

Hmga2 collaborates with *JAK2*^{V617F} in the development of myeloproliferative neoplasms

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Key Points

- In patients with MPNs, repression of *MIRlet-7* and mutations in the polycomb genes *EZH2* and *ASXL1* correlate with *HMGA2* overexpression.
- *Hmga2* overexpression collaborates with *JAK2*^{V617F} to promote lethal MPN in mice, highlighting the crucial role of *Hmga2*.

High-mobility group AT-hook 2 (*HMGA2*) is crucial for the self-renewal of fetal hematopoietic stem cells (HSCs) but is downregulated in adult HSCs via repression by *MIRlet-7* and the polycomb-repressive complex 2 (PRC2) including *EZH2*. The *HMGA2* messenger RNA (mRNA) level is often elevated in patients with myelofibrosis that exhibits an advanced myeloproliferative neoplasm (MPN) subtype, and deletion of *Ezh2* promotes the progression of severe myelofibrosis in *JAK2*^{V617F} mice with upregulation of several oncogenes such as *Hmga2*. However, the direct role of *HMGA2* in the pathogenesis of MPNs remains unknown. To clarify the impact of *HMGA2* on MPNs carrying the driver mutation, we generated $\Delta Hmga2/JAK2^{V617F}$ mice overexpressing *Hmga2* due to deletion of the 3' untranslated region. Compared with *JAK2*^{V617F} mice, $\Delta Hmga2/JAK2^{V617F}$ mice exhibited more severe leukocytosis, anemia and splenomegaly, and shortened survival, whereas severity of myelofibrosis was comparable. $\Delta Hmga2/JAK2^{V617F}$ cells showed a greater repopulating ability that reproduced the severe MPN compared with *JAK2*^{V617F} cells in serial bone marrow transplants, indicating that *Hmga2* promotes MPN progression at the HSC level. *Hmga2* also enhanced apoptosis of *JAK2*^{V617F} erythroblasts that may worsen anemia. Relative to *JAK2*^{V617F} hematopoietic stem and progenitor cells (HSPCs), over 30% of genes upregulated in $\Delta Hmga2/JAK2^{V617F}$ HSPCs overlapped with those derepressed by *Ezh2* loss in *JAK2*^{V617F}/*Ezh2*^{Δ/Δ} HSPCs, suggesting that *Hmga2* may facilitate upregulation of *Ezh2* targets. Correspondingly, deletion of *Hmga2* ameliorated anemia and splenomegaly in *JAK2*^{V617F}/*Ezh2*^{Δ/wild-type} mice, and *MIRlet-7* suppression and PRC2 mutations correlated with the elevated *HMGA2* mRNA levels in patients with MPNs, especially myelofibrosis. These findings suggest the crucial role of *HMGA2* in MPN progression.

Introduction

Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (primary MF [PMF]), are characterized by the chronic proliferation of mature myeloid cells, extramedullary hematopoiesis, and progression to MF or acute leukemia.¹⁻³ Mutations in

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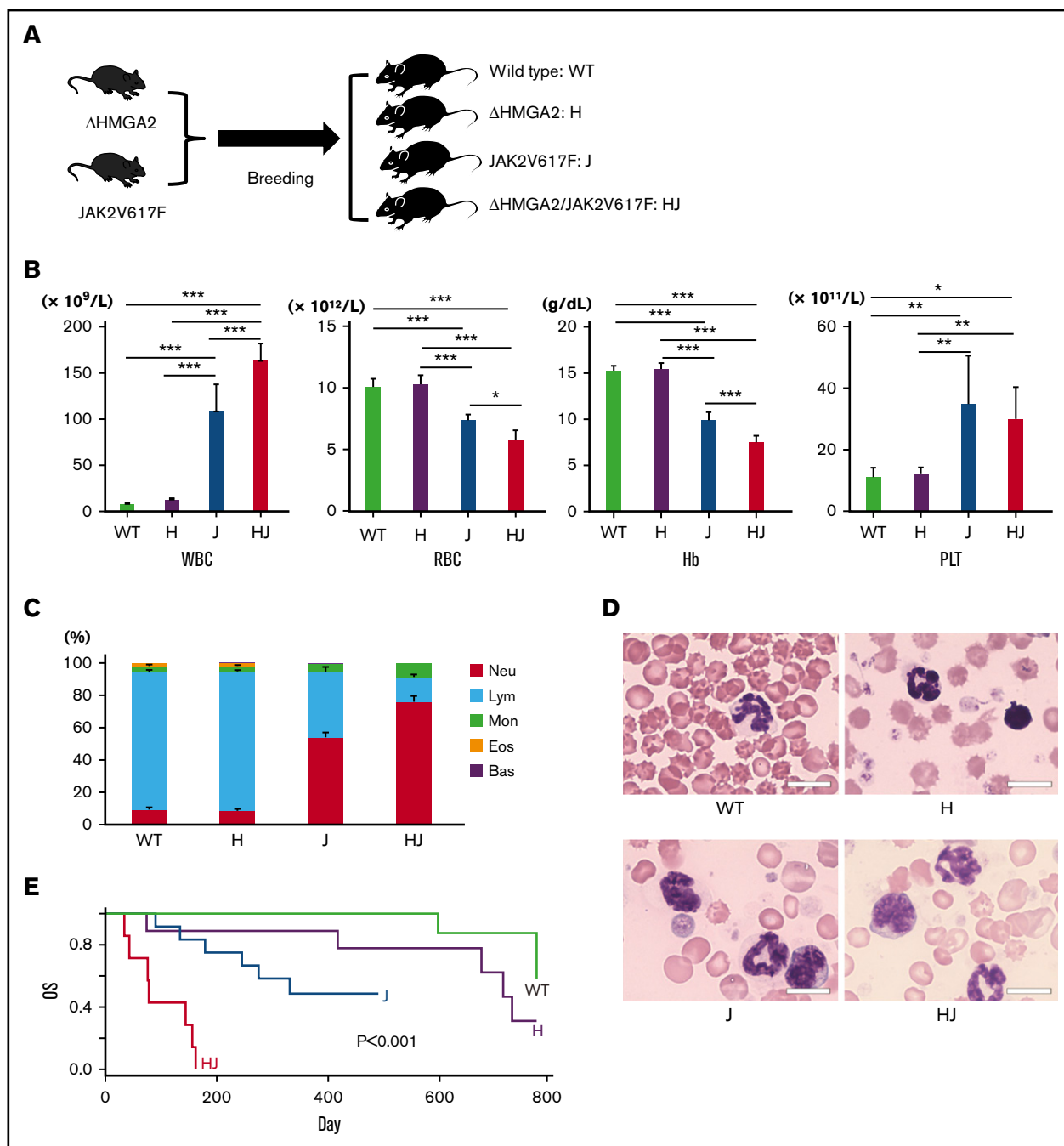


