Loss of Ezh2 synergizes with JAK2-V617F in initiating myeloproliferative neoplasms and promoting myelofibrosis

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Loss of Ezh2 synergizes with JAK2-V617F in initiating myeloproliferative neoplasms and promoting myelofibrosis

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Myeloproliferative neoplasm (MPN) patients frequently show co-occurrence of JAK2-V617F and mutations in epigenetic regulator genes, including EZH2. In this study, we show that JAK2-V617F and loss of Ezh2 in hematopoietic cells contribute synergistically to the development of MPN. The MPN phenotype induced by JAK2-V617F was accentuated in JAK2-V617F;Ezh2+/− mice, resulting in very high platelet and neutrophil counts, more advanced myelofibrosis, and reduced survival. These mice also displayed expansion of the stem cell and progenitor cell compartments and a shift of differentiation toward megakaryopoiesis at the expense of erythropoiesis. Single cell limiting dilution transplantation with bone marrow from JAK2-V617F;Ezh2+/− mice showed increased reconstitution and MPN disease initiation potential compared with JAK2-V617F alone.

RNA sequencing in Ezh2-deficient hematopoietic stem cells (HSCs) and megakaryocytic erythroid progenitors identified highly up-regulated genes, including Lin28b and Hmga2, and chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) analysis of their promoters revealed decreased H3K27me3 deposition. Forced expression of Hmga2 resulted in increased chimerism and platelet counts in recipients of retrovirally transduced HSCs. JAK2-V617F–expressing mice treated with an Ezh2 inhibitor showed higher platelet counts than vehicle controls. Our data support the proposed tumor suppressor function of EZH2 in patients with MPN and call for caution when considering using Ezh2 inhibitors in MPN.

Myeloproliferative neoplasms (MPNs) are a group of diseases characterized by increased proliferation of erythroid, megakaryocytic, and granulocytic lineages. Three clinical entities designated polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (MF [PMF]) are distinguished by clinical, laboratory, and molecular parameters (Tefferi et al., 2009). JAK2-V617F is the most frequently observed somatic mutation in MPN. This mutation augments the tyrosine kinase activity of Jak2 and is found in >95% of patients with PV and in 50–60% of ET and PMF (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Other driver mutations are primarily associated with ET and PMF and target the genes for calreticulin, CALR (Klampfl et al., 2013; Nangalia et al., 2013), and the thymopoietin receptor, MPL (Pardanani et al., 2006; Pikman et al., 2006). Mutations in JAK2, CALR, and MPL are considered phenotypic driver mutations because they reproduce MPN phenotypes upon expression in mouse models. Genes encoding epigenetic regulators are also frequently mutated in MPN (Vainchenker et al., 2011). These mutations often coexist with one of the three driver gene mutations and are thought to promote clonal evolution and disease progression (Lundberg et al., 2014a). Mutations in the histone methyltransferase EZH2 (enhancer of zeste homologue 2) are detected in 5–10% of patients with ET and PMF (Ernst et al., 2010) but are also found in other hematologic malignancies, including myelodysplastic syndromes and acute myeloid leukemia (Bejar et al., 2011; Gerstung et al., 2015). Patients with MPN carry either heterozygous or homozygous EZH2 mutations (Ernst et al., 2010). Most of the EZH2 mutations in MPN result in truncations of the Ezh2 protein and are therefore predicted to be loss of function mutations. In patients with MF, EZH2 mutations are associated with poor prognosis (Guglielmelli et al., 2011).
Several MPN mouse models have been established by expressing \( JAK2-V617F \) as transgenes or knock-in alleles (reviewed in Li et al. [2011]). Here we examined the effects of an \( Ezh2 \) loss of function mutation in a Cre-recombinase-inducible mouse model of \( JAK2-V617F \)-driven MPN. These \( JAK2-V617F \) mice develop ET or PV phenotypes depending on the expression levels of \( JAK2-V617F \) in hematopoietic cells (Tiedt et al., 2008). Recently, we showed that hematopoietic stem cells (HSCs) expressing \( JAK2-V617F \) are capable of inducing clonal MPN in single cell transplantation (Lundberg et al., 2014b), albeit with low efficiency. This suggests that additional mutations, such as in \( Ezh2 \), may synergize with \( JAK2-V617F \) in MPN disease initiation.

\( Ezh2 \) functions as a core component of the polycomb repressive complex 2 (PRC2), which consists of three additional subunits, \( Eed \) (embryonic ectoderm development), \( Suz12 \) (suppressor of zeste 12 homologue), and the histone binding protein \( RBBP4 \). PRC2 methylates lysine 27 on histone H3 (H3K27me3). Ezh2 knockout mice are early embryonic lethal (O’Carroll et al., 2001), but several conditional knockout strains have been generated that allow inactivating \( Ezh2 \) function in adult mice using inducible Cre-loxP systems (Hirabayashi et al., 2009; Neff et al., 2012). Conditional knockout of \( Ezh2 \) in adult hematopoiesis has revealed that \( Ezh2 \) is dispensable for the maintenance of long-term HSCs (LT-HSCs), most likely because of expression of \( Ezh1 \), a close homologue of \( Ezh2 \) that associates with \( Eed \) and \( Suz12 \) to form an alternative PRC2 complex that partially compensates for the loss of \( Ezh2 \) (Xie et al., 2014). Here we studied \( JAK2-V617F \) and \( Ezh2 \) double mutant mice in Cre-inducible conditional systems to assess the functional consequences in hematopoiesis.

