Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders

Tomoya Muto,1,2,3 Goro Sashida,1,4 Motohiko Oshima,1,4 George R. Wendt,1,10 Makiko Mochizuki-Kashio,1,4 Yasunobu Nagata,2 Masashi Sanada,5 Satoru Miyagi,1,4 Atsunori Saraya,1,4 Asuka Kamio,6 Genta Nagae,6 Chiaki Nakaseko,2,3 Koutaro Yokote,2 Kazuya Shimoda,7 Haruhiko Koseki,4,8 Yutaka Suzuki,9 Sumio Sugano,9 Hiroyuki Aburatani,6 Seishi Ogawa,5 and Atsushi Iwama1,4

Polycomb group (PcG) proteins are essential regulators of hematopoietic stem cells. Recent extensive mutation analyses of the myeloid malignancies have revealed that inactivating somatic mutations in PcG genes such as EZH2 and ASXL1 occur frequently in patients with myelodysplastic disorders including myelodysplastic syndromes (MDSs) and MDS/myeloproliferative neoplasm (MPN) overlap disorders (MDS/MPN). In our patient cohort, EZH2 mutations were also found and often coincided with tet methylcytosine dioxygenase 2 (TET2) mutations. Consistent with these findings, deletion of Ezh2 alone was enough to induce MDS/MPN–like diseases in mice. Furthermore, concurrent deletion of Ezh2 and Tet2 established more advanced myelodysplasia and markedly accelerated the development of myelodysplastic disorders including both MDS and MDS/MPN. Comprehensive genome-wide analyses in hematopoietic progenitor cells revealed that upon deletion of Ezh2, key developmental regulator genes were kept transcriptionally repressed, suggesting compensation by Ezh1, whereas a cohort of oncogenic direct and indirect polycomb targets became derepressed. Our findings provide the first evidence of the tumor suppressor function of EZH2 in myeloid malignancies and highlight the cooperative effect of concurrent gene mutations in the pathogenesis of myelodysplastic disorders.

Recent genomic studies have identified a series of recurrent somatic mutations in patients with myeloid malignancies, including myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), chronic myelomonocytic leukemia (CMML)—classified as an MDS/MPN—and acute myeloid leukemia (AML). Notably, these mutations frequently occur in epigenetic regulator genes such as tet methylcytosine dioxygenase 2 (TET2), isocitrate dehydrogenase 1 (IDH1), IDH2, DNA methyltransferase 3A (DNMT3A), and polycomb group (PcG) genes (Chung et al., 2012; Raza and Galili, 2012; Shih et al., 2012). PcG proteins form the polycomb repressive complexes (PRCs) 1 and 2. PRC2 contains three core subunits: SUZ12, one of the EED isoforms, and the histone methyltransferase EZH1 or EZH2, which...
is frequently involved in chromosomal abnormalities such as -7 and 7q-, and inactivating mutations of **EZH2** have also been identified in patients with MDS, MPN, and CMML—all clonal myeloid disorders originating from hematopoietic stem cells (HSCs; Ernst et al., 2010; Nikoloski et al., 2010). Of interest, other components of PRC2, **EED** and **SUZ12** appeared to be mutated in a manner similar to **EZH2**, although the frequencies of their mutations are much lower than those of **EZH2** mutations. The PRC2-related gene **ASXL1**, which plays an important role in the recruitment and/or stability of PRC2 (Abdel-Wahab et al., 2012), has also been shown to carry inactivating mutations in patients with MDS, MPN, and CMML (Abdel-Wahab et al., 2011; Bejar et al., 2011).

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**Figure 2.** Generation of mice reconstituted with Tet2<sup>lox/lox</sup> Ezh2<sup>fl/fl</sup>, and Tet2<sup>lox/lox</sup> Ezh2<sup>fl/fl</sup> hematopoietic cells. (A) A schematic diagram of the experimental process. 10<sup>5</sup> E14.5 fetal liver cells from Cre-ERT;WT, Cre-ERT;Tet2<sup>lox/lox</sup>, Cre-ERT;Ezh2<sup>fl/fl</sup>, and Cre-ERT;Tet2<sup>lox/lox</sup> Ezh2<sup>fl/fl</sup> mice were transplanted into lethally irradiated CD45.1 recipient mice. For deletion of **EZH2**, 100 µl tamoxifen (10 mg/ml) was intraperitoneally injected once a day for 5 consecutive days at 4 wk after transplantation. (B) Levels of H3K27me3 in LSK cells and GMPs. LSK cells and GMPs from BM of WT, Tet2<sup>lox/lox</sup>, Ezh2<sup>fl/fl</sup>, and Tet2<sup>lox/lox</sup> Ezh2<sup>fl/fl</sup> mice were analyzed by Western blotting using an anti-H3K27me3 antibody at 4 mo after transplantation. Levels of H3K27me3 were normalized to the amount of H3 and are indicated relative to WT control values. The levels of H3K27me3 in WT cells were arbitrarily set to 1. The representative data from two independent experiments are presented.
Although these mutations suggest a tumor suppressor function of PRC2-related genes in these diseases, both the impact of each PcG mutation and its interplay with coinciding mutations remain largely unknown.

Loss-of-function mouse models of Tet2, Dnmt3a, and Bap1, as well as gain-of-function mouse models of IDH1, have been reported to recapitulate some aspects of malignancies (Moran-Crusio et al., 2011; Challen et al., 2012; Dey et al., 2012; Sasaki et al., 2012). However, the role of PcG-related mutants has not yet been tested in mice. To understand the contribution of inactivating PcG mutations to the development of myeloid malignancies, we examined the Ezh2-deficient mice over longer periods of time and also tested the impact of concurrent depletion of Ezh2 and Tet2 on hematopoiesis.