Figure 1. Progressive MPN in $\Delta Hmga2/JAK2^{V617F}$ mice. (A) Schematic diagram of the experiment. WT, $\Delta Hmga2$ (H), $JAK2^{V617F}$ (J), and $\Delta Hmga2/JAK2^{V617F}$ (HJ) mice were obtained in predicted Mendelian ratios. The analyses were performed at 12 weeks old. (B) Complete blood cell counts (means \pm standard error of the mean [SEM]) and (C) fractions of white blood cells (%) in 5 WT, 7 H, 5 J, and 4 HJ mice. * $P < .05$; ** $P < .01$; *** $P < .001$ calculated by the Tukey-HSD test. (D) May-Giemsa stains of peripheral blood samples (original magnification $\times 1000$). Scale bars indicate 50 μ m. (E) Survival periods. N = 9 for WT, 9 H, 12 J, and 7 HJ mice. The P values were calculated by a log-rank test. Bas, basophil; Eos, eosinophil; Hb, hemoglobin concentration (g/dL); Lym, lymphocyte; Mon, monocyte; Neu, neutrophil; OS, overall survival; PLT, platelet count ($\times 10^{11}/L$); RBC, red blood cell count ($\times 10^{12}/L$); WBC, white blood cell count ($\times 10^9/L$).

$JAK2$,^{4,5} $CALR$,^{6,7} and MPL ⁸ are established drivers of myeloproliferation. Expression of $JAK2^{V617F}$ in mice can reproduce PV, ET, or MF phenotype,⁹⁻¹⁴ but the role of driver mutations in clonal expansion and disease progression has not been well defined. Mutations in epigenetic modifiers, such as $TET2$,¹⁵ $DNMT3A$,^{16,17} $ASXL1$,¹⁸ and $EZH2$,¹⁹ and the aberrant expression of microRNAs²⁰ might lead to

MPN progression by altering gene expression, but their targets are largely unknown.

We²¹ and others²² recently demonstrated elevation of the proto-oncogene, high-mobility group AT-hook 2 ($HMGA2$) messenger RNA (mRNA) in granulocytes and $CD34^+$ cells of patients with MPNs, especially in PMF with the worst outcome among MPNs.²³

