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Ezh2 loss promotes development of myelodysplastic syndrome but attenuates its predisposition to leukaemic transformation

Goro Sashida^{1,2}, Hironori Harada^{3,†}, Hirotaka Matsui⁴, Motohiko Oshima^{1,2}, Makiko Yui^{1,2}, Yuka Harada^{5,†}, Satomi Tanaka^{1,6}, Makiko Mochizuki-Kashio^{1,2}, Changshan Wang^{1,2}, Atsunori Saraya^{1,2}, Tomoya Muto^{1,6}, Yoshihiro Hayashi^{7,8}, Kotaro Suzuki⁹, Hiroshi Nakajima⁹, Toshiya Inaba⁴, Haruhiko Koseki^{2,10}, Gang Huang^{7,8}, Toshio Kitamura¹¹ & Atsushi Iwama^{1,2}

Loss-of-function mutations of *EZH2*, a catalytic component of polycomb repressive complex 2 (PRC2), are observed in ~10% of patients with myelodysplastic syndrome (MDS), but are rare in acute myeloid leukaemia (AML). Recent studies have shown that *EZH2* mutations are often associated with *RUNX1* mutations in MDS patients, although its pathological function remains to be addressed. Here we establish an MDS mouse model by transducing a *RUNX1S291fs* mutant into hematopoietic stem cells and subsequently deleting *Ezh2*. *Ezh2* loss significantly promotes *RUNX1S291fs*-induced MDS. Despite their compromised proliferative capacity of *RUNX1S291fs/Ezh2*-null MDS cells, MDS bone marrow impairs normal hematopoietic cells via selectively activating inflammatory cytokine responses, thereby allowing propagation of MDS clones. In contrast, loss of *Ezh2* prevents the transformation of AML via PRC1-mediated repression of *Hoxa9*. These findings provide a comprehensive picture of how *Ezh2* loss collaborates with *RUNX1* mutants in the pathogenesis of MDS in both cell autonomous and non-autonomous manners.

¹Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. ²JST, CREST, 7 Gobancho, Chiyoda-ku, Tokyo 102-0076, Japan. ³Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. ⁴Division of Molecular Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. ⁵Division of Radiation Information Registry, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. ⁶Department of Hematology, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. ⁷Division of Pathology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3026, USA. ⁸Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3026, USA. ⁹Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. ¹⁰Laboratory for Lymphocyte Development, RIKEN Center for Integrative Medical Sciences, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. ¹¹Division of Cellular Therapy and Division of Stem Cell Signaling, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato, Tokyo 108-8639, Japan. † Present address: Department of Hematology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Correspondence and requests for materials should be addressed to G.S. (email: gorosashida@faculty.chiba-u.jp) or to A.I. (email: aiwama@faculty.chiba-u.jp).

Recent genome sequencing studies have identified various mutations of epigenetic regulators in patients with myeloid malignancies such as myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN) and acute myeloid leukaemia (AML)^{1–3}. Epigenetic alterations are involved in the establishment of gene expression profiles associated with cancers, and the accumulation of genetic and epigenetic alterations promote tumorigenesis^{4,5}.

Among epigenetic regulators, polycomb-group (PcG) proteins have been implicated in many types of cancer. PcG proteins compose the polycomb repressive complexes 1 and 2 (PRC1 and PRC2), and function to repress the transcription of genes through monoubiquitination at H2AK119 (H2AK119ub1) and trimethylation at H3K27 (H3K27me3), respectively^{6,7}. In the canonical pathway, PRC2 initiates gene silencing by catalysing H3K27me3 modification. PRC1 is then recruited to the target regions by binding to H3K27me3 through the CBX component of PRC1 (ref. 8), and then catalyses the monoubiquitination of H2AK119 to maintain gene silencing⁹. However, recent findings have shown that PRC1 can be recruited to target genes independently of H3K27me3 (refs 10,11). For example, the RUNX1 transcription factor has been shown to physically bind to Bmi1, a component of PRC1, and to recruit PRC1 in a PRC2-independent manner during the differentiation of megakaryocytes¹². Since overexpression or gain-of-function mutations of *EZH2*, a catalytic component of PRC2, are often found in carcinomas and lymphomas^{2,13}, PcG genes have traditionally been characterized as oncogenes⁵. Specifically, overexpression of *Ezh2* has been shown to lead to MPN¹⁴. We also reported that disease caused by MLL-AF9 requires *Ezh2* for progression into AML in a mouse model¹⁵. These findings highlight the oncogenic function of *EZH2* and are consistent with the findings that loss-of-function mutations of *EZH2* are rare in *de novo* AML patients^{2,16}. In contrast, ~10% of MDS patients harbour loss-of-function mutations in *EZH2* (refs 16,17), excluding –7/7q– chromosome anomalies, which involves *EZH2* located at 7q36. *EZH2* mutations in MDS patients are associated with significantly worse prognosis, which is not due to AML transformation^{1,18}. In addition, MDS patients without –7/7q– chromosome anomalies have reduced expression of *EZH2* in CD34⁺ cells¹⁹, suggesting that *EZH2* functions as a tumour suppressor in MDS. Indeed, we have recently shown that concurrent depletion of *Ezh2* and *Tet2* markedly accelerates the development of MDS and MDS/MPN in mice²⁰. However, much remains unknown how *EZH2* loss due to –7/7q– anomalies and loss-of-function mutations of *EZH2* promotes the development of MDS.

RUNX1 (also known as *AML1/CBFA2*), a transcription factor critical for hematopoiesis, is frequently mutated (10–20%) in MDS patients and its mutations are significantly associated with –7/7q– chromosome anomalies^{21,22}. Recent studies have demonstrated that *RUNX1* mutations are one of several alterations significantly associated with *EZH2* mutations in MDS patients and that both mutations independently predict poor prognosis^{1,18,23}. *RUNX1S291fs* mutant retains a DNA binding domain but lacks a transactivation domain in the carboxy terminus²². It has been demonstrated that transduction of *RUNX1S291fs* into bone marrow (BM) progenitor cells is sufficient to induce MDS and AML following MDS (MDS/AML) *in vivo*, although it takes a long latency²⁴.

Given these findings, we set about to understand the molecular mechanism underlying the pathogenesis of MDS. We established a novel MDS mouse model utilizing *Ezh2*-deficient hematopoietic stem cells (HSCs) and the *RUNX1S291fs* mutant. In this study, we found that *Ezh2* loss significantly promoted the transformation of HSCs expressing the *RUNX1S291fs* mutant into MDS cells *in vivo*. Of note, *RUNX1S291fs/Ezh2*-null hematopoietic

stem/progenitor cells (HSPCs) did not activate the expression of potent leukaemic oncogenes such as *Hoxa9* and *Mecom/Evi1* and showed compromised proliferative capacity, consistent with the impaired proliferative capacity of human MDS HSPCs²⁵. We also found *HOXA9* expression to be closely repressed in CD34⁺ HSPCs from the early stage MDS patients. While deregulation of HSC function is an important step for the development of myeloid malignancies²⁶, recent studies indicate that BM microenvironment is another critical factor for the development of myeloid malignancies including MDS^{27,28}. Chronic myeloid leukaemia (CML) cells also affect the function of BM microenvironment to impair normal hematopoiesis and support CML stem cell function^{29,30}. Here, despite the compromised proliferative capacity, *RUNX1S291fs/Ezh2*-null MDS cells outcompete residing wild-type (WT) cells in the MDS BM by activating inflammatory cytokine responses, including the interleukin-6 (IL-6) pathway, which have been shown to impair normal HSCs *in vivo*. Thus, the MDS BM environment negatively impacts normal HSCs, but not the MDS cells, thereby allowing propagation of MDS clones. Finally, we demonstrate that *Ezh2* loss is incompatible with the leukaemic transformation of MDS clones partly due to the inactivation of *Hoxa9* on loss of *Ezh2*.