**RESULTS**

To assess the functional effects of \( Ezh2 \) loss on MPN phenotypes, we interbred mice with a conditional knockout allel of \( Ezh2 \) (Neff et al., 2012) with inducible \( JAK2-V617F \) transgenic mice (hereafter called \( V617F \); Tiedt et al., 2008). We first used the interferon-inducible MxCre system (Kühn et al., 1995) to delete one or both copies of \( Ezh2 \) and simultaneously induce expression of \( JAK2-V617F \). Heterozygous loss of \( Ezh2 \) in \( V617F \) mice resulted in a trend toward more pronounced thrombocytosis and neutrophilia and initially also higher hemoglobin values than \( V617F \) alone (Fig. 1 A). \( V617F \) mice with homozygous loss of \( Ezh2 \) exhibited elevated hemoglobin, thrombocytosis, and neutrophilia already at the time of induction by polyinosine-polycytosine (pIpC), consistent with leaky Cre expression. \( V617F;Ezh2^{Δ/Δ} \) mice showed poor survival, whereas most of the \( V617F;Ezh2^{Δ/Δ} \) mice survived until they were sacrificed at 28 wk (Fig. 1 A, right). Loss of \( Ezh2 \) in the absence of \( V617F \) had no effect on blood counts and did not affect survival. We repeated these experiments with the tamoxifen-inducible \( ScICre^{ER} \) system (Göthert et al., 2005). None of the mice showed an MPN phenotype before tamoxifen administration. The MPN phenotype appeared with a latency of \( \sim 10 \) wk from the start of tamoxifen, and around this time the first deaths occurred (Fig. 1 B). Overall, the steepness of decline in survival of \( V617F;Ezh2^{Δ/Δ} \) was comparable between \( MxCre- \) and \( ScICre-induced mice, although we cannot exclude that pIpC injections in \( MxCre;V617F;Ezh2^{Δ/Δ} \) mice that already displayed MPN at the time of the injections might have had an additional negative impact on survival. The blood counts in \( ScICre-induced mice were comparable or slightly more pronounced than with the \( MxCre \) system (Fig. 1 B). \( JAK2-V617F \) with homozygous loss of \( Ezh2 \) resulted in PMF without a preceding polycythemic phase. \( Ezh2 \) mRNA expression was reduced to \( \sim 50\% \) in \( Ezh2^{Δ/Δ} \) mice and strongly decreased in \( Ezh2^{Δ/Δ} \) mice (Fig. 1 C). This reduction was paralleled by a decrease in \( Ezh2 \) protein and histone H3 methylation on lysine 27 (H3K27me3; Fig. 1 D). Ubiquitination of histone H2A on lysine 119 (H2A-K119Ub), a marker for the activity of the PRC1 complex, was not increased in \( V617F;Ezh2^{Δ/Δ} \) mice. All mice expressing \( V617F \) displayed splenomegaly, but no differences were observed in \( V617F \) mice with or without \( Ezh2 \) deletion (Fig. 1 E). Cellularity in BM was increased in \( V617F \) mice but markedly decreased in \( V617F;Ezh2^{Δ/Δ} \) mice (Fig. 1 F). Furthermore, the numbers of circulating c-kit+ progenitor cells were strongly increased in \( V617F;Ezh2^{Δ/Δ} \) mice (not depicted). The decrease in BM cellularity with an increase in circulating progenitor cells and a fall in hemoglobin over time in \( V617F;Ezh2^{Δ/Δ} \) mice is compatible with MF and is reminiscent of changes typically observed in patients with PMF with increased numbers of circulating CD34+ cells (Barosi et al., 2001). At 16 wk, all \( V617F \) mice, with or without \( Ezh2 \) loss, showed typical histopathological features of MPN, with trilineage hyperplasia and increased numbers of atypical megakaryocytes, together with destruction of normal splenic architecture by infiltrates of highly atypical hematopoiesis (Fig. 1, G and H). Loss of \( Ezh2 \) in \( V617F \) mice accentuated the hyperproliferation of the megakaryocytic lineage and resulted in a marked increase in reticulin fibers in BM and spleen, most prominently seen in \( V617F;Ezh2^{Δ/Δ} \) mice, which also displayed an increase in collagen fibers and osteosclerosis (not depicted). These changes were not observed in \( Ezh2^{Δ/Δ} \) or \( Ezh2^{Δ/Δ} \) mice with WT Jak2. Thus, heterozygous loss of \( Ezh2 \) accelerated the transition from PV to MF and homozgyous loss of \( Ezh2 \) appears to directly lead to PMF, without a preceding polycythemic phase. None of the mice showed transformation to acute leukemia.

Analysis of the progenitor and stem cell compartments by flow cytometry revealed an increase of phenotypic LT-HSCs and short-term HSCs (ST-HSCs) in BM and spleen, most prominently seen in \( V617F;Ezh2^{Δ/Δ} \) mice, which also displayed an increase in collagen fibers and osteosclerosis (not depicted). These changes were not observed in \( Ezh2^{Δ/Δ} \) or \( Ezh2^{Δ/Δ} \) mice with WT Jak2. Thus, heterozygous loss of \( Ezh2 \) accelerated the transition from PV to MF and homozgyous loss of \( Ezh2 \) appears to directly lead to PMF, without a preceding polycythemic phase. None of the mice showed transformation to acute leukemia.

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Figure 1. Analysis of V617F;Ezh2 double mutant mice. (A) Time course of blood counts and survival of MxCre mice induced with pIpC (yellow arrows). The color code for the different genotypes is the same as indicated in C–F. The gray horizontal bars indicate the normal range for the blood values. Survival probability was calculated by the Kaplan-Meier method. The means of the values ± SEM are plotted (n = 4 for MxCre;GFP, n = 6 for MxCre;Ezh2Δ/Δ;GFP,
creased LT-HSCs in spleen and increased ST-HSCs in BM and MPPs in spleen (Fig. 2 A). Loss of Ezh2 on WT Jak2 background caused no significant changes in the HSC compartments. Analysis of the more mature progenitor compartments (Fig. 2 B) showed that V617F alone increased megakaryocyte progenitors (MkPs) and megakaryocytes in spleen, whereas a prominent decrease in erythroid precursors occurred in BM, compensated by a strong increase of erythroid precursors in spleen (Fig. 2 B). Homozygous loss of Ezh2 displayed a significant increase of MkPs and megakaryocytes and also megakaryocyte erythroid progenitors (MEPs) but decreased erythroid precursors in spleen (Fig. 2 B). Thus, heterozygous and homozygous loss of Ezh2 on V617F background expanded the early HSC and progenitor compartment and favored megakaryopoiesis over erythropoiesis. Plasma levels of Tpo were decreased in mice expressing V617F compared with WT (Fig. 2 C). Loss of Ezh2 on V617F background further reduced the Tpo plasma levels, consistent with autoregulation of Tpo by the increased platelet and megakaryocyte mass. Interestingly, TGFβ in plasma showed a trend toward the highest levels in V617F;Ezh2Δ/Δ mice, consistent with the role of TGFβ as a mediator of MF.