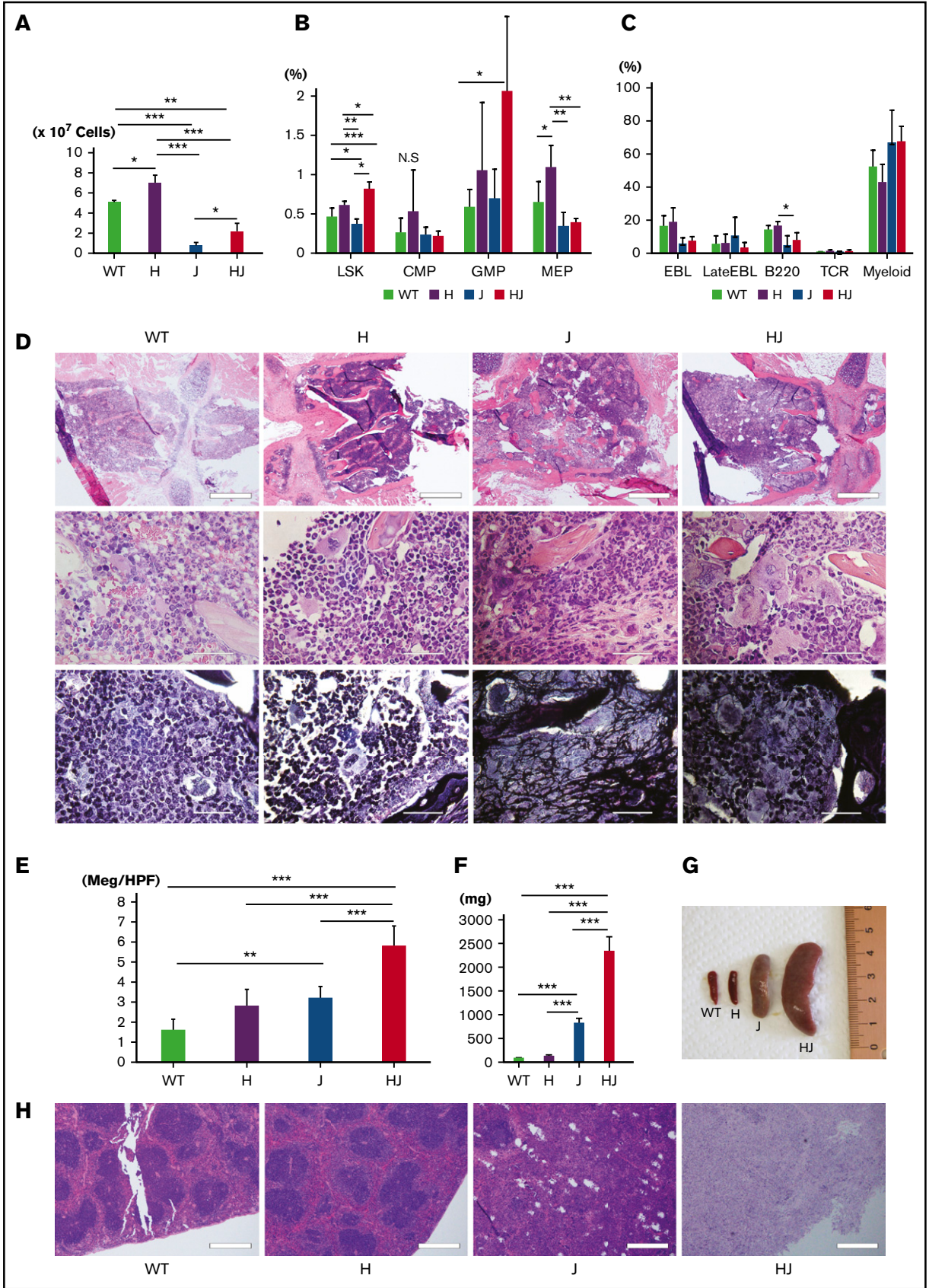


Figure 2.

HMGA2 expression is negatively regulated by *MIRlet-7* microRNAs,²⁴ and high *HMGA2* mRNA levels were correlated with reduced *MIRlet-7* in some patients with MPNs.²¹ We have generated transgenic (tg) mice harboring *Hmga2* complementary DNA (cDNA) with a truncated 3' untranslated region (UTR) ($\Delta Hmga2$), thereby lacking binding sites of *MIRlet-7* that repress *Hmga2* expression.²⁵ $\Delta Hmga2$ mice overexpress *Hmga2*, develop ET-like disease, and their hematopoietic cells exhibit a clonal advantage against wild-type (WT) cells at the hematopoietic stem cell (HSC) level in serial bone marrow (BM) transplants (BMTs). Alternatively, endogenous *Hmga2* expression is upregulated by the loss of *Bmi1* or *Ezh2* in conjunction with *Arf/Ink4a*-knockout (KO)²⁶ or *JAK2V617F*²⁷⁻²⁹ in mice representing more severe MF than mice with *Arf/Ink4a*-KO or *JAK2V617F* alone.

HMGA2 is important for the self-renewal of fetal HSCs, but is not expressed in normal adult tissues and hematopoiesis,³⁰ whereas various cancer cells overexpress *HMGA2*.^{31,32} *HMGA2* mRNA is overexpressed in hematopoietic cells of most patients with PMF,^{21,22,33} but its direct role in the MPNs remains unknown. Here, because most MPN patients with high *HMGA2* mRNA levels harbor a driver mutation represented by *JAK2V617F*,²¹ we studied mice carrying both *JAK2*^{V617F} and overexpression of *Hmga2* due to tg- $\Delta Hmga2$ ($\Delta Hmga2/JAK2^{V617F}) or conditional deletion of *Ezh2* (*JAK2*^{V617F}/*Ezh2*^Δ). The present study revealed that *Hmga2* directly promotes progression of MPN with *JAK2V617F* and, correspondingly, severe MPN of *JAK2*^{V617F}/*Ezh2*^{Δ/WT} mice can be rescued by deletion of *Hmga2*; the study also revealed that *MIRlet-7* suppression and polycomb-repressive complex 2 (PRC2) mutations correlated with the elevated *HMGA2* mRNA levels in patients with PMF.$

Materials and methods

Mice

All mice were on a C57BL6/J background and analyzed 12 weeks after birth or 8 weeks after BMT unless otherwise specified. $\Delta Hmga2$ mice²⁵ and *JAK2*^{V617F} mice (line 2 with MF generated from BDF-1 by backcrossing with WT C57BL6/J mice)^{13,34} were previously described. *Ezh2*^{flox/flox} mice³⁵ were crossed with *Rosa26::Cre-ERT* mice (TaconicArtemis GmbH, Köln, Germany), and deletion of *Ezh2* was induced by intraperitoneal administration of 1 mg per day tamoxifen (Sigma-Aldrich, St. Louis, MO) in corn oil (Sigma-Aldrich) for 5 consecutive days from 4 weeks after BMT. *HMGA2*^{-/WT} mice,³⁶ and *Ly5.2*⁺ and *Ly5.1*⁺ WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME), CLEA (Tokyo, Japan), and RIKEN-BRC (Tsukuba, Japan). Peripheral blood cells were counted by XT-1800i (Sysmex, Kobe, Japan). All

studies were approved by the Animal Study Committee of Fukushima Medical University.

Histopathology

Hematoxylin-eosin, silver, and anti-HMGA2 stains (Spring Bioscience, Pleasanton, CA) were performed for paraffin-embedded samples with standard protocols. Pictures were taken and digitized by a DP73 microscope with CellSens software (Olympus, Tokyo, Japan).