RESULTS
Somatic mutations of PcG genes in myeloid dysplasia
In our cohort of 119 patients with myelodysplastic disorders, which includes MDS, CMML, and AML with myelodysplasia-related changes (AML/MRC), inactivating mutations in EZH2 and ASXL1 were detected in 8.4 and 16.8% of patients, respectively. Moreover, 3.4% of patients had deletion of EZH2 (located at 7q36) associated with -7 and 7q- chromosomal abnormalities (Fig. 1A and Table S1). Notably, 57.1% of these EZH2 mutations coexisted with TET2 mutations. Conversely, 34.8% of patients with TET2 mutations had coexisting EZH2 mutations (Fig. 1B). These findings suggest a link between EZH2 and TET2 mutations in the pathogenesis of myelodysplastic disorders.

Deletion of Ezh2 results in enhanced repopulating capacity of HSCs and promotes myeloid-biased repopulation
To decipher the pathological role of inactivating EZH2 mutations and concurrent inactivation of EZH2 and TET2 genes in malignant stem cell disorders, we crossed Cre-ERT;Ezh2KD/KD mice (Mochizuki-Kashio et al., 2011) and Tet2 gene trap mice (Tet2KD/KD; Shide et al., 2012). Tet2KD/KD mice, in which the gene trap vector is inserted into exon 2 of Tet2 just before the first coding exon, express Tet2 mRNA at levels ~20% of those of the WT mice and frequently die by postnatal day 3 (Shide et al., 2012). Considering the early death of Tet2KD/KD mice and a necessity to exclude the influence of the loss of Tet2 and Ezh2 in BM niche cells, we transplanted E14.5 fetal liver cells from Cre-ERT control (WT), Cre-ERT;Tet2KD/KD, Cre-ERT; Ezh2KD/KD, and Cre-ERT;Tet2KD/KD;Ezh2KD/KD CD45.2 mice into lethally irradiated CD45.1 recipient mice and deleted Ezh2 by intraperitoneal injection of tamoxifen at 4 wk after transplantation (Fig. 2A). We hereafter refer to the recipient mice reconstituted with Tet2KD/KD, Ezh2KD/KD, and Tet2KD/KD;Ezh2KD/KD mice as Tet2KD/KD, Ezh2KD/KD, and Tet2KD/KD;Ezh2KD/KD mice, respectively. EZH2 is a catalytic component of PRC2 that catalyzes the methylation of H3K27. As expected, the levels of H3K27me3 were markedly reduced upon deletion of Ezh2 in both Lineage−“Sca-1”c-Kit+ (LSK) cells, which include HSCs and multipotent progenitor cells (MPPs), and in granulocyte-macrophage progenitors (GMPs; Fig. 2B). TET2, a methylcytosine dioxygenase, catalyzes the oxidation of 5-mC (5-methylcytosine) to 5-hmC (5-hydroxymethyl cytosine), the first step of active demethylation (Ernst et al., 2010; Ko et al., 2010). The levels of 5-hmC in total BM cells were also reduced in Tet2KD/KD and Tet2KD/KD;Ezh2KD/KD, whereas there was no obvious
difference in the levels of 5-mC among each genotype (unpublished data).

To explore the consequence of loss of Ezh2 and/or Tet2 in hematopoietic stem/progenitor cells, we first performed competitive repopulating assays using LSK cells recovered from the recipient mice at 3 mo after deletion of Ezh2. It has been reported that Tet2 loss leads to increased HSC self-renewal and myeloid transformation (Moran-Crusio et al., 2011; Shide et al., 2012). As expected, Tet2KD/KD LSK cells readily established progressively increasing chimerism in the PB over time (Fig. 3A). Similarly, the contribution of Ezh2Δ/Δ and Tet2KD/KD Ezh2Δ/Δ LSK-derived cells to the myeloid compartment of the PB increased over time, although the total chimerism was lower than that of Tet2KD/KD cells because of insufficient production of lymphocytes by Ezh2-deficient LSK cells (Fig. 3, A and B). Indeed, LSK cells of all genotypes established significantly higher chimerism in the BM LSK fraction compared with WT LSK cells (Fig. 3C). All mutant cells taken from the primary recipients continued to propagate in the secondary recipients during serial transplantation and exhibited myeloid-biased differentiation (Fig. 3D). These results reveal that the loss of Ezh2 augments the repopulating capacity of HSCs and activates the production of myeloid cells in much the same way as the loss of Tet2 does.