To eliminate potential effects of Ezh2 deletion in non-hematopoietic tissues and to determine how the double mutant cells compete with WT hematopoiesis, we transplanted noninduced LSK cells together with WT BM competitor cells (Fig. 3). To allow monitoring of hematopoietic cells that carry the JAK2 and/or Ezh2 mutations, we crossed these strains with the UBC-GFP strain (Schaefer et al., 2001) and obtained donor mice that coexpressed GFP in the same cells (V617F;GFP or V617F;Ezh2Δ/Δ;GFP). Both V617F;Ezh2Δ/Δ and V617F;Ezh2Δ/Δ mice showed a competitive advantage in the platelet and neutrophil lineages compared with V617F mice, whereas the differences in the erythroid lineage were minor (Fig. 3 A). The same conclusions were reached in competitive transplantations of BM cells from SclCre;V617F;GFP mice with heterozygous and homozygous loss of Ezh2 (Fig. 3 B).

To determine whether loss of Ezh2 also synergizes with JAK2-V617F in MPN disease initiation from single cells, we performed competitive transplantations at limiting dilutions (Fig. 4). We used the approach previously established for studying V617F mice (Lundberg et al., 2014b). Because the majority of MPN patients with Ezh2 mutations remain heterozygous and the V617F;Ezh2Δ/Δ mice show early lethality, we performed limiting dilutions with BM cells from heterozygous V617F;Ezh2Δ/Δ mice (Fig. 4 A). The numbers of phenotypic LT-HSCs in V617F and V617F;Ezh2Δ/Δ mice were increased two- to threefold compared with WT controls (Fig. 4 B). To obtain on average one HSC per transplant, we therefore reduced the number of BM cells from MxCre;V617F;GFP and MxCre;V617F;Ezh2Δ/Δ;GFP donor mice to 8,000 cells per recipient, whereas 20,000 cells were used for the other genotypes. The time course of the blood counts and chimerism is summarized in Fig. 4 C. Data for each genotype was arranged in vertical columns. The frequencies of reconstitution, defined as chimerism >1% in Gr1-positive peripheral blood cells 22 wk after transplantation, are summarized in Table 1. Recipients of limiting dilutions of BM cells from V617F;GFP mice showed a high degree of tri-lineage chimerism, but only a few of them developed MPN phenotype with mostly ET characteristics. The recipients of BM from V617F;Ezh2Δ/Δ;GFP mice reconstituted at higher frequencies and in most cases showed a PV phenotype (Fig. 4 C and Table 1). Recipients of Ezh2Δ/Δ;GFP BM with WT Jak2 showed increased frequency of reconstitution (Table 1), but only few of them showed chimerism above 20% (Fig. 4 C). BM cellularity between V617F and V617F;Ezh2Δ/Δ mice was comparable (Fig. 4 D), but only the V617F;Ezh2Δ/Δ mice displayed splenomegaly (Fig. 4 E). In line with our previous study (Lundberg et al., 2014b), analysis of the percentages of the V617F;GFP-positive cells in hematopoietic progenitor and stem cell compartments in BM revealed that mice with MPN phenotype retained a high degree of GFP chimerism in the LT-HSC compartment, in contrast to mice without MPN...
Figure 2. Flow cytometric analysis of stem cell and progenitor compartments in BM and spleen of tamoxifen-inducible JAK2-V617F and Ezh2 mutant mice. (A) Frequencies of LT-HSCs, ST-HSCs, MPPs, and LSK cells are shown. The top panel shows analysis of BM, and the bottom panel analysis of spleen cells for the different genotypes, as indicated (n = 5 for SclCre, n = 4 for SclCre;Ezh2Δ/Δ, n = 6 for SclCre;V617F, n = 4 for SclCre;V617F;Ezh2Δ/Δ, and n = 4 for SclCre;V617F;Ezh2Δ/Δ). This experiment was performed three times. (B) Frequencies of MkPs, megakaryocytes, MEPs, and erythroid precursors are shown for BM (top) and spleen (bottom). The same mice as in A were analyzed. (C) Plasma concentrations of Tpo and TGFβ from tamoxifen-inducible JAK2-V617F and Ezh2 mutant mice were measured by ELISA (n = 5 per group for TPO; n = 4 for SclCre;Ezh2Δ/Δ, n = 5 for SclCre;V617F;Ezh2Δ/Δ, n = 3 for SclCre;V617F;Ezh2Δ/Δ, and n = 5 for SclCre;V617F;Ezh2Δ/Δ for TGFβ). Statistical analysis was conducted using the Student’s t test or one-way ANOVA with Bonferroni’s post-hoc multiple comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Results are presented as means ± SEM.
phenotype, where the expansion of V617F;GFP chimera often occurred at later stages of differentiation (Fig. 4 F). The same pattern was also observed in three out of four of the V617F;Ezh2Δ/Δ;GFP mice with MPN phenotype that were analyzed (Fig. 4 F, right). This suggests that in some cases heterozygous loss of Ezh2 may help to overcome restrictions that prevent developing MPN when the V617F clone has not fully expanded in the LT-HSC compartment. Overall, these results show that heterozygous loss of Ezh2 synergized with V617F also in monoclonal MPN disease initiation at limiting dilutions.