Results

***Ezh2* loss promotes *RUNX1* mutant-induced MDS.** Given that *RUNX1* mutations are identified in ~30% of MDS patients harbouring *EZH2* loss-of-function mutations^{1,18,23}, we examined how *Ezh2* loss and *RUNX1* mutants collaborate in the development of MDS *in vivo*. To do so, we transduced a *RUNX1S291fs-IRES-GFP* or a control *empty-IRES-GFP* retroviral construct into either *Cre-ERT;Ezh2^{wt/wt}* or *Cre-ERT;Ezh2^{flox/flox}* (CD45.2⁺) CD34[–] Lin[–] Sca1[–] c-Kit⁺ (LSK) HSCs (Fig. 1a). The transduced cells were transplanted into lethally irradiated CD45.1⁺-recipient mice together with radioprotective CD45.1⁺ BM cells (Fig. 1a). The CD45.2⁺GFP⁺-transduced cells successfully engrafted and established significant levels of chimerism in the peripheral blood (PB) at 1 month post transplantation (Supplementary Fig. 1), and expressed the *RUNX1S291fs* protein (Fig. 1b). We then proceeded to activate *Cre-ERT* recombinase at 6 weeks post transplantation by intraperitoneal injection of tamoxifen (Fig. 1a). We hereafter refer to the recipient mice reconstituted with *Empty/Ezh2^{wt/wt}*, *Empty/Ezh2^{Δ/Δ}*, *RUNX1S291fs/Ezh2^{wt/wt}* and *RUNX1S291fs/Ezh2^{Δ/Δ}*-transduced HSCs as WT, *Ezh2^{Δ/Δ}*, Rx291 and Rx291/*Ezh2^{Δ/Δ}* mice, respectively (Fig. 1a). We confirmed successful deletions of *Ezh2* (Supplementary Fig. 2), and markedly reduced the levels of H3K27me3 in CD45.2⁺GFP⁺ Lin[–] c-Kit⁺ BM cells isolated from *Ezh2^{Δ/Δ}* and Rx291/*Ezh2^{Δ/Δ}* mice (Fig. 1b). Although levels of *RUNX1S291fs* transcripts were comparable between Rx291 and Rx291/*Ezh2^{Δ/Δ}* cells (Supplementary Fig. 3), level of *RUNX1S291fs* protein was much higher in Rx291/*Ezh2^{Δ/Δ}* cells (Fig. 1b), suggesting that *Ezh2* loss somehow impacts the levels of *RUNX1S291fs* protein post transcriptionally.

We first examined whether *Ezh2* loss contributes to the development of MDS in recipient mice. While WT mice ($n = 9$) and *Ezh2^{Δ/Δ}* mice ($n = 9$) did not develop lethal haematological malignancies within 10 months post transplantation, Rx291 and Rx291/*Ezh2^{Δ/Δ}* mice developed MDS. Rx291/*Ezh2^{Δ/Δ}* mice showed a significantly shorter median survival than Rx291 mice did (262 days versus undetermined, $P = 0.038$) (Fig. 1c), indicating that *Ezh2* loss facilitates disease progression. Although Rx291/*Ezh2^{Δ/Δ}* mice did not show any significant changes in the peripheral complete blood counts until 3 months post transplantation, they started to show reduced haemoglobin levels and increased mean corpuscular volume compared with WT mice at

Figure 1 | Ezh2 loss promotes formation of RUNX1S291fs mutant-induced MDS. (a) Experimental scheme of our model mouse utilizing RUNX1S291fs mutant and Ezh2 conditional knockout HSCs. (b) Successful transductions of RUNX1S291fs protein and levels of H3K27me3 in Lin⁺c-Kit⁺ cells detected by western blotting. α-Tubulin and histone H3 were detected as loading controls. (c) Significantly shorter median survival of Rx291/Ezh2^{Δ/Δ} mice ($n = 21$) compared with Rx291 mice ($n = 18$) (262 days versus undetermined, $P = 0.038$ by Log-rank test). (d) Complete blood cell counts of WT ($n = 7$), Ezh2^{Δ/Δ} ($n = 9$), Rx291 ($n = 15$) and Rx291/Ezh2^{Δ/Δ} ($n = 17$) mice at 4 months post transplantation (pre-MDS stage) and moribund Rx291/Ezh2^{Δ/Δ}-MDS mice ($n = 13$). Rx291/Ezh2^{Δ/Δ} mice showed reduced hemoglobin levels and increased mean corpuscular volume (MCV) compared with WT mice (12.4 ± 1.7 versus 13.6 ± 0.9 , $P = 0.047$ and 48.7 ± 3.2 versus 46.5 ± 0.7 , $P = 0.048$ by Student's t -test, respectively). Moribund Rx291/Ezh2^{Δ/Δ}-MDS mice showed significant leukopenia and macrocytic anaemia compared with Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage). Scale bars and asterisks show mean $\pm$ s.d., * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Student's t -test. (e) BM cell counts (from bilateral femurs and tibiae) in WT, Ezh2^{Δ/Δ}, Rx291 and Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage) and moribund Rx291/Ezh2^{Δ/Δ}-MDS mice. Comparable cell counts were seen in Rx291/Ezh2^{Δ/Δ} ($n = 10$) and Rx291/Ezh2^{Δ/Δ}-MDS mice ($n = 10$). Scale bars show mean $\pm$ s.d. P -value was determined by Student's t -test. (f) Appearance of dysplastic blood cells in Rx291/Ezh2^{Δ/Δ}-MDS mice observed by May-Grünwald Giemsa staining. Pseudo-Pelger-Huët cell (PB), hypersegmented neutrophil (PB), a giant platelet (PB), hypolobated megakaryocytes (BM) and nucleated red blood cells (PB) are depicted (clockwise from the upper left picture). Scale bars, 10 μm. ip, intraperitoneal.

4 months post transplantation (Fig. 1d). Ezh2^{Δ/Δ} mice showed a significant leukopenia, in part due to impaired lymphopoiesis as previously reported^{31,32}. While only 2 out of 18 Rx291 mice

developed MDS within 10 months post transplantation, 13 out of 21 Rx291/Ezh2^{Δ/Δ} mice developed MDS including one MDS/myeloid leukaemia (MDS/ML) showing very slow disease

Figure 2 | Ezh2 loss confers a competitive disadvantage to RUNX1S291fs-expressing HSPCs. (a) Chimerism of CD45.2⁺GFP⁺ cells in HSCs, LSK cells and LK cells in the BM and PB cells in WT, Ezh2^{Δ/Δ}, Rx291 and Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage) (*n* = 4–6) and moribund Rx291/Ezh2^{Δ/Δ}-MDS mice (*n* = 12). Scale bars and asterisks show mean ± s.d. and **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 by Student's *t*-test. (b) Chimerism of CD45.2⁺GFP⁺ of Mac1⁺ myeloid cells, B220⁺ B cells and CD4⁺/CD8⁺ T cells in the PB analysed in **a**. Scale bars show mean ± s.d. (c) Proliferative capacity examined with Ki67 in (CD45.2⁺GFP⁺) LSK and LK cells in WT, Ezh2^{Δ/Δ}, Rx291 and Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (*n* = 3–5) and moribund Rx291/Ezh2^{Δ/Δ}-MDS mice (*n* = 3). Scale bars and asterisks show mean ± s.e.m., **P* < 0.05 and ***P* < 0.01 by Student's *t*-test. (d) Enhanced apoptosis in CD71⁺Ter119⁺ Rx291/Ezh2^{Δ/Δ}-MDS erythroblasts but not in MDS LSK/LK stem and progenitor cells in WT, Ezh2^{Δ/Δ}, Rx291 and Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (*n* = 3–6) and moribund Rx291/Ezh2^{Δ/Δ}-MDS mice (*n* = 4). Scale bars and asterisks show mean ± s.e.m. and **P* < 0.05 by Student's *t*-test. (e) Clonogenic capacity of 500 (CD45.2⁺GFP⁺) LSK cells isolated from WT, Ezh2^{Δ/Δ}, Rx291 and Rx291/Ezh2^{Δ/Δ} mice at 4 months post transplantation (*n* = 3). Scale bars and asterisk show mean ± s.e.m. and ****P* < 0.001 by Student's *t*-test.

progression that did not match the criteria of 'acute' leukaemia in the Bethesda proposal (Fig. 1c and Supplementary Table 1)³³. Moribund Rx291/Ezh2^{Δ/Δ} mice with MDS including MDS/ML (Rx291/Ezh2^{Δ/Δ}-MDS mice) showed significant leukopenia and macrocytic anaemia (anaemia with increased mean corpuscular volume) compared with Rx291/Ezh2^{Δ/Δ} mice at pre-MDS stage (Fig. 1d). BM cellularity was variable among Rx291/Ezh2^{Δ/Δ}-MDS mice, implying heterogeneous disease status (Fig. 1e). Rx291/Ezh2^{Δ/Δ}-MDS mice also showed dysplastic cells characteristic of human MDS such as pseudo-Pelger-Huët cells, hypersegmented neutrophils, nucleated red blood cells, giant platelets and hypolobated megakaryocytes (Fig. 1f)³⁴. Thus, our model mice present with leukopenia, anaemia and multilineage dysplasia, phenotypically recapitulating human MDS. In addition, we also confirmed that Ezh2 loss significantly promotes the development of MDS induced by RUNX1D171N, another RUNX1 mutant defective in DNA binding, although RUNX1D171N-mutant cells exhibited a relatively long latency (D171N/Ezh2^{Δ/Δ} (*n* = 5) versus D171N/Ezh2^{wt/wt} (*n* = 7); 353 days versus undetermined, *P* = 0.0133 by Log-rank test). These findings indicate that Ezh2 can function as a tumour suppressor in MDS, especially in the presence of RUNX1 mutants.