Deletion of Ezh2 causes myeloid dysplasia in mice

We next analyzed the hematopoiesis in recipient mice reconstituted with Tet2KD/KD, Ezh2Δ/Δ, and Tet2KD/KDEzh2Δ/Δ hematopoietic cells. (A) PB cell counts in recipients reconstituted with Tet2KD, Ezh2Δ/Δ, and Tet2KD/Ezh2Δ/Δ fetal liver hematopoietic cells at 12 wk after deletion of Ezh2. White blood cell (WBC), hemoglobin (Hb), and platelet (Plt) counts in the PB from WT (n = 19), Tet2KD (n = 23), Ezh2Δ/Δ (n = 13), and Tet2KD/Ezh2Δ/Δ (n = 23) mice are plotted as dots and the mean values are indicated as bars. (B) The hematopoietic compartment of the PB assessed by manual differential cell counts, performed at 12 wk after deletion of Ezh2. WT (n = 5), Tet2KD (n = 7), Ezh2Δ/Δ (n = 5), and Tet2KD/Ezh2Δ/Δ (n = 6) mice are represented as mean ± SEM. Arrowheads indicate statistically significant populations compared with WT (P < 0.05). (C) Smear preparation of PB from WT, Tet2KD, Ezh2Δ/Δ, and Tet2KD/Ezh2Δ/Δ mice at 12 wk after deletion of Ezh2 observed after May–Giemsa staining. Bar, 10 µm. Hyposegmented neutrophils consistent with a pseudo Pelger–Huët anomaly (bottom right), hypersegmented neutrophils (bottom left), and dysplastic monocyte (top right) are depicted in insets. Bar, 5 µm. (D) BM analysis of WT, Tet2KD, Ezh2Δ/Δ, and Tet2KD/Ezh2Δ/Δ mice at 12 wk after deletion of Ezh2. Absolute numbers of total BM cells from a unialteral part of the femur and the tibia of WT (n = 7), Tet2KD (n = 9), Ezh2Δ/Δ (n = 8), and Tet2KD/Ezh2Δ/Δ mice (n = 7) are plotted as dots and mean values are indicated as bars (left). The percentage of LSK cells (middle) and myeloid progenitors (right) are presented as mean ± SEM (WT, n = 7; Tet2KD, n = 6; Ezh2Δ/Δ, n = 6; Tet2KD/Ezh2Δ/Δ, n = 6). (E) Extramedullary hematopoiesis in the spleen of Tet2KD, Ezh2Δ/Δ, and Tet2KD/Ezh2Δ/Δ mice at 12 wk after deletion of Ezh2. Spleen weights of WT (n = 8), Tet2KD (n = 7), Ezh2Δ/Δ (n = 8), and Tet2KD/Ezh2Δ/Δ mice (n = 7) are plotted as dots and mean values are indicated as bars (left). Percentage of LSK cells (middle) and myeloid progenitors (right) are presented as mean ± SEM (WT, n = 5; Tet2KD, n = 5; Ezh2Δ/Δ, n = 7; Tet2KD/Ezh2Δ/Δ, n = 4), * P < 0.05; ** P < 0.001; *** P < 0.0001.
Tet2<sup>KD/KD</sup> mice showed monocytosis in PB as reported previously (Moran-Crusio et al., 2011), whereas Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> mice did not (Fig. 4 B). Cytological analysis of Ezh2<sup>Δ/Δ</sup> and Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> PB revealed morphological abnormalities in myeloid cells, such as delayed maturation of neutrophils, hyposegmented neutrophils consistent with a pseudo Pelger-Huët anomaly, hypersegmented neutrophils, and dysplasia of monocytes (Fig. 4 C). This trend was more evident in Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> PB compared with Ezh2<sup>Δ/Δ</sup> PB but was not obvious in Tet2<sup>KD/KD</sup> PB (Fig. 4 C).

BM analysis at 3 mo after deletion of Ezh2 revealed that although total BM cell numbers were mildly increased only in Tet2<sup>KD/KD</sup> mice, all mutant mice had a greater proportion of LSK cells than the WT mice (Fig. 4 D). Similarly, Tet2<sup>KD/KD</sup>, Ezh2<sup>Δ/Δ</sup>, and Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> mice had a greater proportion of common myeloid progenitors (CMPs) and/or MEPs (Fig. 4 D). Furthermore, extramedullary hematopoiesis was evident in the spleen of all mutant mice as defined by a significant increase in the absolute number of LSK cells and myeloid progenitors, although splenomegaly was evident only in Tet2<sup>KD/KD</sup> mice, likely because of impaired lymphopoiesis in the absence of Ezh2 (Fig. 4 E).

**Ezh2<sup>Δ/Δ</sup> and Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> mice develop myelodysplastic disorders**

During a long observation period, we found that not only Tet2<sup>KD/KD</sup> mice but also Ezh2<sup>Δ/Δ</sup> mice die of hematological disorders. This is the first study to describe the lethal hematologic phenotypes caused by loss of Ezh2. Tet2<sup>KD/KD</sup> mice developed CMMMLike disease accompanied by monocytosis and extramedullary hematopoiesis as reported previously (Moran-Crusio et al., 2011) and half of the mice died by 10 mo after transplantation. Notably, Ezh2<sup>Δ/Δ</sup> mice also developed MDS/MPN-like disease and half of the mice died by 10 mo after transplantation (Fig. 5 A and Table S2). They showed myelo proliferative features characterized by active extramedullary

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**Figure 5. Ezh2<sup>Δ/Δ</sup> mice develop myelodysplastic disorders.**