To further examine the effects of the Ezh2 mutations on the LT-HSC compartment, we transplanted 50 LT-HSCs purified by FACS sorting from donor mice taken 12 wk after tamoxifen induction and assessed their functional capacity to reconstitute and initiate MPN in lethally irradiated recipients (Fig. 5). We used 10^6 WT BM cells as competitors (Fig. 5 A). Because of reduced survival of mice reconstituted with V617F;Ezh2Δ/Δ;GFP BM, we terminated the experiment at 24 wk and the results of analysis at 16 wk are shown (Fig. 5 B). Only 4/10 recipients (40%) that received purified LT-HSCs from V617F;GFP reconstituted with a chimerism of >1% in Gr1-positive cells, and only 2 of the chimeric mice also showed an MPN phenotype. This result confirms our previous findings, which showed that the functional stem cell compartment in V617F mice is shifted toward cells with a
lineage-intermediate immunophenotype (Lundberg et al., 2014b). Interestingly, heterozygous loss of Ezh2 on V617F background increased the frequency of reconstitution to 5/9 recipients (56%), and 4/5 mice with chimerism also displayed an MPN phenotype (Fig. 5B). The effect of homozygous loss of Ezh2 on V617F background was even more pronounced.
and 9/9 recipient showed high chimerism and displayed an ET phenotype (Fig. 5 B), while their survival was reduced (Fig. 5 C). The frequencies of reconstitution and MPN disease initiation is summarized in Fig. 5 D. Homozygous loss of Ezh2 on Jak2 WT background reduced the frequency of reconstitution (5/8 recipients) compared with WT BM cells (8/9 recipients), but at the same time resulted in a shift toward higher percentage of myeloid cells in peripheral blood (Fig. 5 E). V617F alone also resulted in a myeloid bias, and the combination of V617F;Ezh2Δ/Δ shifted the percentages almost completely toward myeloid cells (Fig. 5 E). Comparison of the platelet and erythrocyte chimerism in peripheral blood for all recipients of BM from Ezh2 mutants on Jak2 WT background is shown in Fig. 5 F. Homozygous loss of Ezh2 alone already leads to a shift toward thrombopoiesis.

To study the mechanism of how loss of Ezh2 contributes to enhancement of the MPN phenotype, we compared the mRNA expression profiles of hematopoietic cells with or without mutations in JAK2 and Ezh2 by RNA sequencing. LT-HSCs and MEP cells were FACS sorted from groups of four to six mice per genotype 10 wk after tamoxifen induction, and RNA was used to prepare libraries for sequencing on Illumina NextSeq500. The mutant versus WT Jak2 is the major parameter that divides the heat map of LT-HSCs into two groups (Fig. 6 A), and this influence is also represented in the principal component analysis (PCA) along the principal component 1 axis (Fig. 6 B, left). In MEPs, the influence of the Jak2 genotype is less prominent. Instead, unsupervised clustering reveals a strong influence of the homozygous loss of Ezh2, which defines two distinct groups in the heat map, one with WT Jak2 and one with V617F (Fig. 6 A, right). The effects of homozygous loss of Ezh2 were less prominent in the clustering and the groups of V617F and V617F;Ezh2Δ/Δ MEPs appear to be rather heterogeneous. In PCA of the MEP data, WT Jak2 versus V617F is the dominant factor (principal component 1), explaining 43% of the variation in log2-fold change (Fig. 6 B, right). The second most prominent effect in the PCA (principal component 2) is observed between homozygous loss of Ezh2 versus heterozygous or WT Ezh2, which accounts for 21% of the variation in log2-fold change. Competitive gene set enrichment analysis comparing LT-HSCs from V617F;Ezh2Δ/Δ versus V617F mice revealed interferon-γ and interferon-α signatures (Fig. 6 C). Using self-contained gene set testing, we also found positive enrichment of a fetal liver HSC-specific gene signature (Fig. 6 C, right). In LT-HSCs, the largest number of differentially expressed genes was found between WT cells and cells expressing V617F (Fig. 6 D). There are fewer differentially expressed genes in V617F-expressing cells with different Ezh2 genotypes, and homozygous loss of Ezh2 had a much stronger effect than heterozygous loss. Few differences in gene expression were detected between different Ezh2 genotypes on a WT Jak2 background. In MEPs, the largest number of differentially expressed genes (with P ≤ 0.05) is observed in genotypes that involve comparisons with homozygous loss of Ezh2 both on WT Jak2 and V617F background (Fig. 6 D). The second largest effect is seen in the comparison between V617F and WT Jak2, whereas the heterozygous Ezh2 showed the fewest changes both in comparison with WT Jak2 and in comparison between V617F and V617F;Ezh2Δ/Δ. The top 10 differentially expressed genes for the comparisons between WT and Ezh2Δ/Δ and V617F with V617F;Ezh2Δ/Δ were ordered according to the absolute fold change in expression and are shown in Fig. 6 E. There was no overlap among the top 10 gene lists from LT-HSCs and MEP, except for Lin28b, which showed the highest fold changes in both LT-HSCs and MEPs. The complete list of differentially expressed genes is provided in Tables S1 and S2. Lin28b, together with let-7 and Hmga2, plays an important role in regulating HSC self-renewal (Oguro et al., 2012; Copley et al., 2013). The log2 expression levels of Lin28b and its downstream gene Hmga2 are plotted in Fig. 6 F. In LT-HSCs, Lin28b was significantly elevated only in V617F;Ezh2Δ/Δ cells, whereas in MEPs both WT Jak2;Ezh2Δ/Δ and V617F;Ezh2Δ/Δ cells showed increased Lin28b (Fig. 6 F). Hmga2 expression was unchanged in LT-HSCs (not depicted). However, Hmga2 expression was significantly elevated in MEPs from mice with Ezh2Δ/Δ on WT Jak2 or V617F backgrounds (Fig. 6 F, right).

Up-regulated expression of Hmga2 was previously linked to increased megakaryopoiesis and MF (Oguro et al., 2012). We determined the mRNA expression levels of HMG2 in granulocytes from MPN patients with EZH2 mutations (Fig. 6 G). A trend toward higher expression of HMG2 was observed in four patients with mutations in CALR that carried heterozygous EZH2 mutations and were very high in one patient with mutated CALR and a homozygous EZH2 mutation. Patients with JAK2-V617F and homozygous EZH2 mutations also showed a trend toward higher HMG2 expression. We also

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<th>Genotype of donor mice</th>
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<th>Frequency of long-term reconstitution</th>
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<td>8,000</td>
<td>6/41 (15%)</td>
<td>2/6 (33%)</td>
</tr>
<tr>
<td>MxCre;V617F;Ezh2Δ/Δ;GFP</td>
<td>41</td>
<td>8,000</td>
<td>16/41 (39%)</td>
<td>8/16 (50%)</td>
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The indicated numbers of BM cells were injected into lethally irradiated recipients.

Reconstitution was scored based on the presence of >1% GFP-positive cells in the peripheral blood G1+ cells at 22 wk after transplantation.