Ezh2 loss provokes ineffective hematopoiesis. MDS patients show ineffective hematopoiesis characterized by impaired

differentiation and enhanced apoptosis of BM cells that leads to cytopenia³⁵. To evaluate hematopoiesis in Rx291/Ezh2^{Δ/Δ}-MDS mice, we first examined chimerism of CD45.2⁺GFP⁺ cells in HSCs (CD34⁺LSK), HSPCs (LSK) and Lin⁺c-Kit⁺Sca1⁺ (LK) myeloid progenitor cells in the BM and myeloid cells and lymphoid cells in the PB. While Ezh2^{Δ/Δ} and Rx291 mice showed comparable chimerism of CD45.2⁺GFP⁺ mutant cells in both BM and PB, both Rx291/Ezh2^{Δ/Δ} (at pre-MDS stage) and Rx291/Ezh2^{Δ/Δ}-MDS (at terminal stage) mice showed higher chimerism in BM HSCs and HSPCs, but contradictory lower chimerism in PB (Fig. 2a), due to impaired production of Mac1⁺ myeloid cells, CD4⁺/CD8⁺ T cells and B220⁺ B cells (Fig. 2b). These findings indicate that on deletion of Ezh2, RUNX1S291fs-expressing HSCs acquire a competitive disadvantage in terms of their ability to generate PB cells.

We then examined proliferative capacity of stem/myeloid progenitor cells by staining with Ki67, a marker for cellular proliferation. Transduction of RUNX1S291fs appeared to reduce Ki67⁺ frequencies in LK myeloid progenitor cells and in LSK cells, and this trend was even more evident in LK cells in Rx291/Ezh2^{Δ/Δ}-MDS mice (Fig. 2c). Thus, the proliferative capacity of Rx291/Ezh2^{Δ/Δ} cells was attenuated during the development of MDS, especially in myeloid progenitor cells. As Rx291/Ezh2^{Δ/Δ}-MDS mice also showed anaemia, we next examined the apoptosis of BM cells by Annexin V staining. Ezh2^{Δ/Δ} mice showed

Table 1 | Frequency of repopulating cells in RUNX1S291fs-induced cells.

	2×10^4 Lin ⁻ c-Kit ⁺	2000 Lin ⁻ c-Kit ⁺	200 Lin ⁻ c-Kit ⁺	Frequency of repopulating cells	95% CI
Rx291	0/6 mice	0/3 mice	0/3 mice	Undetermined	Undetermined
Rx291/Ezh2 ^{Δ/Δ}	2/7 mice	0/5 mice	0/6 mice	1/65,089	1/259,626-1/16,318

BM, bone marrow; CI, confidence interval; GFP, green fluorescence protein; MDS, myelodysplastic syndrome; PB, peripheral blood.
Frequency of repopulating cells was determined by transplantation using limiting doses of RUNX1S291fs-expressing (CD45.2⁺ GFP⁺) Lin⁻ c-Kit⁺ cells (2×10^4 , 2×10^3 and 200) isolated from Rx291 and Rx291/Ezh2^{Δ/Δ} mice at pre-MDS stage together with 2×10^5 competitor CD45.1⁺ BM cells. Mice showing chimerism of CD45.2⁺ GFP⁺ cells >1% in PB at 4 months post transplantation were considered to be positively repopulated mice.

Figure 3 | Ezh2 loss promotes initiation and propagation of RUNX1S291fs-induced MDS. (a) Representative flow cytometric profiles of LSK cells in recipient mice infused with 2×10^4 Rx291 or Rx291/Ezh2^{Δ/Δ} Lin⁻ c-Kit⁺ cells at 6 months post transplantation. Proportion of CD45.2⁺ GFP⁺ cells within LSK cells was indicated (%). (b) Dysplastic neutrophils, red blood cells and megakaryocytes in the BM of the positively repopulated recipient mice in a that developed MDS. Scale bar, 10 μ m. (c) Significantly shorter median survival of recipients of Rx291/Ezh2^{Δ/Δ}-MDS cells ($n=8$), compared with those of Rx291-MDS cells ($n=8$) (72.5 days versus 165 days; $P<0.0001$ by Log-rank test). Lethally irradiated recipient mice were infused with 1×10^6 CD45.2⁺ GFP⁺ RUNX1S291fs-expressing BM cells isolated from Rx291-MDS mice or Rx291/Ezh2^{Δ/Δ}-MDS mice together with 2×10^5 competitor CD45.1⁺ BM cells. (d) Chimerism of CD45.2⁺ GFP⁺ cells in Mac1⁺ myeloid cells, B220⁺ B cells and CD4⁺/CD8⁺ T cells in the PB in Rx291-MDS ($n=4$) and Rx291/Ezh2^{Δ/Δ}-MDS recipient mice ($n=7$) at the advanced MDS stage. Scale bars show mean $\pm$ s.e.m.

enhanced apoptosis especially in LSK cells (Fig. 2d). Rx291/Ezh2^{Δ/Δ}-MDS mice showed enhanced apoptosis in (CD45.2⁺ GFP⁺) CD71⁺ Ter119⁺ erythroblasts, but not in LK and LSK cells, compared with WT mice (Fig. 2d), implying that RUNX1S291fs mutant suppressed the Ezh2 loss-induced apoptosis in LSK cells. Previous studies have shown that the *in vitro* colony-forming capacity of HSPCs isolated from MDS patients and MDS mouse models is impaired^{25,36}. Consistent with these findings, we found that Rx291/Ezh2^{Δ/Δ} LSK cells generated significantly fewer colonies *in vitro* than WT and Rx291 LSK cells (Fig. 2e). Taken together, the impaired proliferative capacity of myeloid progenitor cells and enhanced apoptosis in erythroblasts may account for the cytopenia observed in Rx291/Ezh2^{Δ/Δ}-MDS mice. Thus, our mouse model also recapitulates ineffective hematopoiesis, one of the pathological features of MDS.

Ezh2 loss promotes initiation and propagation of MDS. Since Rx291/Ezh2^{Δ/Δ} HSPCs were capable of potent induction of MDS despite their impaired proliferative capacity and competitive disadvantage in generating PB cells, we examined the

repopulating capacity of Rx291 and Rx291/Ezh2^{Δ/Δ} cells in detail by employing a limiting dilution transplantation assay utilizing 2×10^4 , 2×10^3 and 200 CD45.2⁺ GFP⁺ Lin⁻ c-Kit⁺ cells isolated from the primary Rx291 and Rx291/Ezh2^{Δ/Δ} mice at pre-MDS stage together with 2×10^5 competitor CD45.1⁺ BM cells. We considered the recipient mice showing chimerism of CD45.2⁺ GFP⁺ cells >1% in PB at 4 months post transplantation as positively repopulated mice. Rx291 Lin⁻ c-Kit⁺ cells did not repopulate any mice in any experiments, whereas Rx291/Ezh2^{Δ/Δ} Lin⁻ c-Kit⁺ cells at a dose of 2×10^4 cells positively repopulated two out of seven recipient mice, allowing estimation of the frequency of repopulating cells to be between 1 in 259,626 and 1 in 16,318 Lin⁻ c-Kit⁺ cells (95% confidence interval)(Table 1). Notably, these Rx291/Ezh2^{Δ/Δ} cells repopulated in the positive mice outcompeted the WT competitor cells in the BM (Fig. 3a), and induced MDS by 6 months post transplantation as indicated by dysplastic neutrophils, nucleated red blood cells, hypolobated megakaryocytes in the BM (Fig. 3b), as well as mild macrocytic anaemia. Thus, loss of Ezh2 promotes the expansion of MDS-initiating cells in HSPCs expressing RUNX1S291fs at the ‘pre-MDS phase’.

Table 2 | GSEA for Ezh2 targets and AML-related targets.