(A) Kaplan-Meier survival curve of WT (n = 24), Tet2<sup>KD/KD</sup> (n = 28), Ezh2<sup>Δ/Δ</sup> (n = 23), and Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup> (n = 32) mice. Some of the mice (WT, n = 11; Tet2<sup>KD/KD</sup>, n = 11; Ezh2<sup>Δ/Δ</sup>, n = 11; Tet2<sup>KD/KD</sup>Ezh2<sup>Δ/Δ</sup>, n = 13) were sacrificed for analyses in the middle of observation (indicated by short vertical bars on the curves). These survival curves were generated from two to four independent experiments, n.s., not significant. (B) Hematoxylin and Eosin (H&E) staining of paraffin-embedded sections of spleen. Bars, 500 µm. (C) Spleen weights plotted as dots with the mean values indicated as bars (WT, n = 3; Tet2<sup>KD/KD</sup>, n = 4; and Ezh2<sup>Δ/Δ</sup>, n = 3). (D) The percentage of LSK cells and myeloid progenitors in the spleen presented as mean ± SEM (WT, n = 5; Tet2<sup>KD/KD</sup>, n = 4; and Ezh2<sup>Δ/Δ</sup>, n = 3). (E) Absolute numbers of total BM cells from a unilateral pair of the femur and the tibia are plotted as dots and mean values are indicated as bars. (F) Percentage of LSK cells and myeloid progenitors in the BM presented as mean ± SEM. The numbers of mice analyzed are WT, n = 3; Tet2<sup>KD/KD</sup>, n = 4; and Ezh2<sup>Δ/Δ</sup>, n = 3. (G) Hemoglobin contents in PB are plotted as dots and mean values are indicated as bars (left). Percentage of Annexin V<sup>+</sup> cells in Ter119<sup>hi</sup>CD71<sup>hi</sup> BM erythroblasts is also shown as mean ± SEM from three independent experiments. The numbers of mice analyzed are WT, n = 3; Tet2<sup>KD/KD</sup>, n = 3; and Ezh2<sup>Δ/Δ</sup>, n = 3 (right). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
hematopoiesis in the spleen as evident from splenomegaly with a marked increase in LSK cells and MEPs resulting in effacement of lymphoid follicles (Fig. 5, B–D). They also showed a significant increase in LSK cells and MEPs in the BM, whereas they did not show obvious monocytosis in the PB (Fig. 5, E and F; and Table S2). They were anemic and showed increased apoptosis in Ter119highCD71high erythroblasts (Fig. 5 G) and LSK cells (Fig. 6 E) in the BM, suggesting ineffective erythropoiesis, a feature compatible with myelodysplastic disorders. Ezh2ΔΔ mice also showed dysplasia of myeloid cells as described above (Fig. 4 C).

To our surprise, concurrent deletion of Tet2 and Ezh2 significantly shortened the latency of disease development and all Tet2KD/KDEzh2ΔΔ mice died by 10 mo after transplantation (Fig. 5 A). Histological analysis of moribund Tet2KD/KDEzh2ΔΔ mice revealed lethal pneumonia in all mice analyzed (n = 6; unpublished data). Tet2KD/KDEzh2ΔΔ mice developed heterogeneous diseases which were classified into two types of myeloid neoplasms: (1) MDS/MPN including CMML (n = 8) and (2) MDS (n = 6; Table S2). MDS/MPN mice showed myeloproliferative features, including CMML-like monocytosis in the PB (Fig. 6 A and Table S2) and/or splenomegaly with extramedullary hematopoiesis (Fig. 6 B), and an increase in LSK cells in the BM (Fig. 6 C). In contrast, MDS mice did not show obvious myeloproliferative features but showed a trend of pancytopenia (Fig. 6 A and Table S2). Myeloid dysplasia, including delayed maturation of neutrophils, a pseudo Pelger-Huët anomaly, hypersegmented neutrophils, and dysplasia of monocytes, was evident in Tet2KD/KDEzh2ΔΔ mice compared with Tet2ΔΔ and Ezh2ΔΔ mice as described above (Fig. 4 C). Furthermore, MDS and MDS/MPN mice had a lower proportion of Ter119highCD71high BM erythroblasts shown as mean ± SEM from three independent experiments (WT, n = 3; MDS/MPN, n = 2; MDS, n = 2). The proportion of Annexin V+ cells in LSK cells from the indicated mice at 8 mo after transplantation shown as mean ± SEM from three independent experiments (WT, n = 3; Tet2KD/KD, n = 3; Ezh2ΔΔ, n = 3; and Tet2KD/Ezh2ΔΔ, n = 3), *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Figure 6.** Tet2KD/KDEzh2ΔΔ mice develop myelodysplastic disorders. (A–C) Hematological data from moribund Tet2KD/KDEzh2ΔΔ mice and those surviving at 300 d after transplantation. PB cell counts and the number of monocytes in the PB (A), spleen weights (B), and absolute numbers of total BM cells from a unilateral pair of the femur and the tibia (C, left) are plotted as dots and mean values are indicated as bars. Percentage of LSK cells (C, middle) and myeloid progenitor cells (C, right) are presented as mean ± SEM. Data were collected from WT (n = 8), MDS/MPN (n = 8), and MDS (n = 6) mice for spleen weights and WT (n = 11), MDS/MPN (n = 7), and MDS (n = 6) mice for others. (D) Percentage of Annexin V+ cells in Ter119highCD71high BM erythroblasts shown as mean ± SEM from three independent experiments (WT, n = 3; MDS/MPN, n = 2; MDS, n = 2). (E) Percentage of Annexin V+ cells in LSK cells from the indicated mice at 8 mo after transplantation shown as mean ± SEM from three independent experiments (WT, n = 3; Tet2KD/KD, n = 3; Ezh2ΔΔ, n = 3; and Tet2KD/Ezh2ΔΔ, n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
understand the consequences of the loss of Ezh2 and/or Tet2, we next performed gene set enrichment analysis (GSEA; Subramanian et al., 2005). Embryonic stem (ES) cell–like signatures, which have been widely used in assessment of cancer gene signatures, are subdivided into a PRC module, a Myc module, and a core ES module (Kim et al., 2010). Among these, the Myc module, which is active in various cancers and predicts cancer outcome, was significantly enriched in Ezh2Δ/Δ LSK cells and became highly enriched in Tet2Δ/ΔΔ Ezh2Δ/Δ LSK cells during the development of MDS/MPN and MDS (Fig. 8 A). Of note, the Myc module became enriched in LSK cells but not in GMPs, suggesting the existence of pathological stem cells in the LSK fraction. Several modules related to enhanced cell cycle were also positively enriched in MDS/MPN and MDS LSK cells (unpublished data). We next analyzed gene sets of Ezh2 targets that lose H3K27me3 upon deletion of Ezh2 in ES cells (ES_Ezh2 targets) and Ezh1 targets profiled in Ezh2Δ/−/− ES cells (ES_Ezh1 targets; Shen et al., 2008). As expected, all of the PRC2 gene sets showed a trend of positive enrichment (derepression) in Ezh2Δ/Δ and Tet2Δ/ΔΔ Ezh2Δ/Δ LSK cells and GMPs (Fig. 8 A). Notably, however, Ezh1 targets were negatively enriched (repressed) and the degree of positive enrichment of Ezh2 targets was attenuated in Tet2Δ/ΔΔ Ezh2Δ/Δ LSK cells from mice which developed myelodysplastic disorders. These data suggest compensatory action by Ezh1 in LSK cells during the development of myelodysplastic disorders. Of interest, both the Ezh1 and Ezh2 targets were positively enriched even in Tet2Δ/ΔΔ LSK cells, suggesting some involvement of Tet2 in PRC2-mediated gene silencing in HSC/MPPs.