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Table 1. Summary of competitive limiting dilution transplantation experiments
examined expression of two other genes in patients’ granulocytes: IGF2BP3, a downstream target of Lin28b pathway, and Pcolce2, an up-regulated gene in the top 10 list of MEPs from mice with Ezh2Δ/Δ on WT and V617F backgrounds. Both showed a similar trend toward higher expression in MPN patients with EZH2 mutations. These data are consistent with a model in which the decrease of PRC2 activity through loss of Ezh2 ultimately results in gene expression changes that favor megakaryopoiesis but alone are insufficient to cause a thrombocytosis phenotype, unless JAK2-V617F is coexpressed.

The Lin28b–Let7–Hmga2 axis is an attractive candidate pathway to mediate the synergistic effect of loss of Ezh2 and expression of JAK2-V617F and on megakaryopoiesis. To determine whether the promoters of Lin28b and Hmga2 are directly affected by the loss of Ezh2 activity, we performed chromatin immunoprecipitation (ChIP) with antibodies against H3K27me3 followed by quantitative PCR (qPCR) analysis using primers for the promoter regions of Lin28b and Hmga2 (Fig. 7 A). We found that both the Lin28b and Hmga2 promoters exhibited significantly reduced H3K27me3 depo-
Figure 6. Gene expression analysis by RNA sequencing of LT-HSCs and MEPs. (A) Unsupervised clustering of differentially expressed genes (P ≤ 0.05; log2-fold changes >1.5). Each column represents data from one individual mouse. The color code for the genotypes of the individual mice is the same as in B. (B) PCA. The data for LT-HSCs and MEPs were derived from two independent experiments and combined (n = 4 or 5 for SclCre, n = 7 or 4 for...
Expression of Hmga2 in LT-HSCs with WT Jak2
FACS-sorted LT-HSCs from WT or V617F-expressing BM
likely to be mediated by a concerted action of several genes
of EZH2, $n = 1$ for CALR mutation with homozygous mutation of EZH2, $n = 8$ for JAK2V617F mutation with WT EZH2, $n = 12$ for JAK2V617F mutation with heterozygous mutation of EZH2, and $n = 4$ for JAK2V617F mutation with h homzygous mutation of EZH2. [F and G] Horizontal lines indicate the mean of the values. *, $P < 0.05$, ***, $P < 0.001$. 

DISCUSSION

Our data provide evidence for functional synergism between JAK2-V617F and heterozygous or homzygous loss of EZH2 in hematopoiesis in vivo. The MPN phenotype induced by V617F alone was accentuated in double mutant mice with V617F and heterozygous or homzygous loss of EZH2. Peripheral blood counts, notably platelets and neutrophils, were increased, survival was reduced, and a higher degree of MF was observed in heterozygous V617F;Ezh2$^{+/−}$ mice, and these alterations were even more pronounced in homozygous V617F;Ezh2$^{+/−}$ mice (Fig. 1). Loss of Ezh2 on a Jak2 WT background did not affect blood counts, survival, or histology.

Comparison of the progenitor and stem cell compartments between V617F and V617F;Ezh2 mutant mice revealed a slight increase of LT-HSCs in homozygous V617F;Ezh2$^{+/−}$ mice (Fig. 2 A), whereas a strong increase in MKPs and a decrease in erythroid precursors were noted (Fig. 2 B). These changes are in line with the alterations observed in peripheral blood of these mice. Although expansion of MEPs was observed in spleens of homozygous V617F;Ezh2$^{+/−}$ mice, this alteration may reflect increased activity of megakaryopoiesis.
of this bipotent progenitor population. Loss of Ezh2 on Jak2 WT background had no significant effect on the progenitor and stem cell compartment compared with WT controls, except for an increase of MEPs in BM (Fig. 2 B).

Synergism between JAK2-V617F and heterozygous loss of Ezh2 was also evident in BM transplantation experiments at limiting dilutions. BM grafts from heterozygous V617F/Ezh2<sup>−/−</sup> mice that contained on average only a single HSC engrafted at higher frequencies and showed more frequently a MPN phenotype in lethally irradiated recipients than grafts from V617F mice (Fig. 4 and Table 1). Although expression of V617F alone resulted in a decrease of functional HSC activity when 50 FACS-sorted LT-HSCs were transplanted in competition with WT BM cells, loss of Ezh2 on V617F background restored the reconstitution capacity and increased disease initiation potential of FACS-sorted LT-HSCs (Fig. 5). The reduced efficiency of phenotypic LT-HSCs from V617F mice for long-term reconstitution in transplantation experiments was noted in our previous study and was accompanied by an expansion of stem cell activities in cells with lineage-intermediate Sca1<sup>+</sup> c-kit<sup>+</sup> immunophenotype (Lundberg et al., 2014b). Overall, the synergistic effects of V617F and loss of Ezh2 manifested in expansion of the stem cell and progenitor cell compartment with increased reconstitution and MPN disease initiation potential and shift of differentiation toward megakaryopoiesis at the expense of erythropoiesis.

The effects of conditional Ezh2 knockout in hematopoiesis have been previously studied. The tendency of increasing megakaryopoiesis was reported in conditional Ezh2 knockout on a WT Jak2 background. In one report, loss of Ezh2 resulted in subtle thrombocytosis (Mochizuki-Kashio et al., 2011). Thrombocytopenia in Mpl<sup>−/−</sup> mice was partially improved in Mpl<sup>−/−</sup>;Ezh2<sup>−/−</sup> double mutant mice (Majewski et al., 2010). We did not observe thrombocytosis in our Ezh2<sup>−/−</sup> mice on a WT Jak2 background, but we noted increased MEPs in BM and a trend toward more megakaryocytes (Fig. 2 B) as well as a shift toward increased platelet chimerism in Ezh2<sup>−/−</sup> mice compared with WT controls (Fig. 5 F). Loss of Ezh2 attenuated but did not prevent MLL-AF9-mediated acute myeloid leukemia (Neff et al., 2012). Although loss of Ezh2 in the context of V617F accelerated disease, overexpression of Ezh2 has also been implicated in the progression of various types of human cancers (reviewed by Sauvageau and Sauvageau [2010] and Margueron and Reinberg [2011]), and a recurrent gain of function mutation in EZH2 (EZH2-Y641) has also been reported to occur in lymphomas (Morin et al., 2010; Yap et al., 2011). Paradoxically, overexpression of Ezh2 also enhances HSC self-renewal (Kamminga et al., 2006) and results in a myeloproliferative disorder with leukocytosis and splenomegaly (Herrera-Merchan et al., 2012). These findings are difficult to reconcile and likely reflect the complex and cell type-dependent roles of PRC2 complex. Indeed, several compensatory mechanisms have been described that come into effect when Ezh2 function is abolished, including compensation by Ezh1, as well as noncanonical PRC2 functions that do not depend on H3K27me3 (Xie et al., 2014).