Cells

From mice, peripheral leukocytes were obtained by lysing samples with Pharm Lyse (BD, Franklin Lakes, NJ). BM nuclear cells were collected by grinding bones, and mononuclear cells (MNCs) were separated by centrifugation through Ficoll Histopaque (Sigma-Aldrich). The patients' granulocytes were separated by centrifugation through Ficoll Lymphosepar I (IBL, Gunma, Japan).²¹

Flow cytometry

Flow cytometry was performed using a FACSCanto II (BD). Cells were stained with fluorescein isothiocyanate (FITC)-Ter119, phycoerythrin (PE)-CD71, eFluora 450-B220, peridinin chlorophyll (PerCP)/Cy5.5-T-cell receptor β (TCR β), and allophycocyanin (APC)-Gr1; or PerCP/Cy5.5-Sca-1, PE/Cy7-cKit, Alexa647-CD34, PE-CD16/32, FITC-CD41, and Biotin-Lineage (CD3e, CD4, CD5, CD8a, CD11, B220, Ter119, and Gr1) followed by visualization with APC/Cy7-streptavidin. For chimerism analyses, eFluora450-B220, PerCP/Cy5.5-TCR β , APC-CD45.1, and PE-CD45.2 (all from eBioscience, San Diego, CA) were used. Phosflow with phosphorylated STAT3 (pSTAT3) antibody (BD) was performed according to the manufacturers' protocol.

Colony assay

On a 35-mm plate, BM MNCs were cultured in 1 mL of semisolid methylcellulose medium. For colony-forming unit-erythroid (CFU-E), 1×10^5 cells were cultured in M3234 (StemCell Technologies, Vancouver, BC, Canada) with or without human erythropoietin (EPO; Bioworld Technology, St. Louis Park, MN), and the colony numbers were counted after 3 days. For myeloid colonies, 1×10^4 cells were cultured in M3434 (StemCell Technologies) and colonies were counted after 5 days.

Western blotting

The total protein was extracted from 1×10^7 BM MNCs using CellLytic M buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich). The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a

Figure 2. *Hmga2* expression augments BM cells and HSCs with enhancing extramedullary hematopoiesis in *JAK2*^{V617F}-induced MPN. (A) The total nuclear cell numbers from the ground right femur ($\times 10^7$). N = 3 for each. (B) The ratio of each fraction in BM by fluorescence-activated cell sorter (FACS; percentage in all gated cells) is shown. N = 5 for WT, 5 H, 4 J, and 3 HJ mice. (C) Ratios of differentiated cells in BM (percentage in all gated cells). N = 3 for each genotype. (D) Sternal BM histology. Hematoxylin and eosin (top and middle) and silver (bottom) stains. Scale bars indicate 500 μ m (top; original magnification $\times 40$) and 50 μ m (middle and bottom; original magnification $\times 1000$). (E) Mean megakaryocyte counts in high-powered field (Meg/HPF; $\times 400$). Mean of 5 fields are shown in each genotype. (F) Spleen weight (N = 4, each genotype). (G) Representative picture of the spleens from the littermates. (H) Histology of spleens (hematoxylin and eosin stain). Scale bars indicate 500 μ m (original magnification $\times 40$). (A-C, E-F) Bars show the means \pm SEM. **P* < .05; ***P* < .01; ****P* < .001 (Tukey-HSD test). B220, B220⁺ B cell; CMP, common myeloid progenitor cell; EBL, CD71⁺Ter119⁺ erythroblast; GMP, granulocyte-macrophage progenitor cell; H, $\Delta Hmga2$; HJ, $\Delta Hmga2/JAK2^{V617F}; J, *JAK2*^{V617F}; LateEBL/Ret, CD71⁺Ter119^{dim} late erythroblast/reticulocyte; MEP, megakaryocyte-erythroid progenitor cell; Myeloid, Gr-1⁺ myeloid cell; N.S., not significant; T, TCR⁺ T cell.$