	<i>Ezh2</i> ^{Δ/Δ}	<i>Rx291</i>	<i>Rx291/Ezh2</i> ^{Δ/Δ}	<i>Rx291/Ezh2</i> ^{Δ/Δ} -MDS	<i>Rx291/Ezh2</i> ^{Δ/Δ} -MDS/ML
Ezh2 targets	1.37/0.001	1.15/0.118	1.15/0.142	-1.41/0.001	-1.27/0.031
HSCs fingerprints	0.85/0.764	-2.03/0.000	-2.67/0.000	-2.73/0.000	-2.61/0.000
<i>MLL-AF9</i>	-1.62/0.000	-0.91/1.0	1.17/0.671	-1.52/0.009	-1.51/0.009
<i>NUP98-HOXA9</i>	1.20/0.104	-1.32/0.000	-1.85/0.000	-1.94/0.000	-1.97/0.000

AML, acute myeloid leukemia; FDR, false discovery rate; GSEA, gene set enrichment analysis; HSC, hematopoietic stem cell; MDS, myelodysplastic syndrome; ML, myeloid leukemia; NES, normalized enrichment score; WT, wild type.
GSEA for Ezh2 targets, HSC fingerprints, AML-related targets of *MLL-AF9* and *NUP98-HOXA9* in CD45.2⁺ GFP⁺ LSK cells from *Ezh2*^{Δ/Δ}, *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage) and moribund *Rx291/Ezh2*^{Δ/Δ}-MDS and MDS/ML mice, compared with those from WT mice. NES and FDR q-value are shown in each cell. Red and blue colours represent positive (upregulated in the given genotype relative to WT) and negative (downregulated in WT relative to the given genotype) enrichment, respectively. Concentrated colours show that the nominal P-value is less than 0.05 and FDR is less than 0.05, which suggest meaningful enrichment for the given gene sets. Pale colours show borderline enrichment with FDR q-value between 0.05 and 0.25. The white colour cells do not meet these criteria.

Since both *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice developed MDS albeit with different latencies (Fig. 1c), we next investigated whether *RUNX1S291fs*-induced MDS cells could propagate in secondary recipients in the absence of *Ezh2*. We transplanted 1 × 10⁶ CD45.2⁺ GFP⁺ *RUNX1S291fs*-expressing BM cells isolated from *Rx291*-MDS and *Rx291/Ezh2*^{Δ/Δ}-MDS mice independently into lethally irradiated CD45.1⁺ recipient mice together with 2 × 10⁵ CD45.1⁺ BM cells. We found that all secondary recipients infused with *Rx291/Ezh2*^{Δ/Δ}-MDS cells died from MDS within 100 days, whereas those with *Rx291*-MDS cells took much longer to develop lethal MDS (median survival, 72.5 days versus 165 days; *P* < 0.0001) (Fig. 3c). Furthermore, production of peripheral myeloid cells by *Rx291/Ezh2*^{Δ/Δ}-MDS cells appeared to be less efficient compared with *Rx291*-MDS cells at the advanced MDS stage (Fig. 3d), again suggesting ineffective hematopoiesis by *Rx291/Ezh2*^{Δ/Δ}-MDS cells. These findings indicate that *Ezh2* loss promotes both initiation and propagation of *RUNX1S291fs*-induced MDS.

Ezh2 target genes were repressed in *Ezh2*-null MDS HSPCs. To understand how *Ezh2* loss promotes *RUNX1S291fs*-induced MDS, we began to investigate gene expression profiles in CD45.2⁺ GFP⁺ LSK cells isolated from WT, *Ezh2*^{Δ/Δ}, *Rx291*, *Rx291/Ezh2*^{Δ/Δ} (at 4 months post transplantation), and two *Rx291/Ezh2*^{Δ/Δ}-MDS mice (MDS and MDS/ML). Since *Ezh2* functions to repress transcription via H3K27me3 modification, its absence is supposed to activate transcription of its target genes. As expected, gene set enrichment analysis (GSEA) revealed that *Ezh2*^{Δ/Δ} LSK cells significantly de-repressed canonical *Ezh2* target genes (Table 2 and Supplementary Fig. 4), defined in murine ES cells (GSE15388)³⁷. However, *Ezh2* targets were negatively enriched in both *Rx291/Ezh2*^{Δ/Δ}-MDS and MDS/ML LSK cells, compared with WT LSK cells (Table 2). Repression of *Ezh2* target genes was also evident when we compared *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells to *Rx291/Ezh2*^{Δ/Δ} LSK cells at the pre-MDS phase (Fig. 4a), indicating that *Ezh2* targets underwent transcriptional repression during the development of MDS despite the absence of *Ezh2*. This finding is similar to our previous observations on MDS developed in *Tet2*^{KD/KD} *Ezh2*^{Δ/Δ} mice compound for *Tet2* hypomorphic gene trap (*Tet2*^{KD/KD}) and conditional deletion of *Ezh2*, where repression of *Ezh2* target genes was attributed to the compensatory function of *Ezh1*, another catalytic component of PRC2 (ref. 20).

We next examined the expression of ‘HSC fingerprint genes’, a gene set specific to HSCs defined by extensive microarray

analyses³⁸. *RUNX1S291fs* repressed expression of ‘HSC fingerprint genes’ and this trend was more evident in *Rx291/Ezh2*^{Δ/Δ} and *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells (Table 2 and Supplementary Fig. 4). RT-PCR analysis confirmed down-regulation of ‘HSC fingerprint genes’ such as *Mecom/Evi1* (Fig. 4b), which is a well-known oncogene that plays a critical role for both HSCs and AML³⁹. Of note, expression of *Mecom/Evi1* became further repressed in *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells, compared with *Rx291/Ezh2*^{Δ/Δ} LSK cells (Fig. 4b), suggesting that some epigenetic events took place during the disease progression at the *Mecom/Evi1* region.

Promoter DNA hypermethylation is one of the most well-known epigenetic events that propagates during tumour development⁴⁰. We postulated that promoter DNA hypermethylation might also be involved in the transcriptional repression of *Ezh2* targets and ‘HSC fingerprint genes’ in the absence of *Ezh2*. To evaluate promoter DNA hypermethylation in LSK cells, we performed reduced representation bisulphite sequencing (RRBS). Compared with WT (CD45.2⁺ GFP⁺) LSK cells, we found 232, 512 and 204 hypermethylated promoters in *Rx291*, *Rx291/Ezh2*^{Δ/Δ} and *Rx291/Ezh2*^{Δ/Δ}-MDS (CD45.2⁺ GFP⁺) LSK cells, respectively (annotated gene lists are shown in Supplementary Data 1). Notably, the promoter regions of *Mecom/Evi1* acquired DNA hypermethylation in *Rx291/Ezh2*^{Δ/Δ} and *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells, but not in *Rx291* LSK cells, (Fig. 4c). This hypermethylation was coincided loss of H3K27me3 at the promoter on deletion of *Ezh2* (Fig. 4d). These findings imply that *Mecom/Evi1* is a direct target of *Ezh2* in this context. We next examined the correlation between canonical *Ezh2* targets in ES cells and genes that underwent DNA hypermethylation identified in this study. Some DNA hypermethylated genes overlapped with the canonical *Ezh2* targets in *Rx291/Ezh2*^{Δ/Δ} LSK cells (16 out of 374 genes, *P* = 0.886) and *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells (16 out of 168 genes, *P* = 0.0284), but not in *Rx291* LSK cells (Fig. 4e), and appeared to be developmental regulators of hematopoietic and non-hematopoietic lineages (for example, *Mecom/Evi1*, *Pdx1* and *Hand1*) (Supplementary Table 2), suggesting that aberrant promoter DNA hypermethylation is an alternative epigenetic mechanism that represses *Ezh2* targets in the absence of *Ezh2*.

***RUNX1S291fs/Ezh2*-null cells compromise normal HSPCs function.** Although *Rx291/Ezh2*^{Δ/Δ} HSPCs did not show enhanced proliferative capacity, *Rx291/Ezh2*^{Δ/Δ} cells were able to induce MDS at a lower dose compared with *Rx291* cells.

Figure 4 | *RUNX1S291fs/Ezh2*-null MDS HSPCs represses expression of *Ezh2* target genes. (a) Gene set enrichment plots for the canonical *Ezh2* target gene set comparing *Rx291/Ezh2*^{Δ/Δ}-MDS (left panel) and *Rx291/Ezh2*^{Δ/Δ}-MDS/ML (right panel) LSK cells with *Rx291/Ezh2*^{Δ/Δ} LSK cells at pre-MDS stage. (b) Quantitative RT-PCR analysis of the expression of *Mecom/Evi1* in CD45.2⁺ GFP⁺ LSK cells from in WT, *Ezh2*^{Δ/Δ}, *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage) and moribund *Rx291/Ezh2*^{Δ/Δ}-MDS mice (*n* = 4–5). Scale bars and asterisks show mean ± s.e.m., **P* < 0.05 and ***P* < 0.01 by Mann–Whitney *U*-test. (c) DNA methylation status at promoter and intron regions of *Mecom/Evi1* detected by RRBS in LSK cells from WT, *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice at 4 months post transplantation (pre-MDS stage) and from moribund *Rx291/Ezh2*^{Δ/Δ}-MDS mouse. The proportion of methylated (in blue) and non-methylated (in red) cytosines at mm9 position chr3:29911333 (downstream intron), chr3:30409294 and 30409295 (promoter) and chr3:30407526 (upstream) are depicted in circles. Blue arrowheads indicate regions amplified by ChIP–QPCR in d. (d) The levels of H3K27me3 at the promoter and intron regions of *Mecom/Evi1* in *Rx291* and *Rx291/Ezh2*^{Δ/Δ} LSK cells as detected by ChIP–QPCR. The relative amounts of immunoprecipitated DNA are depicted as a percentage of input DNA. Scale bars and asterisks show mean ± s.e.m. (*n* = 4), and **P* < 0.05 by Mann–Whitney *U*-test. (e) Venn diagrams of overlaps between canonical *Ezh2* target genes and DNA hypermethylated genes in *Rx291*, *Rx291/Ezh2*^{Δ/Δ} or *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells. Total number of genes was 13146. *P*-value of intersection was determined by using *R* code.