Gene expression analysis in LT-HSC and MEP cells revealed strong effects of V617F over WT Jak2 and also strong effects of homozygous loss of Ezh2<sup>−/−</sup> (Fig. 6). In contrast, heterozygous Ezh2<sup>−/+</sup> had little influence on gene expression levels, despite showing functional synergism with V617F on the MPN phenotype in V617F;Ezh2<sup>−/−</sup> double mutant mice. Several genes highly up-regulated in MEPs from Ezh2<sup>−/−</sup> mice on Jak2 WT, as well as on a V617F background, have established functions in hematopoiesis, including Lin28b, a negative regulator of let-7 microRNA biogenesis (Viswanathan et al., 2008; Piskounova et al., 2011), which is up-regulated in many human tumors and cancer cell lines (Viswanathan et al., 2009). The Lin28b–let–7 pathway is involved in the regulation of HSC self-renewal in fetal and adult hematopoiesis (Copley et al., 2013). Hmga2 is an established target of let-7 and, when up-regulated, is linked to increased megakaryopoiesis and MF (Oguro et al., 2012). Hmga2 is highly expressed in some patients with PMF (Andrieux et al., 2004). We also found Hmga2 to be up-regulated in MEPs from Ezh2<sup>−/−</sup> and V617F;Ezh2<sup>−/−</sup> mice (Fig. 6 F) and also in granulocytes from MPN patients with homozygous loss of EZH2 (Fig. 6 G). These data suggested a possible function of the Lin28b–let–7–Hmga2 axis in mediating the observed hyperproliferation of the megakaryocytic lineage and acceleration of MF in our mouse model.

ChIP-qPCR data demonstrated that the Lin28b and Hmga2 promoters in LSK and MEP cells exhibit reduced
H3K27me3 deposition upon Ezh2 loss (Fig. 7 A), suggesting that both are normally repressed by Ezh2. Although forced expression of Lin28b in V617F-expressing cells increased cell numbers and megakaryocyte numbers during growth in vitro (Fig. 7 B), this effect was not observed in mice transplanted with retrovirally transduced BM cells in vivo (Fig. 7 C). In contrast, overexpression of Hmga2 promoted megakaryopoiesis both in vitro and in vivo, suggesting that increased expression of Hmga2 is a downstream event mediating synergism between V617F and loss of Ezh2.

Our data support the proposed tumor suppressor function of EZH2 in patients with MPN. The synergism between mutant JAK2-V617F and reduced Ezh2 activity in hematopoiesis is evident in heterozygous V617F;Ezh2Δ/Δ mice, which is consistent with the finding that most MPN patients with EZH2 mutations do not progress to homozygosity. The prospect of using Ezh2 inhibitors in the context of MPN may carry the risk of aggravating the disease.

MATERIALS AND METHODS

Transgenic mice. Mice with Cre-recombinase–inducible human JAK2-V617F transgene were generated directly in the C57BL/6 background in our laboratory (Tiedt et al., 2008). These V617F mice were crossed with mice with a conditional Ezh2 knockout allele, in which exons 16 and 17 are flanked by loxP sites (Ezh2fl/fl, Neff et al., 2012) to generate double mutant mice (V617F;Ezh2Δ/Δ). These mice were crossed with MxCre transgenic mice (Kühn et al., 1995) or SclCre transgenic mice (Göthert et al., 2005) to obtain offspring that allow inducible deletion of the floxed Ezh2 alleles and activation of the V617F transgene. Ezh2Δ/Δ, MxCre, SclCrefl/fl, and UBC-GFP mice had been backcrossed into the C57BL/6 background for 12 generations, and the purity of the C57BL/6 background was verified using a 1449 SNP Illumina BeadChip for 12 generations, and the purity of the C57BL/6 background was verified using a 1449 SNP Illumina BeadChip panel and the SNaP-Map Software at the DartMouse Speed Congenic Core Facility at The Geisel School of Medicine at Dartmouth. Cre expression in MxCre mutant mice was induced by intraperitoneal injection of 300 µg pIpC three times every second day, and Cre expression in SclCre mutant mice was induced by intraperitoneal injection of 2 mg tamoxifen once daily for 4 wk, with a 1-wk break in the middle. The recipient C57BL/6 mice used for competitive transplantations were purchased from Janvier Labs. All mice in this study were kept under specific pathogen–free conditions with free access to food and water in accordance to Swiss Federal Regulations, and all experiments were performed in strict adherence to Swiss laws for animal welfare and approved by the Swiss Cantonal Veterinary Office of Basel-Stadt.

Competitive BM transplantations. For competitive transplantations, mice were intercrossed with the UBC-GFP strain, and whole BM or fractionated hematopoietic cells were used as donor cells. This system allows tracking donor-derived hematopoiesis (chimerism) in all hematopoietic lineages in recipient mice. Competitive whole BM transplantation was performed with 0.25 × 10⁶ BM cells from 10-wk-old SclCre mutant mice without tamoxifen injection. Whole BM cells were transplanted intravenously into 10-wk-old female recipient mice (C57BL/6/N) irradiated with 12 Gray (Gy), together with 0.75 × 10⁶ BM cells of WT mice. Tamoxifen was injected for 5 wk, starting 8 wk after transplantation, with 1-wk break in the middle of the treatment. LT-HSC transplantations were performed with 50 purified LT-HSCs from SclCre mutant mice 12 wk after tamoxifen injection together with 10⁶ BM cells of WT mice. LSK transplantation was performed with 1,500 purified LSK cells from MxCre mutant mice at 8 wk of age without pIpC injection together with 1.5 × 10⁶ BM cells of WT mice. For competitive transplantations at limiting dilution, 20,000 total BM cells from MxCre;GFP or MxCre;Ezh2Δ/Δ;GFP donormice 5 wk after pIpC injection were mixed with 2.0 × 10⁶ WT BM cells (1:100 ratio) and transplanted into lethally irradiated female recipients. For competitive transplantations at limiting dilution from MxCre;V617F;GFP or MxCre;V617F;Ezh2Δ/Δ;GFP donor mice, 8,000 total BM cells were harvested 5 wk after pIpC injection and transplanted with 10⁶ WT BM cells (1:250 ratio). Blood samples were taken every 6–8 wk to determine chimerism and for complete blood analysis. Secondary transplantations were performed with BM cells from primary recipients harvested at 30 wk after transplantation.