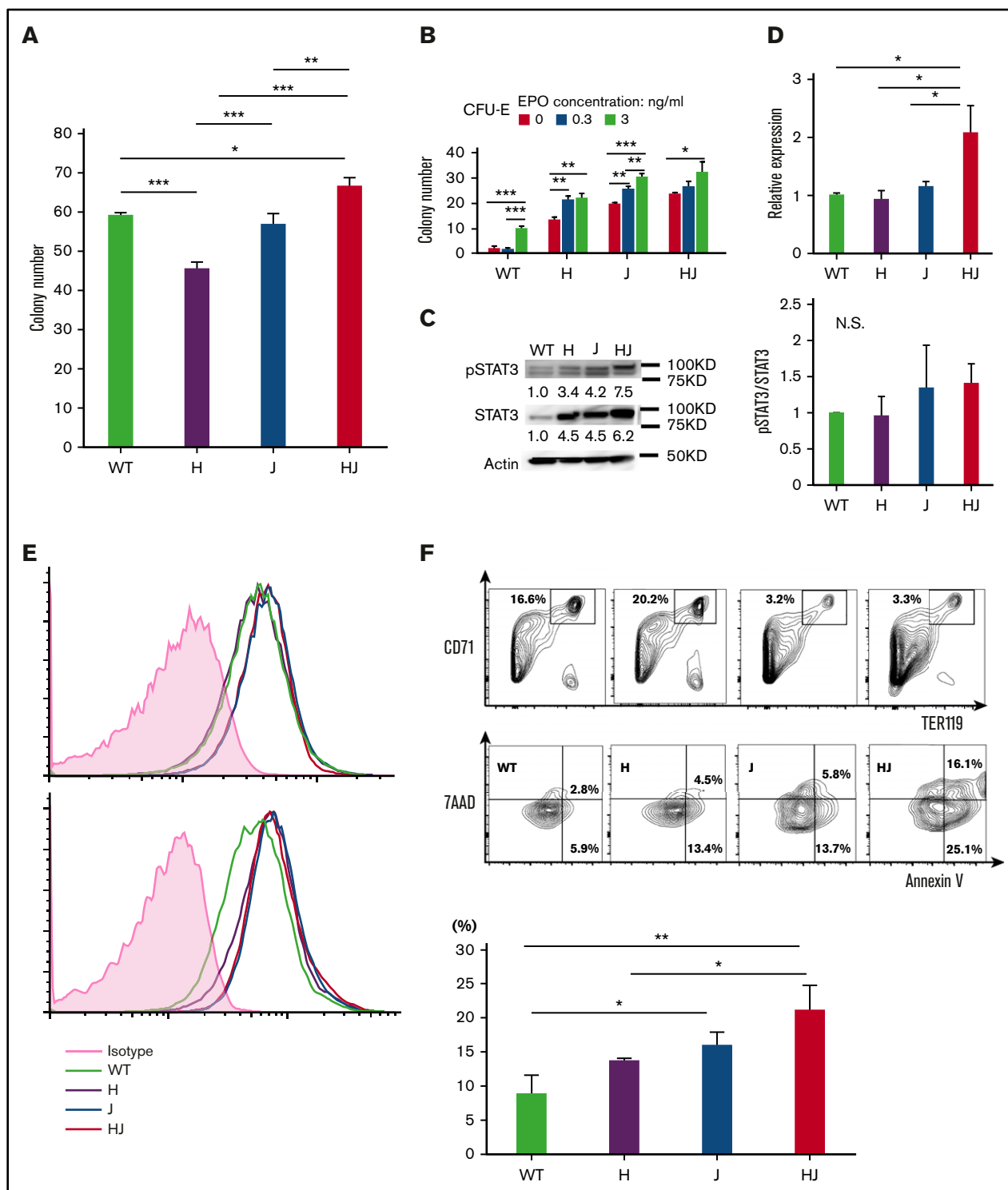


Figure 3. Stat3 activation and apoptotic erythroblasts in $\Delta Hmga2/JAK2^{V617F}$ mice. (A) Myeloid colonies from 1×10^4 BM MNCs ($N = 3$ for each genotype, means \pm SEM). $*P < .05$; $**P < .01$; $***P < .001$ (Tukey-HSD tests). (B) Numbers of CFU-E colonies (means \pm SEM) from 1×10^5 BM MNCs ($N = 3$ for each genotype). $*P < .05$; $**P < .01$; $***P < .001$ (Tukey-HSD tests). The P values calculated by ANOVA were $<.01$ for both EPO concentrations and mouse transgenes. (C) Western blotting of Stat3 and pStat3 in BM MNCs without cytokine stimulation in each tg mouse. Left panel, A representative result; right panel, mean pSTAT3/STAT3 concentration (from 2 independent experiments) relative to WT. (D) Stat3 mRNA in BM MNCs by qRT-PCR (means \pm SEM; $N = 3$ for each). $*P < .05$ (Tukey-HSD test). (E) FACS for pStat3 in BM MNCs incubated in the absence (top) and presence (bottom) of IL-3. The horizontal and vertical axes, respectively, indicate the brightness of pStat3 and the cell counts. (F) FACS for apoptosis of CD71⁺Ter119⁺ erythroblasts by Annexin V/7-Aminoactinomycin D (7AAD) staining. Top panel, A representative result; bottom panel, mean ratio of Annexin V⁺/7AAD⁻ apoptotic erythroblasts from 3 independent experiments. $*P < .05$; $**P < .01$; $***P < .001$ (Tukey-HSD tests). (A-F) H, $\Delta Hmga2$; J, $JAK2^{V617F}$; HJ, $\Delta Hmga2/JAK2^{V617F}$.

nitrocellulose membrane (GE Healthcare Life Sciences, Uppsala, Sweden), blocked with 5% bovine serum albumin (Wako, Tokyo, Japan), and probed with primary antibodies to Stat3, pStat3 (Cell Signaling Technology, Beverly, MA), actin, and anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX). The signals were detected with the LAS3000 (Fujitsu, Tokyo, Japan), and densities of bands were measured using ImageJ (version 1.50i; NIH, Bethesda, MD).

BMT

Recipient mice were lethally irradiated (9.0 Gy) 24 hours before BMT and cells were injected IV under anesthesia with isoflurane (Intervet, Osaka, Japan). In the competitive repopulations, 2.5×10^6 Ly5.2⁺ BM cells with the same number of Ly5.1⁺ WT BM cells were injected into Ly5.1⁺ mice. Serial BMTs were performed from these recipients to other lethally irradiated Ly5.1⁺ mice. For phenotypic reconstitution, 5.0×10^6 BM cells were injected.

