counts of each gene were calculated by HTSeq (v0.11.2) (49). Then, the count matrix was used to identify differentially expressed genes by DESeq2 (50) with a cutoff of FDR < 0.05 and |fold change| > 1.8. The fragments per kilobase of transcript per million mapped (FPKM) matrix transformed by count matrix was used for GSEA (51).

**scRNA-seq and data analysis**

For scRNA-seq, the cKit<sup>+</sup> cells were pooled from two mice of each genotype. The libraries for each single-cell sample were prepared using the Chromium Single Cell 3’ Reagent Kit (10X Genomics) with the Chromium Controller. The libraries were sequenced on the Illumina NovaSeq 6000 sequencing system. Sequencing results were demultiplexed and converted to FASTQ format by CellRanger (v3.0.2; www.10xgenomics.com) with a parameter of mkfastq. The sequencing reads were aligned to the mouse reference genome (mm10) and quantified using CellRanger (v3.1.3) (66). Peak calling was performed with MACS2 (v2.1.2) (66) with the following options: -g mm -B -q 0.05. For histone modifications, peaks with high confidence (treatment/input > 5) were retained. The BAM files were normalized using deepTools (v3.1.3) (67) to generate a BigWig file for visualization with the following options: --normalize Using CPM --binSize 50. Differential peaks were detected by DiffBind (68) using the embedded method DESeq2 with the threshold of FDR < 0.05. ChIPpeakAnno (69) and ChiPseeker (70) were used to annotate the total or differential peaks. All figures were visualized in R and GraphPad Prism software.

**Statistical analysis**

Differences between experimental groups were determined by the log-rank test, unpaired two-tailed Student’s t test, or one-way analysis of variance followed by an appropriate post hoc correction. P values of less than 0.05 were considered significant. The statistical methods used for comparisons are indicated in the relevant figure legends.
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