Alterations in H3K27me3 upon deletion of Ezh2

To assess the genome-wide effects of loss of Ezh2 and/or Tet2 on epigenome, we conducted chromatin immunoprecipitation sequencing (ChIP-seq) for H3K27me3 in hematopoietic cells from WT, Tet2Δ/ΔΔΔ Ezh2Δ/Δ, and Tet2Δ/ΔΔΔ Ezh2Δ/Δ mice at 4 mo after transplantation. Due to the paucity of LSK HSC/MPPs, we used GMPs for ChIP-seq analysis and characterized the H3K27me3 levels over the region from 5.0 kb upstream to 0.5 kb downstream of transcriptional start sites (TSSs) of Reference Sequence (RefSeq) genes (Pruitt et al., 2007). As expected, drastic reductions in the levels of H3K27me3 were observed in GMPs from Ezh2Δ/Δ and Tet2Δ/ΔΔΔ Ezh2Δ/Δ mice, although considerable levels of H3K27me3 remained even in the absence of Ezh2 (Fig. 8 B). Among PcG targets (greater than fourfold enrichment of H3K27me3 compared with input in WT GMPs) that showed greater than twofold reduction of H3K27me3 levels in Tet2Δ/ΔΔΔ Ezh2Δ/Δ GMPs, 121 genes were derepressed greater than twofold in both MDS/MPN and MDS LSK cells compared with WT. These genes included 24 potential oncogenes such as Hmg2 (Oguro et al., 2012), Pbx3 (Li et al., 2013), and Lmo1 (Tremblay et al., 2010; Table S3).

Surprisingly, H3K27me3 marks around TSSs became more enriched (greater than onefold compared with WT) in a small portion of genes upon deletion of Ezh2, particularly in Ezh2Δ/Δ GMPs (Fig. 8 B–D), suggesting a major role for Ezh1 on these gene promoters. Indeed, these genes overlapped considerably with the targets of Ezh1 in ES cells (Shen et al., 2008; Fig. 8 E), and this trend was more obvious with genes that showed high levels of H3K27me3 (>35-fold enrichment of H3K27me3 compared with input in Ezh2Δ/Δ GMPs). More importantly, the genes that maintained H3K27me3 levels upon deletion of Ezh2 were overlapped markedly with genes marked with bivalent histone domains in ES cells (Bernstein et al., 2006; Ku et al., 2008; Fig. 8 F). This trend was again more obvious with the 124 genes that showed high levels of H3K27me3 (>35-fold enrichment of H3K27me3 in Ezh2Δ/Δ GMPs), and most of these genes appeared to be canonical developmental regulators, including genes encoding homeobox, paired-box, T-box, forkhead, and Gata family transcription factors and zinc finger DNA-binding proteins (Table S4). Because the amounts of DNA immunoprecipitated from Ezh2Δ/Δ and Tet2Δ/ΔΔΔ Ezh2Δ/Δ GMPs are less than those from WT and Tet2Δ/ΔΔΔ GMPs, we

Figure 7. Gene expression alterations associated with disease progression. (A) Venn diagram of genes up-regulated or down-regulated in LSK cells from Tet2Δ/ΔΔΔ, Ezh2Δ/Δ, and Tet2Δ/ΔΔΔ Ezh2Δ/Δ mice at 4 mo after transplantation and MDS/MPN (ID19) and MDS (ID18) mice (>2.0- or <0.5-fold compared with the WT control, respectively). The numbers of genes in each group are indicated. The overlaps between every two gene sets are statistically significant (P < 0.01). (B) A heat map showing the correlation of overall gene expression profiles among LSK cells from the indicated mice. Black, red, and blue colors represent no, positive, and negative correlation, respectively. The score of correlation coefficient between LSK cells from each genotype or diseased mice was defined with Pearson’s correlation and is shown on the right.
may somewhat overestimate the H3K27me3 levels in Ezh2\textsuperscript{−/−} GMPs. The proportions in all RefSeq genes are shown in the first lane. PRC2 target genes were subdivided into genes with reduced H3K27me3 levels (<0.5-fold compared with WT), genes that maintained H3K27me3 levels (>1-fold compared with WT), and genes with the H3K27me3 enrichment >35-fold compared with input in Ezh2\textsuperscript{−/−} GMPs in B.