RNA sequencing

Each preparation of RNA extracted from flow-sorted BM lineage⁺ Sca1⁺cKit⁺ cells (LSKs) and CD71⁺Ter119⁺ erythroblasts using an RNeasy Mini kit (Qiagen, Hilden, Germany) was subjected to reverse transcription (RT) and amplification for 14 cycles, respectively, with the SMARTer Ultra Low Input RNA kit for Sequencing v3 (Clontech, Palo Alto, CA). The double strand-cDNA was fragmented using the S220 Focused ultrasonicator (Covaris), then cDNA libraries were generated using a NEBNext Ultra DNA Library Prep kit (New England BioLabs, Beverly, MA). Sequencing was performed using HiSeq1500 (Illumina) with a single-read sequencing length of 60 bp. After mapping the reference genes to mm10 with TopHat v2.0.13, fragments per kilobase of exon per million reads mapped (FPKM) and reads per kilobase of exon per million reads mapped (RPKM) values were calculated using Cufflinks v2.2.1. After omitting the genes with <0.3 FPKM/RPKM values, values were used for evaluation of gene expressions and detections of affected gene sets by gene set enrichment analysis (GSEA; Broad Institute, Cambridge, MA). Genes with >1.5-fold upregulation were also applied to the pathway analysis using Ingenuity Pathways Analysis (IPA; Qiagen). Principal component analysis and statistical significance for overlapping genes among genotypes were evaluated according to the RPKM values with 0.5 of the pseudo count using R 3.3.0 (R Foundation, Vienna, Austria). Gene sets with both <.05 nominal *P* values and <0.25 false discovery rate (FDR) *q* values were determined as significantly affected sets in GSEA. Pathways with a *z* score > |2| were considered to be significant for IPA.

Patients

We studied 45 patients (27 ET, 18 PMF), including 15 patients who were enrolled in our previous study.²¹ The diagnosis was made according to World Health Organization (WHO) 2008 criteria,³⁷ and peripheral blood samples were collected at the time of examination. Samples from 13 healthy volunteers served as controls. The present

study was approved by the Ethics Review Board of Fukushima Medical University, which is guided by local policy, national law, and the Declaration of Helsinki. Written informed consent was obtained from each patient and healthy volunteer.

qRT-PCR

The total RNA extracted from granulocytes was used for RT with RevarTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and the TaqMan MicroRNA RT kit (Life Technologies, Carlsbad, CA) for mRNA and microRNA, respectively. Quantitative RT-polymerase chain reaction (qRT-PCR) was performed using a TP800 (Takara, Otsu, Japan) followed by data analysis with Multiplate RQ software (Takara) using the comparative cycle threshold method. RNA levels were determined as relative to *HPRT1* and *U6* noncoding RNA for mRNA and microRNA, respectively. The upper limit of the *HMG2* mRNA level in granulocytes (1.0) was determined in our former study according to +2 standard deviations (SDs) of the mean value in 13 healthy volunteers.²¹ The lower limit level of *MIRlet-7c* in granulocytes was determined according to -2SD of the mean value of 15 healthy volunteers. The primers and probes are listed in supplemental Table 1.

Mutation analysis

Genomic DNA was extracted from the patients' peripheral leukocytes using the QIAamp DNA Mini kit (Qiagen) for screening of mutations in *JAK2*, *MPL*, and *CALR*³⁸⁻⁴⁰ and the target sequence for 50 genes⁴¹ in a GeneRead DNaseq panel for human myeloid neoplasms (Qiagen) using a MiSeq (Illumina). The data were analyzed by the Qiagen NGS Data Analysis web portal. Silent mutations and known germ line polymorphisms in the National Center for Biotechnology Information single nucleotide polymorphism public database were excluded.

Statistics

Statistical significance was determined by an unpaired 2-tailed Student *t* test, the Tukey-honest significant difference (HSD) test, or 2-way factorial analysis of variance. Survival differences were measured by a log-rank test. All statistical analyses were performed using R3.3.0.

Results

Hmga2 promotes progression of MPN in *JAK2*^{V617F} mice

First, we crossed $\Delta Hmga2$ mice with *JAK2*^{V617F}-tg (*JAK2*^{V617F}) mice (Figure 1A). WT, $\Delta Hmga2/JAK2$ ^{WT} ($\Delta Hmga2$), *Hmga2*^{WT}*JAK2*^{V617F} (*JAK2*^{V617F}), and $\Delta Hmga2/JAK2$ ^{V617F} mice were born at expected Mendelian ratios. At 12 weeks old, *JAK2*^{V617F} mice showed leukocytosis and anemia; we previously reported the similar trend in mice with *JAK2*^{V617F} of BDF-1¹³ and C57BL6/J^{27,34} backgrounds. However, leukocytosis and anemia were most severe in $\Delta Hmga2/JAK2$ ^{V617F} mice (Figure 1B). Neutrophilia without proliferation of blasts was prominent in $\Delta Hmga2/JAK2$ ^{V617F} mice

Figure 4. (continued) for recipients of the secondary BMT. Among *JAK2*^{V617F} mice (N = 7), BM cells from mice that achieved engraftment at the first BMT (red lines, N = 3) were exclusively referred to the second BMT. (B) Kaplan-Meier survival curves, (C) sternal BM histology, and (D) representative picture of the spleens of recipient mice at 12 weeks after the BMT, in BMT from the indicated transgenic mice without competitors (N = 6 for each). (B) *P* values were calculated by a log-rank test. (C) Hematoxylin-eosin (HE) and silver stains are shown (original magnification $\times 400$). Scale bars indicate 50 μ m. H, $\Delta Hmga2$; J, *JAK2*^{V617F}; HJ, $\Delta Hmga2/JAK2$ ^{V617F}.