However, the mechanism by which the *Rx291/Ezh2*^{Δ/Δ} cells outcompeted WT cells was unclear. Given that BM environment is critical for the development of myeloid malignancies^{27,28}, we postulated that CD45.1⁺ WT cells in *Rx291/Ezh2*^{Δ/Δ} mice might be depleted via indirect mechanisms associated with MDS. We did not see obvious differences in the frequencies of phenotypic LSK HSPCs in CD45.1⁺ cells between *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice (*n* = 4; mean ± s.d. 0.0495 ± 0.0668% versus 0.0661 ± 0.0449%; *P* = 0.694 by Student's *t*-test). However, colony-forming cells were significantly reduced in CD45.1⁺ WT LSK cells isolated from *Rx291/Ezh2*^{Δ/Δ} mice, compared with those from *Rx291* mice and the primary WT LSK cells (Supplementary Fig. 5). To assess the repopulating capacity of CD45.1⁺ WT HSPCs *in vivo*, 2 × 10⁵ CD45.1⁺ WT BM cells

isolated from *Rx291* mice or *Rx291/Ezh2*^{Δ/Δ} mice were transplanted with an equal number of 8-week-old CD45.2⁺ competitor BM cells into lethally irradiated CD45.2⁺-recipient mice (Fig. 5a). CD45.1⁺ cells from *Rx291/Ezh2*^{Δ/Δ} mice scarcely contributed to LSK cells in BM and mature hematopoietic cells in PB at 4 months post competitive transplantation, whereas CD45.1⁺ cells from *Rx291* mice reconstituted hematopoiesis efficiently (Fig. 5b). These findings indicate that the function of WT HSPCs in *Rx291/Ezh2*^{Δ/Δ} mice is markedly impaired. To understand the underlying mechanism, we examined gene expression profiles of CD45.1⁺ LSK cells isolated from *Rx291* and *Rx291/Ezh2*^{Δ/Δ} mice. Recent studies indicated that inflammatory cytokines are critical factors to regulate normal HSCs under both homeostatic and stress conditions such as

Figure 5 | Impaired normal HSPC functions in RUNX1S291fs/Ezh2-null mice. (a) Experimental scheme of analysing function of CD45.1⁺ WT BM cells in Rx291 mice and Rx291/Ezh2 Δ/Δ mice. CD45.1⁺ WT (2×10^5) BM cells isolated from Rx291 and Rx291/Ezh2 Δ/Δ mice were transplanted into lethally irradiated CD45.2⁺ mice along with the same number of freshly isolated CD45.2⁺ WT (2×10^5) BM cells. (b) Repopulation capacity of CD45.1⁺ WT cells from Rx291 and Rx291/Ezh2 Δ/Δ mice. Chimerism of CD45.1⁺ Mac1⁺ myeloid cells in PB (left panel) and LSK cells (right panel) in BM at 4 months post transplantation is depicted ($n = 3$). Scale bars and asterisk show mean $\pm$ s.d. and $*P < 0.05$ by Student's *t*-test. (c) Gene set enrichment plots for IL-6 (ref. 54) (left panel) and TNF α (TNF receptor signalling, Pathway Interaction Database)(right panel) pathway gene sets comparing CD45.1⁺ WT LSK cells to CD45.2⁺ GFP⁺ mutant LSK cells in Rx291/Ezh2 Δ/Δ mice. (d) Quantitative RT-PCR analysis of the expression of *IL-6* in CD45.2⁺ GFP⁺ LSK cells from WT, Ezh2 Δ/Δ , Rx291 and Rx291/Ezh2 Δ/Δ mice at 4 months post transplantation (pre-MDS stage) and from moribund Rx291/Ezh2 Δ/Δ -MDS mice ($n = 4-5$). Scale bars and asterisk show mean $\pm$ s.d. and $*P < 0.05$ by Mann-Whitney *U*-test. (e) Levels of IL-6 and TNF α proteins in CD45.2⁺ GFP⁺ BM cells from WT, Ezh2 Δ/Δ , Rx291 and Rx291/Ezh2 Δ/Δ mice (at pre-MDS stage) ($n = 3-6$) when cultured with or without 10 ng ml^{-1} LPS for 5 hours *in vitro*. Expression levels were examined by flow cytometry and shown in mean fluorescence intensity (MFI). Scale bars and asterisk show means $\pm$ s.e.m and $*P < 0.05$ by Student's *t*-test. (f) Levels of IL-6 protein in the PB serum from WT, Ezh2 Δ/Δ , Rx291 and Rx291/Ezh2 Δ/Δ mice (at pre-MDS stage) and from moribund Rx291/Ezh2 Δ/Δ -MDS mice ($n = 6-8$). Expression levels were examined by enzyme-linked immunosorbent assay utilizing anti-IL-6 antibody and are shown as box and whisker plots, $*P < 0.05$ by Mann-Whitney *U*-test.

infection^{41,42}. GSEA revealed upregulation of genes related to interferon- γ and IL-6 pathways in CD45.1⁺ WT LSK cells from Rx291/Ezh2 Δ/Δ mice compared with WT LSK cells from WT mice (Supplementary Table 3), suggesting activation of inflammatory cytokine responses in residual normal HSPCs in Rx291/Ezh2 Δ/Δ mice. Furthermore, upregulation of IL-6 and tumor necrosis factor alpha (TNF α) pathways was evident when we compared CD45.1⁺ WT LSK cells with CD45.2⁺-mutant LSK

cells from the same Rx291/Ezh2 Δ/Δ mice (Fig. 5c), but not from Rx291fs mice (Supplementary Fig. 6). Indeed, Rx291/Ezh2 Δ/Δ -MDS (CD45.2⁺ GFP⁺) LSK cells significantly upregulated *IL-6*, compared with WT LSK cells (Fig. 5d). Given that IL-6 and TNF α impair normal HSC function *in vivo*⁴³, we examined levels of IL-6 and TNF α proteins in BM cells and circulating amount of IL-6 in PB serum. Levels of IL-6, but not TNF α , were significantly elevated in Rx291/Ezh2 Δ/Δ BM cells (Fig. 5e).

Stimulation of BM cells with an endotoxin, lipopolysaccharide (LPS), for 5 h *ex vivo*, increased the amount of not only IL-6 but also TNF α to levels significantly higher in *Rx291/Ezh2 Δ/Δ* BM cells compared with *Rx291* BM cells (Fig. 5e). In addition, *Rx291/Ezh2 Δ/Δ* mice at the MDS stage showed significantly elevated levels of circulating IL-6 protein in the PB serum (Fig. 5f). These findings indicate that *Rx291/Ezh2 Δ/Δ* -MDS cells, despite their compromised proliferative capacity, outcompete WT HSPCs by impeding their function via an inflammation-related mechanism in the MDS BM environment.

RUNX1S291fs helps PRC1 repress *Hoxa9* in the absence of *Ezh2*. MDS patients with *EZH2* mutations show low risk of transformation to AML and *EZH2* mutations are rare in *de novo* AML². Indeed, none of the *Rx291/Ezh2 Δ/Δ* mice developed typical *de novo* AML in this study. We therefore examined expression of target genes of *MLL-AF9* and *NUP98-HOXA9* fusions^{44,45} in LSK cells by GSEA, and found that *Rx291/Ezh2 Δ/Δ* -MDS LSK cells had negative enrichment of canonical AML target genes (Table 2 and Supplementary Fig. 7). Among these AML target genes, *Hoxa9* is critical for both HSCs and *MLL-AF9*-positive AML cells^{44,46}.