Figure 8. Alterations in gene expression and H3K27me3 levels upon deletion of Ezh2. (A) Gene expression alterations associated with disease progression. Normalized enrichment score (NES) from overall gene expression profiles of LSK cells and GMPs derived from GSEA is shown as the number in each cell. Red and blue colors represent positive (up-regulated in the given genotype relative to WT) and negative (up-regulated in WT relative to the given genotype) enrichment, respectively. Concentrated colors show that the nominal p-value is <0.05 and false discovery rate (FDR) is <0.05, which suggests meaningful enrichment for the given gene sets. Pale colors show borderline enrichment. The cells which do not meet these criteria show white color. (B) Summary of H3K27me3 enrichment detected by ChIP-seq analysis. GMPs isolated from primary recipient mice at 4 mo after transplantation were subjected to ChIP-seq analysis using an anti-H3K27me3 antibody. The fold enrichment values of H3K27me3 signals were calculated against the input signals (ChIP/input) from 0.5 kb upstream to 0.5 kb downstream of the TSSs of RefSeq genes. The red boxes indicate genes with the H3K27me3 enrichment >35-fold in Ezh2\textsuperscript{−/−} and Tet2\textsuperscript{−/−} Ezh2\textsuperscript{−/−} GMPs. (C) Scatter plots of H3K27me3 enrichment in Tet2\textsuperscript{−/−}, Ezh2\textsuperscript{−/−}, and Tet2\textsuperscript{−/−} Ezh2\textsuperscript{−/−} GMPs in comparison with that in WT GMPs. Gray lines indicate 35-fold enrichment, which is the value defined as high enrichment in A. The numbers of genes with higher and lower fold enrichment than that of WT (divided by red lines) are indicated. (D) Scatter plots of 124 genes enclosed by a red square in B showing the H3K27me3 enrichment >35-fold relative to WT in Ezh2\textsuperscript{−/−} and Tet2\textsuperscript{−/−} Ezh2\textsuperscript{−/−} (DKO) GMPs. Gray lines indicate 1.0, which is the value indicating that Ezh2\textsuperscript{−/−} and Tet2\textsuperscript{−/−} Ezh2\textsuperscript{−/−} GMPs showed the same fold enrichment as WT. (E and F) The proportion of Ezh1 target genes (E) and genes that are marked with univalent (H3K4me3 or H3K27me3) or bivalent histone domains (H3K4me3 and H3K27me3; F) identified in ES cells were categorized by the changes in H3K27me3 levels in Ezh2\textsuperscript{−/−} GMPs. The proportions in all RefSeq genes are shown in the first lane. PRC2 target genes were subdivided into genes with reduced H3K27me3 levels (<0.5-fold compared with WT), genes that maintained H3K27me3 levels (>1-fold compared with WT), and genes with the H3K27me3 enrichment >35-fold compared with input in Ezh2\textsuperscript{−/−} GMPs in B.

DISCUSSION
In this study, we first demonstrated that the deletion of Ezh2 confers a growth advantage to HSCs and results in an MDS/MPN-like disease in mice, similar to the depletion of Tet2. Ezh2\textsuperscript{−/−} mice developed a lethal MDS/MPN-like disease with myeloproliferative features, including enhanced repopulating capacity of HSCs and extramedullary hematopoiesis in the spleen, and myelodysplastic features, such as anemia accompanied by enhanced apoptosis in erythroblasts and dysplasia in myeloid cells. These findings are compatible with the recurrent inactivation of EZH2 genes in myelodysplastic disorders and strongly support the tumor suppressor function of Ezh2.
of EZH2. Although no mutations have been identified in PRC1 genes, we recently reported that concurrent loss of the PRC1 gene Bmi1 along with Ink4a/Arf in mice causes pathological hematopoiesis that mimics human primary myelofibrosis, a disease categorized as an MPN (Oguro et al., 2012). Together, inactivation of PcG genes could contribute to the pathogenesis of malignant stem cell disorders.

We further demonstrated that inactivating Ezh2 and TET2 mutations coexist in 6.7% of patients with myelodysplastic disorders, and concurrent deletion of Ezh2 and TET2 in mice accelerates the development of heterogeneous myelodysplastic disorders including MDS and MDS/MPN. Of note, Tet2KD/KD;Ezh2ΔΔ mice showed more advanced myelodysplastic features compared with Tet2KD/KD and Ezh2ΔΔ mice. This is the first study to describe the cooperative effects of the two independent mutations in the pathogenesis of myelodysplastic disorders. Extensive mutation analyses of the myeloid malignancies have revealed that most MDS and MDS/MPN patients have several concurrent genetic mutations, but their pathological meanings remain to be addressed. Our findings highlight the cooperative effects of the independent mutations in the pathogenesis of myelodysplastic disorders. It is assumed that the deregulated gene expression upon concurrent depletion of TET2 and Ezh2 accounts for enhancement in the
myelodysplastic features, such as dysplasia of myeloid cells, functional defects in neutrophils, and enhanced apoptosis in erythroid cells, although these details require further investigation. On the contrary, EZH2 mutations are very rare in de novo AML and chronic myeloid leukemia in blast crisis (CML-BC; Ernst et al., 2010; Chung et al., 2012; Raza and Galili, 2012; Shih et al., 2012), and MDS patients with EZH2 mutations have much lower risk of transformation to AML compared with those with other gene mutations (Chung et al., 2012; Raza and Galili, 2012; Shih et al., 2012). Indeed, no Ezh2^{Δ/Δ} and Tet2^{KD/KD} Ezh2^{Δ/Δ} mice developed AML in this study. We and other groups have previously reported that deletion of Ezh2 largely attenuates the leukemogenicity of AML in a mouse model (Neff et al., 2012; Tanaka et al., 2012). These findings suggest differential roles of EZH2 in AML versus MDS and MDS/MPN.