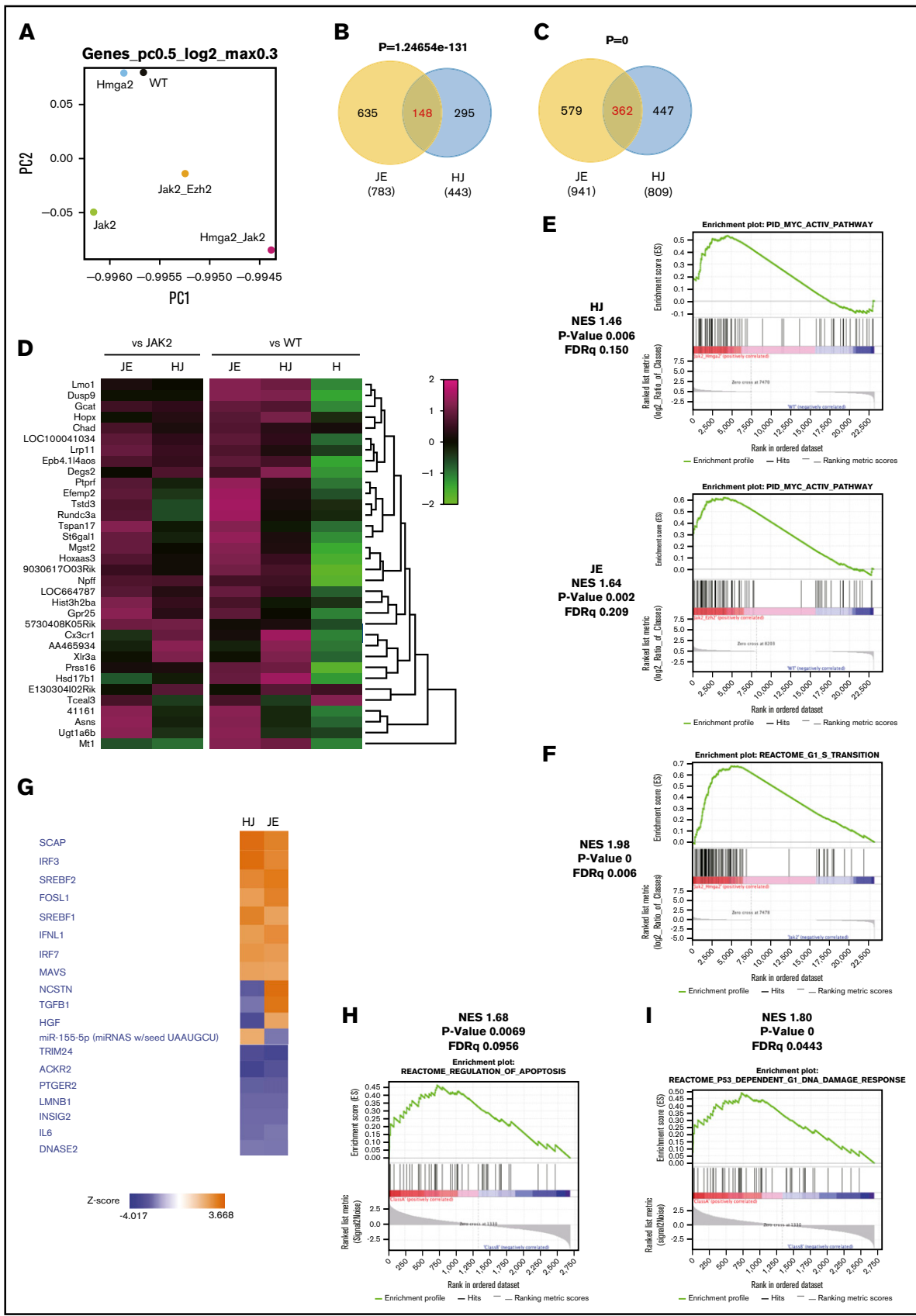


Figure 5.

(Figure 1C-D). The $\Delta Hmga2/JAK2^{V617F}$ mice died within 150 days, whereas other mice survived for over 1 year (Figure 1E). The number of BM nuclear cells were increased in $\Delta Hmga2$ mice but decreased in $JAK2^{V617F}$ mice, and they were partially restored in $\Delta Hmga2/JAK2^{V617F}$ mice (Figure 2A). In the presence of $JAK2^{V617F}$, $Hmga2$ augmented the ratio of hematopoietic stem and progenitor cell (HSPC)-enriched LSKs (Figure 2B), but not erythroblasts (Figure 2C). Modest increases of granulocyte-macrophage progenitor and $Gr1^+$ cells were observed in the $JAK2^{V617F}$ and $\Delta Hmga2/JAK2^{V617F}$ mice (Figure 2B-C). Megakaryocytes were more proliferative in $\Delta Hmga2$ and $JAK2^{V617F}$ mice compared with WT mice, and were further increased in $\Delta Hmga2/JAK2^{V617F}$ mice without morphologic change (Figure 2D-E). $JAK2^{V617F}$ and $\Delta Hmga2/JAK2^{V617F}$ BM were equally fibrotic; there was no MF in the WT or $\Delta Hmga2$ BM (Figure 2D). Splenomegaly was more remarkable and the splenic structures were destroyed in $\Delta Hmga2/JAK2^{V617F}$ mice compared with both $\Delta Hmga2$ and $JAK2^{V617F}$ mice (Figure 2F-H). BM cells showed slight increases in myeloid colonies in $\Delta Hmga2/JAK2^{V617F}$ mice (Figure 3A). All of the $\Delta Hmga2$, $JAK2^{V617F}$, and $\Delta Hmga2/JAK2^{V617F}$ BM showed EPO-independent erythroid-colony formation, an important feature of MPN. The colony numbers were the highest in $\Delta Hmga2/JAK2^{V617F}$ for each EPO concentration, and the difference became smaller as the EPO concentration increased (Figure 3B).