Figure 6 | RUNX1S291fs helps PRC1 repress *Hoxa9* expression in the absence of *Ezh2*. (a) Heat map showing expression profiles of *Hoxa* genes in CD45.2⁺GFP⁺ LSK cells from WT, *Ezh2 Δ/Δ* , *Rx291* and *Rx291/Ezh2 Δ/Δ* mice at 4 months post transplantation (pre-MDS stage) and from moribund *Rx291/Ezh2 Δ/Δ* -MDS and MDS/ML mice. (b) Quantitative RT-PCR analysis of the expression of *Hoxa9* and *Hoxa10* in LSK cells from WT, *Ezh2 Δ/Δ* , *Rx291* and *Rx291/Ezh2 Δ/Δ* mice at 4 months post transplantation (pre-MDS stage) and from moribund *Rx291/Ezh2 Δ/Δ* -MDS mice ($n = 4-5$). Scale bars and asterisk show mean $\pm$ s.d., * $P < 0.05$ and *** $P < 0.001$ by Student's *t*-test. (c,d) Levels of H3K27me3 (c) and H2AK119ub1 (d) at the promoters of *Hoxa* genes in *Rx291* and *Rx291/Ezh2 Δ/Δ* LSK cells as detected by ChIP-QPCR. The relative amounts of immunoprecipitated DNA are depicted as a percentage of input DNA. Scale bars and asterisks show mean $\pm$ s.e.m., * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by Student's *t*-test ($n = 4$). (e) Detection of RUNX1S291fs protein in RUNX1S291fs-expressing 32D cells by western blotting using an anti-RUNX1 antibody. α -Tubulin was detected as a loading control. Arrows indicate endogenous Runx1 and RUNX1S291fs proteins. (f) Recruitment of Bmi1 to the *Hoxa9* promoter in RUNX1S291fs-expressing 32D cells. Localization of Bmi1 at the *Hoxa9* promoter was evaluated in 32D cells and RUNX1S291fs-expressing 32D cells by ChIP-QPCR. The relative amounts of immunoprecipitated DNA are depicted as a percentage of input DNA. Scale bars and asterisks show means $\pm$ s.e.m., * $P < 0.05$ and ** $P < 0.01$ by Student's *t*-test ($n = 3$). (g) Derepression of *Hoxa9* by knockdown of *Bmi1* detected by quantitative RT-PCR. 32D cells expressing RUNX1S291fs were infected with lentiviruses expressing shRNA directed to a *Luciferase* control or *Bmi1*. Scale bars and asterisk show mean $\pm$ s.e.m., * $P < 0.05$ and *** $P < 0.001$ by Student's *t*-test ($n = 4$).

Expression of *Hoxa9* was downregulated in LSK cells expressing RUNX1S291fs mutant and further depressed by *Ezh2* loss at the MDS stage (Fig. 6a). RT-PCR confirmed that expression of *Hoxa9* as well as that of *Hoxa10*, another oncogene characteristic of AML, was significantly downregulated in *Rx291/Ezh2*^{Δ/Δ}-MDS LSK cells compared with WT LSK cells (Fig. 6b).

In contrast to *Mecom/Evi1*, promoter DNA hypermethylation was not involved in the repression of *Hoxa9* expression in *Rx291/Ezh2*^{Δ/Δ} LSK cells (Supplementary Fig. 8). We and other group recently reported that RUNX1 recruit PRC1 via direct binding to Bmi1 to establish transcriptional repression of genes in a context-dependent manner^{12,47}. We therefore hypothesized that the RUNX1S291fs mutant might collaborate with Bmi1 to suppress expression of *Hoxa9*. We first performed a chromatin immunoprecipitation (ChIP)-QPCR assay for H3K27me3 and H2AK119ub1 at promoters of the *Hoxa* gene cluster using *Rx291* and *Rx291/Ezh2*^{Δ/Δ} LSK cells. We found that levels of H3K27me3 were markedly reduced at *Hoxa9* and *Hoxa10* promoters in *Rx291/Ezh2*^{Δ/Δ} cells (Fig. 6c). By contrast, H2AK119ub1 was significantly enriched at the *Hoxa9* promoter in *Rx291/Ezh2*^{Δ/Δ} cells compared with *Rx291* cells (Fig. 6d). To determine whether Bmi1 binds to the promoter region of *Hoxa9*, we performed a ChIP-QPCR assay using an anti-Bmi1 antibody in murine 32D cells expressing RUNX1S291fs (Fig. 6e) due to the paucity of primary LSK cells. As expected, Bmi1 significantly bound to the *Hoxa9* promoter in RUNX1S291fs-expressing 32D cells compared with the control 32D cells (Fig. 6f). To determine whether the recruitment of Bmi1 contributed to transcriptional repression of *Hoxa9*, we transduced short hairpin RNA (shRNA) directed to *Bmi1* or *luciferase*, a negative control, in RUNX1S291fs-expressing 32D cells. Knockdown of *Bmi1* induced a significant elevation of *Hoxa9* expression in RUNX1S291fs-expressing 32D cells (Fig. 6g). We also observed that RUNX1S291fs mutant physically associates with Bmi1 in RUNX1S291fs-expressing 32D cells (Supplementary Fig. 9). Taken together, the RUNX1S291fs mutant promotes PRC1-mediated transcriptional repression of *Hoxa9* in the absence of *Ezh2*. While *Ezh2* loss alone did not significantly affect the levels of H3K27me3 at the promoter of *Hoxa9*, it induced a mild increase in the levels of H2AK119ub1 and moderately repressed the expression of *Hoxa9* (Fig. 6b and Supplementary Fig. 10). These findings imply that *Ezh2* loss could also lead to the transcriptional repression of *Hoxa9*, although the underlying mechanism is obscure.

Inactivation of *Hoxa9* restrains MDS from leukaemic formation.

We next interrogated the pathophysiological relevance of reduced *Hoxa9* expression to MDS. We first examined expression of *HOXA9* in CD34⁺ stem/progenitor cells isolated from RCMD (refractory cytopenia with multilineage dysplasia), RAEBI/II (refractory anaemia with excess blasts I/II), MDS/AML, *de novo* AML patients and healthy volunteers by quantitative RT-PCR. While the level of *HOXA9* expression in MDS-RCMD and *de novo* AML patients was comparable to the control CD34⁺ cells, it was significantly elevated to the levels higher than those of *MLL*-AF9-positive AML cell lines in both MDS-RAEBI/II and MDS/AML patients (Fig. 7a). Thus, *HOXA9* expression is robustly activated in high-risk MDS and MDS/AML patients, suggesting that the level of *HOXA9* expression is critical to the leukaemic transformation of MDS.

To test this hypothesis, we transduced ectopic *Hoxa9* into HSCs from WT and *Rx291/Ezh2*^{Δ/Δ} mice. To evaluate the proliferative capacity of *Hoxa9*-expressing *Rx291/Ezh2*^{Δ/Δ} HSCs, we first conducted serial replating assays *in vitro*. While *Rx291/Ezh2*^{Δ/Δ} cells did not form colonies after the second plating,

Hoxa9-expressing *Rx291/Ezh2*^{Δ/Δ} cells acquired the capability to give rise to colonies and efficiently generated colonies even after the fourth replating, similar to *Hoxa9*-expressing WT cells (Figs 7b,c). We next transplanted *Hoxa9*-expressing HSCs into lethally irradiated recipient mice together with life-saving 2×10^5 BM cells. Notably, two out of four recipients transplanted with *Hoxa9*-expressing *Rx291/Ezh2*^{Δ/Δ} cells developed AML with increased myeloblast cells (Fig. 7d), and died by 6 months post transplantation, while *Hoxa9*-expressing WT cells developed neither MPN nor AML within the observation period, possibly due to a longer latency⁴⁸. *Hoxa9*-expressing *Rx291/Ezh2*^{Δ/Δ} AML cells in the BM highly expressed both Mac1 and c-Kit antigens (Fig. 7e), similar to *MLL*-AF9-induced AML cells¹⁵. This phenotype was clearly distinct from *Rx291/Ezh2*^{Δ/Δ}-MDS cells in the BM (Fig. 7e). Taken together, the repressed *Hoxa9* expression inhibits the leukaemic transformation of *Rx291/Ezh2*^{Δ/Δ}-MDS cells.