Although deletion of Ezh2 results in a drastic reduction in H3K27me3 levels, considerable levels of H3K27me3 are retained. The genes that maintained H3K27me3 levels after loss of Ezh2 largely overlap with the targets of Ezh1 in ES cells, and most of these genes are developmentally regulated genes that are marked with bivalent histone domains (Bernstein et al., 2006; Ku et al., 2008). Furthermore, tumor suppressor genes such as Ink4a/Arf, a critical PcG target for the maintenance of the self-renewal capacity of HSCs (Oguro et al., 2006), and PcG-targeted developmental regulator genes, such as Pax5 (Oguro et al., 2010), were kept closely repressed by reduced but significant levels of H3K27me3 modification (Fig. 9). These findings indicate that Ezh1 is capable of repressing developmental regulator genes and tumor suppressor genes, acting to maintain pathological stem cells in the absence of Ezh2. Of interest, Ezh1 was recently reported to maintain HSCs in a slow-cycling, undifferentiated state (Hidalgo et al., 2012). Together, our findings suggest that Ezh1 plays a critical role in pathological stem cells as well as HSCs.

In contrast, several PcG targets became derepressed in Ezh2^{Δ/Δ} and Tet2^{KD/KD} Ezh2^{Δ/Δ} LSK cells. These included many direct PcG targets that are potential oncogenes, such as Hmga2, Pbx3, and Lmo1. Furthermore, the Myc module, which is active in various cancers, was positively enriched in Ezh2^{Δ/Δ} LSK cells and further enriched in MDS/MPN and MDS LSK cells. The derepression of PcG-targeted oncogenes in conjunction with the up-regulation of the Myc module could function as the major drivers in HSCs for the induction of myelodysplastic and myeloproliferative disorders in the absence of Ezh2.

Of interest, Ezh2 knock-in (Ezh2-KI) mice overexpressing Ezh2 specifically in hematopoietic cells develop MPN (Herrera-Merchan et al., 2012). Ezh2-KI mice show an enhanced HSC activity similarly to Ezh2^{Δ/Δ} mice in this study. GSEA showed significant positive enrichment of the Myc module in both Ezh2^{Δ/Δ} and Ezh2-KI LSK cells (unpublished data). However, Ezh2-KI mice do not show myelodysplastic features. Apoptosis in LSK cells is suppressed in Ezh2-KI mice but enhanced in Ezh2^{Δ/Δ} mice. Ezh2-KI LSK cells show enhanced colony-forming capacity, whereas Ezh2^{Δ/Δ} LSK cells show severely compromised proliferation in culture probably due to failure in the transcriptional repression of tumor suppressor genes such as p19Arf (unpublished data), as we have previously reported in transformed myeloid progenitors (Tanaka et al., 2012). These findings clearly discriminate the effects of overexpression and loss/hypomorphism of Ezh2 on hematopoiesis, even though both results in clonal propagation of HSCs.

Finally, the functional cross talk between DNA and histone modifications is an intriguing issue to understand the epigenetics of myeloid malignancies. Tet2^{KD/KD} Ezh2^{Δ/Δ} mice developed not only MDS/MPN but also MDS, whereas Ezh2^{Δ/Δ} mice developed only MDS/MPN. Moreover, myelodysplastic features were more pronounced in Tet2^{KD/KD} Ezh2^{Δ/Δ} mice compared with Ezh2^{Δ/Δ} mice. These findings suggest that loss of Ezh2 and Tet2 cooperate to generate a MDS phenotype in mice. However, enhanced apoptosis in the absence of Ezh2 was significantly attenuated in LSK cells, but not in erythroblasts, upon down-regulation of the Tet2 expression, suggesting an opposing impact of loss of Ezh2 and Tet2 on apoptosis in MDS-initiating hematopoietic stem and progenitor cells.

The understanding of the pathological significance of each somatic gene mutation in myeloid malignancies is just starting to be revealed. Nevertheless, our findings clearly indicate that mouse models, including our own, are capable of faithfully mimicking myelodysplastic disorders and can serve as valuable tools for the analysis of epigenomics of myelodysplastic disorders and preclinical therapeutic studies.

**MATERIALS AND METHODS**

**Sequencing analysis of primary samples with MDS and related myeloid neoplasms.** Genomic DNAs from 119 BM samples with MDS and related neoplasms were analyzed for mutations of TET2, IDH1, IDH2, EZH2, and ASXL1 genes by high-throughput sequencing of SureSelect-enriched target exons (Agilent Technologies) according to manufacturer's protocol. Genome-wide copy number lesions were also analyzed using GeneChip SNP-genotyping microarrays (Affymetrix) as previously described (Chen et al., 2008). Allelic imbalances were detected from the allele-specific copy numbers calculated using CNAG/ACNAR software (http://www.genome.unimn.jp; Nannya et al., 2005; Yamamoto et al., 2007). All mutations were confirmed by Sanger-sequencing. All patient samples were appropriately anonymized before the analysis according to the protocol approved by the Ethics Boards of the University of Tokyo.

**Mice and generation of fetal liver chimeras.** Tet2^{KD/KD} and Ezh2^{Δ/Δ} mice, which had been backcrossed at least eight times onto a C57BL/6 (CD45.2) background, were used (Mochizuki-Kashio et al., 2011; Shide et al., 2012). For conditional deletion of Ezh2, the mice were crossed with Rosa::Cre-ERT mice (Taconic). C57BL/6 (CD45.2) mice were purchased from Japan SLC. 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). To generate fetal liver chimeras, we intravenously transplanted E14.5 fetal liver from CD45.2 mice into 8-wk-old CD45.1 recipients irradiated at a dose of 9.5 Gy. After 1 mo, the recipient mice were injected with 100 µl tamoxifen dissolved in corn oil at a concentration of 10 mg/ml intraperitoneally once a day for 5 consecutive days to induce Cre activity.