Purification of mouse LT-HSCs and MEP. Mouse LT-HSCs were purified from BM cells of SclCre mutant mice 12 wk after tamoxifen injection. BM mononuclear cells were stained with an allophycocyanin (APC)–conjugated anti–c-Kit antibodies (BioLegend). c-Kit–positive cells were isolated with goat anti–APC microbeads (Miltenyi Biotec) through an LS column (Miltenyi Biotec). The c-Kit–positive cells were further stained with antibody cocktail consisting of biotinylated anti–Gr-1, Mac-1, IL-7Rα, B220, CD4, CD8α, and Ter119 monoclonal antibodies (lineage-marker cocktail), PE-conjugated anti–CD150, PE-Cy7–conjugated anti–CD48, and brilliant violet 421 (BV421)–conjugated anti–Sca-1 antibodies. Biotinylated antibodies were detected with streptavidin–APC-Cy7 (BioLegend). Mouse MEPs were also purified from spleen cells of SclCre mutant mice 12 wk after tamoxifen injection. In brief, c-Kit–positive cells were isolated using PE-conjugated anti–c-Kit antibody and anti-PE microbeads with LS column (Miltenyi Biotec). The c-Kit–positive cells were further stained with lineage-marker cocktail followed by PE-Cy7–conjugated anti–CD16/32 (BioLegend), APC-conjugated anti–CD34 (BD), streptavidin-APC-Cy7, and BV421–conjugated anti–Sca-1 antibodies. Cell sorting was performed by Influx (BD), and results were analyzed with FlowJo software (Tree Star).

Transduction of mouse LT-HSCs and competitive repopulation assays. Recombinant retrovirus vectors, MIGR1-IRES-GFP, Mys-Hmga2-IRES-GFP, and Lin28b-IRES-GFP were provided by A. Iwama (Chiba University, Chuo-ku, Japan). To the limiting dilution, 20,000 BM cells from MxCre;GFP or MxCre;Ezh2Δ/Δ;GFP donor mice were transplanted into lethally irradiated female recipients. For competitive transplantations at limiting dilution from MxCre;V617F;GFP or MxCre;V617F;Ezh2Δ/Δ;GFP donor mice, 8,000 total BM cells were harvested 5 wk after pIpC injection and transplanted with 10⁶ WT BM cells (1:250 ratio). Blood samples were taken every 6–8 wk to determine chimerism and for complete blood analysis. Secondary transplantations were performed with BM cells from primary recipients harvested at 30 wk after transplantation.
Chiba, Japan). LT-HSCs were sorted into 96-well microtiter plates coated with the recombinant fibronectin fragment CH-296 (RetroNectin; Takara Bio Inc.) at 300 cells/well and were incubated in StemSpan (STEMCELL Technologies) supplemented with 100 ng/ml mouse SCF (PeproTech) and 100 ng/ml human Tpo (PeproTech) for 24 h. The cells were transduced with a retrovirus vector at a multiplicity of infection of 800 in the presence of 10 µg/ml protamine sulfate (Sigma-Aldrich) and 1 µg/ml RetroNectin for 24 h. After transduction, cells were further incubated in StemSpan supplemented with 10 ng/ml SCF and 10 ng/ml TPO. 150 LT-HSCs transduced with the indicated retrovirus were transplanted intravenously into 8-wk-old female C57BL/6 mice irradiated at a dose of 9.5 Gy, together with 2.5 × 10^5 BM competitor cells from 8-wk-old C57BL/6 mice.

**Peripheral blood analysis.** Blood was collected from the tail vein or by cardiac puncture, and complete blood counts (CBCs) were determined on an Advia 120 Hematology Analyzer using Multiprimer version 5.9.0-MS software (Bayer).

**Flow cytometry analysis.** The whole blood was stained with APC-conjugated anti-Ter119 and PE-conjugated anti-CD61 for erythrocyte and platelet analysis. White blood cells after RBC lysates were stained with PE-conjugated anti-CD8, PE-Cy7-conjugated anti-B220, APC-conjugated anti–Mac-1, APC-Cy7-conjugated anti–CD4, and Pacific blue (PB)–conjugated anti–Gr1 (BioLegend). Single cell suspensions from BM and spleen were stained with lineage-marker cocktail followed by FITC-conjugated anti–CD41, PE-conjugated anti–CD150, PE-Cy7–conjugated anti–CD48, APC–conjugated anti–c–Kit, BV421–conjugated anti–Sca-1, and streptavidin–APC–Cy7. For progenitor analysis, cells were stained with lineage-marker cocktail followed by FITC-conjugated anti–CD34 (BD), PE-conjugated anti–CD16/32, APC–conjugated anti–c–Kit, BV421–conjugated anti–Sca-1, and streptavidin–APC–Cy7 antibodies (BioLegend). For megakaryocytes and erythrocyte precursor analysis, cells were stained with FITC–conjugated anti–CD42c (Emfret), Dylight–647–conjugated anti–αIbβ3 (CD41/61; Emfret), PE–conjugated anti–CD71, and APC–conjugated anti–Ter119. Analyses were performed on an LSRFortessa (BD) or CyAn ADP Analyzer (Beckman Coulter), and results were analyzed by FlowJo software.