Discussion

Our studies show that the loss of *Ezh2* promotes initiation and propagation of RUNX1 mutant-induced MDS, which supports a tumour-suppressive role for *EZH2* in MDS. Our mouse model with concurrent *Ezh2* loss and RUNX1S291fs expression developed MDS-like features such as anaemia, leukopenia, impaired myeloid cells differentiation and multilineage dysplasia. In addition, our mouse model showed an obvious resistance to transform into AML as reported that a few *de novo* AML patients show mutations of *EZH2* (refs 1,2,18). Indeed, our MDS mice showed a gene expression profile distinct from AML induced by *MLL*-AF9 or *NUP98-HOXA9* (refs 44,45). Interestingly, expression of *Hoxa9*, one of the most well-known leukaemic oncogenes, was dramatically repressed in our MDS mice in contrast to AML. It has been reported that *Hoxa9* deficiency impairs the proliferative capacity of HSCs, delays myeloid cell differentiation and decreases neutrophil counts⁴⁶, a phenotype that is quite similar to the ineffective hematopoiesis observed in *Rx291/Ezh2*^{Δ/Δ}-MDS mice, except for the dysplasia we observed. Although it remains unknown how downregulation of *Hoxa9* contributes to the development of MDS, we found that level of *HOXA9* expression in human CD34⁺ cells was clearly correlated with the disease progression of MDS in patients. Given that AML transformation was induced by the ectopic expression of *Hoxa9* in *Rx291/Ezh2*^{Δ/Δ} cells, we propose that the resistance to AML transformation in the absence of *Ezh2* is at least in part dependent on the repression of *Hoxa9* expression. Another prototypical HSC regulator that has also been shown to drive leukaemia is *MECOM/EVI1* (ref. 39). In line with our observations in this study, however, expression of *EVI1* is generally low in MDS patients⁴⁷. Downregulation of *Mecom/Evi1* expression may also contribute to the resistance to AML transformation in the absence of *Ezh2*. These findings indicate that our mouse model utilizing *Ezh2* loss and the RUNX1S291fs mutant accurately recapitulates many aspects of MDS harbouring *EZH2* mutations.

In MDS patients, mutant cells propagate in the BM despite impaired proliferative capacity and are thought to eventually outcompete normal hematopoietic cells³⁵. Our findings in *Rx291/Ezh2*^{Δ/Δ}-MDS mice provided a clue to understand the mechanism of clonal competition between MDS clones and normal cells in MDS patients. *Rx291/Ezh2*^{Δ/Δ}-MDS cells appeared to be defective in proliferative capacity as evidenced by the reduced expression of two potent leukaemic oncogenes, *Hoxa9* and *Mecom/Evi1*. Of interest, inflammatory cytokine pathways such as IL-6 pathway, which are inhibitory towards HSCs⁴² were significantly activated in residing normal HSPCs,

Figure 7 | *Hoxa9* expression level is critical for transformation from MDS to AML. (a) Quantitative RT-PCR analysis of the expression of *HOXA9* in CD34⁺ cells of seven MDS-RCMD, seven MDS-RAEB/II, five MDS/AML, four *de novo* AML patients, four *MLL-AF9*-positive AML cell lines (THP-1, NOMO-1, MOLM-13 and MONO-MAC1) and six healthy controls. Scale bars and asterisks show mean $\pm$ s.d., ** $P < 0.01$, and *** $P < 0.001$ by Mann-Whitney *U*-test. (b,c) Clonogenic capacity of *Rx291/Ezh2*^{Δ/Δ} cells overexpressing *Hoxa9*. WT and *Rx291/Ezh2*^{Δ/Δ} HSCs were transduced with a *Hoxa9* retrovirus and plated in methylcellulose media, then replated into the same medium every 14 days. The data are shown as mean $\pm$ s.e.m. ($n = 3$) in b. Expression of exogenous Flag-tagged *Hoxa9* protein in colony cells was detected by western blotting using an anti-Flag antibody in c. β -Actin was detected as a loading control. (d) Morphology of AML cells from BM of recipients infused with *Hoxa9*-expressing *Rx291/Ezh2*^{Δ/Δ} cells observed by May-Grünwald Giemsa staining. Scale bar, 20 μ m. (e) Fluorescence-activated cell sorting profiles of BM cells from mouse in d and *Rx291/Ezh2*^{Δ/Δ}-MDS mouse. (f) A proposed model of the role of *Ezh2* loss in the development of *RUNX1S291fs*-induced MDS.

but not in *Rx291/Ezh2*^{Δ/Δ}-mutant HSPCs in the same *Rx291/Ezh2*^{Δ/Δ} mice. Indeed, expression of IL-6 was significantly elevated in *Rx291/Ezh2*^{Δ/Δ} BM cells compared with *Rx291* BM cells at the level of both transcript and protein. *Rx291/Ezh2*^{Δ/Δ} BM cells exhibited heightened sensitivity to LPS stimulation, and the level of circulating IL-6 protein in the PB serum was significantly elevated in *Rx291/Ezh2*^{Δ/Δ} mice during the development of MDS. Excessive IL-6 production by MDS cells has also been shown to promote leukopenia and thrombocytosis through the activation of *Traf6* in a 5q-MDS mouse model⁴⁹. In addition, combined exposure of IL-6, CCL2 and TNF α has been reported to impair normal HSC function *in vivo* and inhibition of these cytokines rescues the impaired HSC function⁴³. Recent studies indicated that CML cells alter the BM microenvironment to impair normal HSCs, but favour CML stem cells function, at least in part, via activation of inflammatory cytokines^{29,30}. All these findings support that activation of inflammatory

cytokine responses largely accounts for compromised normal hematopoiesis in our MDS model. In our model, *Rx291/Ezh2*^{Δ/Δ}-MDS cells outcompete normal cells by activating inflammatory responses, thereby achieving clonal expansion despite their compromised proliferative capacity (Fig. 7f). Our findings provide additional evidence of non-autonomous cells mechanisms that promote the expansion of MDS clones in the MDS BM environment.

Since *Ezh2* functions to repress transcription via H3K27me3 modification, its absence could lead to the activation of its target genes⁷. However, we observed that *Ezh2* target genes were mostly kept transcriptionally repressed in *Rx291/Ezh2*^{Δ/Δ} HSPCs at the MDS stage. We have recently shown that *Tet2*^{KD/KD}*Ezh2*^{Δ/Δ} MDS LSK cells maintain the transcriptional repression of *Ezh2* targets via the complementary function of *Ezh1* (ref. 20). Similarly, we observed residual enrichments of H3K27me3 at specific genes (for example, *Hoxa11*), so the same mechanism may also operate

in *RUNX1S291fs/Ezh2*-null MDS cells. In this study, however, we identified two alternative epigenetic mechanisms underlying the repression of *Ezh2* targets in *RUNX1S291fs*-expressing cells (Fig. 7f). First, *Rx291/Ezh2*^{Δ/Δ} HSPCs showed promoter DNA hypermethylation at some *Ezh2* target genes, and this appeared to repress expression of important developmental regulator genes of both hematopoietic and non-hematopoietic lineages (for example, *Mecom/Evi1*, *Pdx1* and *Hand1*). As described above, expression of *MECOM/EVI1* is generally low in MDS patients and its upregulation may trigger leukaemic transformation^{24,47}. Our findings raise the possibility that promoter DNA hypermethylation is a major molecular mechanism that keeps *MECOM/EVI1* transcriptionally repressed in MDS patients. Second, we found that expression of *Hoxa9* was repressed through *RUNX1S291fs*-directed PRC1 recruitment in the absence of *Ezh2*. Importantly, *HOXA9* expression is closely repressed in MDS-RCMD patients irrespective of the *RUNX1* mutations. Given that repression of *Hoxa9* is critical to attenuate the predisposition of MDS to develop into AML, there are presumably several different ways to establish repression of *Hoxa9*, and it would be intriguing to address this issue. Nonetheless, the repressed *Hoxa9* expression appears to be a hallmark of MDS-specific epigenetic alteration.

In conclusion, our findings provide a comprehensive picture of how *Ezh2* loss functions to collaborate with *RUNX1* mutant in the pathogenesis of MDS in both cell autonomous and non-autonomous manners, and how it attenuates the predisposition of MDS clones to transform into AML. While many studies have demonstrated that *EZH2* functions as an oncogene in various tumours, we have experimentally demonstrated the tumour-suppressive function of *EZH2* in MDS *in vivo*. Since there are only a few mouse models available for human MDS³⁴, we hope that this MDS model will contribute to our understanding of the role of altered epigenetic regulations in the pathogenesis of MDS and to the innovation of novel therapies for MDS.

Methods

Mice. *Ezh2* conditional knockout (*Ezh2*^{lox/lox}) mice were previously described, and crossed with *Rosa26-Cre-ERT* mice for conditional deletion³². C57BL/6 mice congenic for the *Ly5* locus (CD45.1) were purchased from Sankyo-Lab Service. All experiments using the mice were performed in accordance with our institutional guidelines for the use of laboratory animals and approved by the Review Board for animal experiments of Chiba University (approval ID: 25-104) or Cincinnati Children's Hospital Medical Center.