**Serial transplantation assay.** 600 CD45.2 LSK cells were sorted from the BM of the primary recipient mice reconstituted with WT, Tet2^{KD/KD}, Ezh2^{Δ/Δ},
and Tet2(KO)/Ezh2(A−) cells at 4 mo after transplantation and injected into lethally irradiated CD45.1 mice (secondary recipient mice) along with 4 × 10^5 BM cells (CD45.1) for competitor cells. For tertiary transplantation, 2 × 10^5 whole BM cells taken from the secondary recipient mice 4 mo after transplantation were injected into lethally irradiated CD45.1 mice.

Western blot analysis of H3K27me3. Samples were separated by SDS-PAGE, transferred to a PVDF membrane and detected by Western blotting using the following antibodies: anti-H3 (Abcam) and anti-H3K27me3 (Millipore).

Flow cytometry and antibodies. mAbs recognizing the following antigens were used in flow cytometry and cell sorting: CD45.2 (104), CD45.1 (A20), Gr-1 (RB6-8C5), CD11b/Mac-1 (M1/70), Ter-119, CD127/IL-7Rα (A7R34), B220 (RA3-6B2), CD4 (L3T4), CD8α (53-6.7), CD117/c-KIT (BB8), Sca-1 (D7), and CD16/32/FcyRII-III (93). The mAbs were purchased from BD, eBioscience, or BioLegend. For Annexin V staining, cells were suspended in 1× Annexin binding buffer (BD) and stained with FITC-Annexin V (BD) according to the manufacturer’s protocol. Dead cells were eliminated by staining with 1 µg/ml propidium iodide (Sigma-Aldrich). All flow cytometric analyses and cell sorting were performed on a FACSARia II or FACSCanTo II (BD).

Microarray analysis. A one-color microarray-based gene expression analysis system (Agilent Technologies) containing 39,429 clones was used (SurePrint G3 Mouse GE 8 × 60K array; Agilent Technologies), according to the manufacturer’s instructions. Total RNA was extracted from 5 × 10^5 sorted LSK cells or GMPs. A total of 10 ng of total RNA was mixed with spike-in controls using a One Color Spike Mix kit (Agilent Technologies), amplified and labeled with Cyanine 3 using a Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer’s instructions, which generated single-color labeled cRNA. A total of 1,650 ng of the labeled cRNA was used for each hybridization. The process of hybridization and washing was performed using a Hi-RPM Gene Expression Hybridization kit (Large; Agilent Technologies) according to the manufacturer’s instructions, which generated single-color labeled cRNA. A total of 1,650 ng of the labeled cRNA was used for each hybridization. The process of hybridization and washing was performed using a Hi-RPM Gene Expression Hybridization kit (Large; Agilent Technologies) and a Gene Expression Wash Pack (Agilent Technologies), according to the manufacturer’s instructions. A total of 170,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences (mm9) from University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/) using the Eland program. Sequences allowing no more than two mismatches per sequence were used for the analysis. The ChIP-Seq signal was quantified as total number of reads per million. To enumerate genes with H3K27me3 modification, genes with cumulative tag numbers in the region 5.0 kb upstream of the TSS to 0.5 kb downstream of the TSS greater than twofold was selected.

Statistical analysis. Heat map was drawn by Matrix2pg (Pavlidis and Noble, 2003). Statistical tests were performed using Prism (version 5; GraphPad Software). For analysis of survival curves, log rank (Mantel-Cox) test was performed.

Deposition of the data. Microarray and ChIP-seq sequence data were deposited in Gene Expression Omnibus (accession no. GSE42666) and DNA Data Bank of Japan (DDBJ, accession no. DRA000485-488), respectively.

Online supplemental material. Table S1 lists the gene mutations and abnormalities in chromosome 7 in patient samples. Table S2 provides the detailed hematological characteristics of diseased mice. Table S3 lists the potential oncogenes of direct PcG targets derepressed in the absence of Ezh2. Table S4 lists the genes with a fold enrichment of H3K27me3 >35 compared with BSA and salmon (Invitrogen), and then incubated with an anti-H3K27me3 antibody (07–449; Millipore) for 2 h at 4°C. Chromatin was immunoprecipitated overnight at 4°C with antibody-conjugated Dynabeads. The immunoprecipitates were extensively washed with the following combination of wash buffers: high salt RIPA buffer (10 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC, and protease inhibitor cocktail), LiCl wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, and 0.5% DOC), and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Bound chromatin and input DNA were placed in elution buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 300 mM NaCl, and 0.5% SDS) and reverse cross-linked. Immunoprecipitated DNA and input DNA were treated with RNase A (Sigma-Aldrich) and proteinase K (Roche), and purified with a QIAquick PCR purification kit (Qiagen). Adaptor-ligated DNA fragments were size fractionated by 12% acrylamide gel, and the 170- to 250-bp fraction was recovered. DNA thus obtained was amplified by 18 cycles of PCR. 1 ng DNA was used for the sequencing reaction of the GAIIx (Illumina), according to the manufacturer’s instructions. A total of 170,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences (mm9) from University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/) using the Eland program. Sequences allowing no more than two mismatches per sequence were used for the analysis. The ChIP-Seq signal was quantified as total number of reads per million. To enumerate genes with H3K27me3 modification, genes with cumulative tag numbers in the region 5.0 kb upstream of the TSS to 0.5 kb downstream of the TSS greater than twofold was selected.

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