**Quantitative RT-PCR analysis.** Total RNA was extracted from 0.5 × 10^6 BM cells from SclCre mutant mice 12 wk after tamoxifen injection using TRIzol solution (Thermo Fisher Scientific) and reverse transcribed by High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific) with an oligo dT primer. Real-time qPCR was performed with 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), using the Power SYBR Green PCR Master mix and normalized with mouse Gusb. Sequences of primers are as follows: 5′–ACTTACTGCTGGCACCAGCCT–3′ and 5′–GTTGAACAGAAAGCTGCACA–3′ for mouse Ezh2 and 5′–ATAAGACGGATCAAGCCCG–3′ and 5′–ACTCCT CACTGGAACATGCGA–3′ for mouse Gusb. Total RNA from granulocytes of MPN patients were extracted and reverse transcribed using the same method (Lundberg et al., 2014a). Real-time qPCR was performed with 7500 Fast Real-Time PCR System, using the TaqMan gene expression system (Thermo Fisher Scientific) and normalized by human GUSB. The collection of blood samples was performed at the study center in Basel, Switzerland approved by the local Ethics Committee (Ethiskommission Beider Basel) and at the study center in Florence, Italy according to a research protocol approved by local IRB in Florence (#2011/0014777 and #2015/0015136). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of MPN was established according to the revised criteria of the World Health Organization (Vardiman et al., 2009).

**Western blotting.** Total BM cells (20 × 10^6) from SclCre mutant mice 12 wk after tamoxifen injection were lysed and immediately boiled in 400 µl SDS sample buffer. The lysates were subjected to electrophoresis on NuPAGE 4–12% Bis-Tris Protein Gels (Invitrogen) and transferred to nitrocellulose membranes by Western blotting. The membranes were probed with antibodies against Ezh2 (CST), β-actin, histone H3 (Abcam), H3K27me27 (EMD Millipore), histone H2A (CST), and H2AK119Ub (CST). Signals were detected by HRP-conjugated anti–mouse IgG or HRP-conjugated anti–rabbit IgG antibodies, followed by ECL detection.

**ChIP assay.** ChIP was performed using a True MicroChIP kit from Diagenode. In brief, 10,000 LSK cells or MEP cells isolated by FACS were cross-linked with 1% formaldehyde for 8 min at room temperature and incubated for 5 min after addition of 0.125 M glycine. Cells were washed with PBS and lysed by lysis buffer, and sonicated to the DNA fragments were 200–500 bp by Bioruptor Pico (Diagenode). Immuno-precipitation was performed by using an anti–H3K27me27 (EMD Millipore), and DNA was purified using MicroChIP DiaPure columns (Diagenode). qPCR analysis was performed using Power SYBR Green PCR Master mix with 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are as follows: promoter region of Hmg2a, 5′–CGAATCTGGACGACTT–3′ and 5′–GCCAAAGCCGTGACAA–3′; promoter region of Lin28b, 5′–GGGAGAGAGAGGGAAG–3′ and 5′–GGTGTGACCCGAAGATACAA–3′.

**Next generation sequencing and bioinformatics analysis.** Total RNA was isolated from 1,000 FACS-sorted LT-HSCs using Pico–Pure RNA isolation kit (ARCTURUS) or from 230,000 FACS-sorted MEP cells using TRIzol–LS solution. Total RNA was quality-checked on the Bioanalyzer instrument (Agilent Technologies) using the RNA 6000 Pico.
The library of LT-HSCs was prepared by using SMARTer Low Input RNA kit with Low Input Library Prep kit (Takara Bio Inc.). The library of MEPs was prepared using by TruSeq Stranded mRNA Library Prep kit (Illumina). Samples were pooled to equal molarity and run on the Fragment Analyzer for quality check and used for clustering on the NextSeq 500 instrument (Illumina). Samples were sequenced using the NextSeq 500 High Output kit 75-cycles (Illumina), and primary data analysis was performed with the Illumina RTA version 2.1.3 and bcl2fastq-2.16.0.10. Raw data were deposited in Gene Expression Omnibus under the accession no. GSE82074.

Reads were mapped against the mouse genome (version mm9; NCBI build 37) using the spliced-read aligner STAR (Dobin et al., 2013). The mean fraction of mapped reads was 80%. All subsequent gene expression data analysis was performed within the R software (R Foundation for Statistical Computing). Raw reads and mapping quality were assessed by the qQCReport function from the R/Bioconductor software package QuasR (Gaidatzis et al., 2015). Expression of RefSeq genes was quantified by counting reads mapping into exons using the qCount function of QuasR. For gene expression visualization, the resulting count table was adjusted by library size and log transformed (log2 expression) in figures. The full expression set was visualized as a heat map (unsupervised clustering of row and columns); it only includes genes that showed at least a 1.5 log2-fold change (relative to the mean) on an absolute scale in one of the samples and that were also significant (P ≤ 0.05) in one of the genotype comparisons. The heat map was generated using the NMF package (Gaujoux and Seoighe, 2010) with default clustering settings. For the PCA, genes were ordered by their absolute log2-fold change, and the top 1/4 (n = 4708) were retained for the analysis. PCA was performed with the R function “pccomp.”

The R/Bioconductor software package edgeR (McCarthy et al., 2012) was used for detecting differentially expressed genes between genotypes. The model fitted by edgeR included both Ezh2 and Jak2 genotypes as well as their interaction effect and additionally adjusted for gender as a possible confounder. P-values for the contrasts between genotypes were calculated by likelihood ratio tests and adjusted for multiple testing by controlling the expected false discovery rate (Benjamini and Hochberg, 1995). Competitive gene set enrichment analysis was performed on all categories of the Molecular Signature Database (MSigDB) using the function CAMERA of the edgeR package. The self-contained gene set testing on gene signature of fetal liver HSCs (Copley et al., 2013) was conducted using the function ROAST of the edgeR package.

Statistical analysis. Results are presented as means ± SEM. To assess the statistical significance among individual cohorts, one-way ANOVA with subsequent Bonferroni’s post-hoc multiple comparison test (Prism version 6 software; GraphPad Software) or unpaired Student’s t test was used. P ≤ 0.05 was considered significant.

Online supplemental material. Table S1, available as an Excel file, contains the complete gene list of differentially expressed transcripts found in LT-HSCs. Table S2, available as an Excel file, contains the complete gene list of differentially expressed transcripts found in MEP cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151136/DC1.

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