Retroviral transduction and transplantation. *pMYs-empty control-IRES-GFP*, *pMYs-RUNX1S291fs-IRES-GFP* and *pMYs-RUNX1D171N-IRES-GFP* vectors were previously described²⁴. *CS-H1-shRNA-EF-1α-EGFP* vector expressing shRNA-directed *Bmi1* or *luciferase* were previously described⁵⁰. *Hoxa9* cDNA was kindly provided by Dr Takuro Nakamura, and the cDNA was subcloned into pMYs vector. Retrovirus infection into CD34⁺ LSK HSCs was performed as previously described⁵¹. Briefly, retrovirus-infected 50 HSCs were cultured in SF-O3 medium containing 50 ng ml⁻¹ mouse SCF and 50 ng ml⁻¹ human TPO for 3 days, and then these HSCs were intravenously injected in 8.5 Gy or 9 Gy irradiated CD45.1⁺ mice together with radioprotective 2 × 10⁵ CD45.1⁺ BM cells. At 6 weeks post transplantation, 1 mg tamoxifen was administered via intraperitoneal injection for 5 consecutive days to completely delete *Ezh2* alleles³².

Colony assay. Colony assays were performed in Methocult M3234 (Stem Cell Technologies) supplemented with 20 ng ml⁻¹ mouse SCF, 20 ng ml⁻¹ mouse IL-3, 20 ng ml⁻¹ human TPO and 2 units ml⁻¹ human EPO. The number of colonies was counted at day14 of culture. For replating assay, colonies were scored at day14 and pooled 3 × 10⁴ cells were replated into the same medium.

Cell culture. BM cells were stimulated with or without 10 ng ml⁻¹ LPS (*Escherichia coli* 0127:B8; Sigma-Aldrich) in 10% fetal bovine serum RPMI supplemented with 10 ng ml⁻¹ mouse IL-3 and GolgiStop (BD 554724) containing monensin, which inhibits intracellular protein transport processes.

Flow cytometry and antibodies. Flow cytometry and cell sorting were performed by utilizing the following monoclonal antibodies; CD45.2 (104), CD45.1 (A20), Gr1 (RB6-8C5), CD11b/Mac1 (M1/70), Ter119, CD71 (R17217), CD127/IL-7Rα (A7R34), B220 (RA3-6B2), CD4 (L3T4), CD8α (53-6.7), CD117/c-Kit (2B8), Sca1 (D7), CD34 (MEC14.7) and FcγRII-III (93)³². These antibodies were purchased from eBioscience or BioLegend. Lineage mixture solution contains Gr1, Mac1, B220, CD4, CD8α, Ter119 and IL-7Rα biotin-conjugated antibodies. Apoptotic cells were stained with anti-Annexin V-APC antibody (BD 550474) and with propidium iodide following staining cell surface markers to discriminate Lineage⁻ Sca1⁺ c-Kit⁺ cells or CD71⁺ Ter119⁺ cells. To evaluate cellular proliferation, cells were fixed and permeabilized (FIX & PERM, Invitrogen) according to the manufacturer's instruction, and then detected by using anti-Ki67-PE antibody (BD 556027) following staining cells by lineage mixture, streptavidin-APC-Cy7, Sca1 PE-Cy7 and c-Kit APC antibodies. To evaluate intracellular amounts of IL-6 and TNFα proteins, BM cells were fixed and permeabilized and then detected by using anti-IL-6 (BD 554401) and anti-TNFα (BD 557730) antibodies following staining cells with CD45.2 antibody. All flow cytometry analyses and cell sorting were performed on FACSARIAII or FACSCantoII (BD).

Quantitative RT-PCR. Quantitative RT-PCR was performed on a StepOnePlus Real time PCR System (Life Technologies) by using SYBR Premix Ex TaqII (Takara) or FastStart Universal Probe Master (Roche) with a Universal Probe Library (Roche) and primers shown in Supplementary Table 4. The expression levels of *HOXA9* and *GAPDH* were examined by using TaqMan Gene Expression Assays (Hs03929097_g1 and Hs00266821_m1, respectively; Life Technologies). All data are presented as relative expression levels normalized to either *Gapdh* or *GAPDH* expression.

Microarray analysis. Total RNA was extracted from ~2 × 10⁵ pooled BM LSK cells (isolated from two to four mice per each genotype except diseased mouse) by using an RNeasy Plus Mini Kit (Qiagen). A quantity of 20 ng of total RNA was mixed with spike-in controls using an Agilent One Colour Spike Mix Kit (Agilent), amplified and labelled with Cyanine 3 using a Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer's instructions. Microarray analysis using a SurePrint G3 Mouse GE Microarray 8 × 60 K kit (Agilent) was performed according to the manufacturer's instructions. The raw data were deposited in Gene Expression Omnibus under the accession number GSE50537.

Western blotting ChIP and quantitative RT-PCR. Antibodies for western blotting and immunoprecipitation were as follows: anti-H3 (Abcam, ab1791), anti-H3K27me3 (Millipore 07449), anti-Bmi1 (clone 8A9, kindly provided by Dr N. Nozaki, MAB Institute), anti-RUNX1 (Abcam, ab23980), anti-FLAG (M2, Sigma), anti-β-Actin (Santa Cruz, sc-47778), and anti-α-Tubulin (Santa Cruz, sc-5286). Uncropped raw scans of blots used in the figures are presented in Supplementary Fig. 11. ChIP assays were performed as previously described¹⁵. Briefly, 1 × 10⁵ pooled BM LSK cells were used for each immunoprecipitation. The following antibodies were used for the immunoprecipitation reactions: anti-H3K27me3 (Millipore 07449), anti-H2AK119ub1 (Cell Signaling, 8240), anti-Bmi1 (Bethyl A301694A) and pre-immune immunoglobulin G. DNA was amplified by quantitative RT-PCR on a StepOnePlus Real time PCR System (Life Technologies) using primers shown in Supplementary Table 4.

Cytokine assay. Amounts of mouse IL-6 in PB serum were measured by ELISA kit (BD Bioscience) according to the manufacturer's protocol.

RRBS. Total 500 ng genomic DNA extracted from LSK cells (isolated from two to three mice per each genotype and a disease mouse) were digested with MspI restriction enzyme that recognizes and cleaves CCGG tetranucleotide sequences irrespective of their methylation. DNA fragments were then subjected to end-repair and dA-tailing using an NEBNext DNA Library Prep Master Mix Set (NEB, Ipswich, MA), followed by the ligation with methylated adaptor oligo-DNA (Illumina, San Diego, CA). After the bisulphite conversion from un methylated cytosine to uracil by using an EZ DNA Methylation kit (Zymo Research, Irvine, CA) followed by agarose gel excision of fragments ranging from 150 bp to 400 bp, DNA fragments ligated with adaptors in both ends were amplified by 18 cycles of PCR using PE1.0 and PE2.0 oligo primers (Illumina). The generated library was sequenced by the 36 bp single-end protocol of GAIIX (Illumina) using two lanes of a flow cell. As un methylated cytosine of the library is converted to thymine during the PCR amplification, mapping of sequenced tags to murine reference genome (mm9, UCSC Genome Browser) was performed by Bismark software⁵², which can align bisulphite-converted tags to genome and evaluate their methylation simultaneously. In this study, methylation level was estimated in CpGs with ≥ 10 reads (713,353 CpGs per sample in an average). Statistical analysis was done by methyl kit⁵³. As promoters were defined as regions spanning - 2.5 to + 0.5 Kb from transcription start sites and containing ≥ 5 CpGs that can be evaluated as ≥ 10 reads, these promoter regions were taken into account for differential methylation analysis. The raw data were deposited in DDBJ under the accession numbers, DRA001143, DRA001144, DRA001145 and DRA001146.

MDS patient samples. This study comprises MDS–RCMD ($n = 7$), MDS–RAEBI/II ($n = 7$), MDS/AML ($n = 5$) and *de novo* AML ($n = 4$) patients at the Hiroshima University Hospital. CD34⁺ blood cells were isolated using a CD34 MicroBead kit and autoMACS system (Milteny Biotec, Germany). Patient anonymity was ensured, and the study was approved by the Institutional Review Committee at Hiroshima University. Patients gave written informed consent for the study according to the Declaration of Helsinki.

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Author contributions

G.S. designed the research, performed experiments, analysed results and wrote the manuscript; H.H., H.M. and Y.H. performed experiments and analysed results; M.Y., S.T., A.S. and Y.H. performed experiments; M.O., M.M.-K., C.W., T.M., K.S., H.N., T.I., G.H. and T.K. assisted in the experiments; H. K. provided mice; and A.I. designed the research, analysed results and wrote the manuscript.

Additional information

Accession codes: The microarray data has been deposited in the Gene Expression Omnibus under accession code GSE50537. The bisulphite sequencing data have been deposited in the DDBJ under the accession codes DRA001143, DRA001144, DRA001145 and DRA001146.

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