We sought the basis of the severe phenotype in $\Delta Hmga2/JAK2^{V617F}$ mice. Based on a recent finding on the importance of STAT3 in MPNs,⁴² we investigated the Stat3 status. We found increased expression of *Stat3* mRNA and protein in BM MNCs of $\Delta Hmga2/JAK2^{V617F}$ mice compared with $\Delta Hmga2$ and $JAK2^{V617F}$ mice (Figure 3C-D), whereas the basal Stat3 phosphorylation was comparable among these genotypes (Figure 3C,E). We also evaluated the apoptosis of erythroblasts because $\Delta Hmga2/JAK2^{V617F}$ mice presented with more severe anemia despite EPO-independent erythroid-colony formation and similar MF compared with $JAK2^{V617F}$ mice. The ratios of erythroblasts were equally altered in $JAK2^{V617F}$ and $\Delta Hmga2/JAK2^{V617F}$ BM, but $\Delta Hmga2/JAK2^{V617F}$ erythroblasts were more apoptotic (Figure 3F).

Hmga2 expands the $JAK2^{V617F}$ clone and reproduces the severe MPN after BMT

To investigate repopulating ability, we transplanted $Ly5.2^+$ $\Delta Hmga2$, $JAK2^{V617F}$, or $\Delta Hmga2/JAK2^{V617F}$ BM cells with $Ly5.1^+$ competitor cells into lethally irradiated mice. After the initial BMT, $\Delta Hmga2$, and $\Delta Hmga2/JAK2^{V617F}$ cells were engrafted in all recipients, whereas $JAK2^{V617F}$ cells were <10% in 4 of 7 recipients (Figure 4A). The proportion of the $Ly5.2^+$ donor cells were the highest in recipients of $\Delta Hmga2$ followed by $\Delta Hmga2/JAK2^{V617F}$ in B cells and total cells, and the opposite was found in myeloid cells. After a second BMT, $\Delta Hmga2/JAK2^{V617F}$ cells were less dominant than $\Delta Hmga2$ cells

but were restored, whereas $JAK2^{V617F}$ cells were completely rejected (Figure 4A). $\Delta Hmga2$ cells fully expanded after a third BMT (not shown), confirming the strong repopulating and self-renewal abilities of HSCs overexpressing $Hmga2$,^{25,30} which likely conferred a clonal dominance to $JAK2^{V617F}$ cells. Moreover, the BMT from $\Delta Hmga2/JAK2^{V617F}$ mice to WT mice reproduced splenomegaly and proliferation of megakaryocytes in BM, despite not inducing MF (Figure 4C-D). However, mice transplanted with $\Delta\Delta Hmga2/JAK2^{V617F}$ cells survived for a shorter time compared with the recipients from single-tg mice (Figure 4B).

Long-term Hmga2 expression causes anemia without MF

The impact of $Hmga2$ on MF was not evident in BMT. To clarify whether $Hmga2$ alone induces fibrosis in the long-term, we studied old $\Delta Hmga2$ mice (supplemental Figure 1). Eventually, 60-week-old $\Delta Hmga2$ mice exhibited anemia, but ratios of their erythroid progenitors and erythroblasts were not different from old WT mice. They showed moderate splenomegaly, but never developed MF, as confirmed in a considerable number (N = 26). Moreover, the erythroblasts from old $\Delta Hmga2$ mice were more apoptotic compared with old WT mice. However, serial BMT revealed that cells from old $\Delta Hmga2$ mice retain a clonal advantage against young WT cells. Taken together, the long-term expression of $Hmga2$ induces anemia possibly due to the apoptosis of erythroblasts without MF, whereas their HSCs possess the repopulating ability of young $\Delta Hmga2$ mice.

Gene expression profile in $JAK2^{V617F}$ cells with Hmga2 overexpression

HMGA2 changes chromatin structure and binds to AT-rich DNA sequences via AT-hook domains, leading to the upregulations of targets.⁴³ To identify HMGA2 targets in the setting of MPNs, we performed RNA sequencing (RNA-seq) of LSKs from WT, $\Delta Hmga2$, $JAK2^{V617F}$, and $\Delta Hmga2/JAK2^{V617F}$ mice, as well as $JAK2^{V617F}/Ezh2^{\Delta\Delta}$ mice based on recent reports showing that *Ezh2* KO deteriorates MPNs with upregulating endogenous *Hmga2* expression in HSPCs of mice with $JAK2^{V617F}$.²⁷⁻²⁹ Relative to WT, $\Delta Hmga2/JAK2^{V617F}$ mice showed the greatest change in the gene expression profile among genotypes (Figure 5A). We found that 148 of 443 genes (33.4%) or 362 of 809 genes (44.7%) were upregulated in $\Delta Hmga2/JAK2^{V617F}$ mice compared with $JAK2^{V617F}$ mice or WT mice, respectively, overlapped with those of $JAK2^{V617F}/Ezh2^{\Delta\Delta}$ mice (Figure 5B-C; supplemental Table 2), and that 35 genes, including known oncogenes targeted by *Ezh2*, *Lmo1*, *Gcat*, and *Prss16*,⁴⁴ were common (Figure 5D). There were some commonly enriched gene sets, including those involved in activation of the MYC pathway between $\Delta Hmga2/JAK2^{V617F}$ and $JAK2^{V617F}/Ezh2^{\Delta\Delta}$ mice (Figure 5E). $\Delta Hmga2/JAK2^{V617F}$ LSKs had frequent upregulation of gene

Figure 5. Hmga2 overexpression alters transcription of genes including the targets of Ezh2. (A) A Principal component (PC) analysis based on total gene expression in LSK cells (LSKs) isolated from WT, H, J, HJ, or JE recipient mice (5 for each and mixed for RNA-seq) 4 weeks after tamoxifen injection. (B) Venn diagrams showing upregulated genes between HJ and JE LSKs relative to J. (C) Venn diagrams showing upregulated genes between HJ and JE LSK cells relative to WT. (D) Heatmaps showing the expression of representative genes upregulated in panels B and C. (E) MYC pathway enriched in HJ (top) and JE (bottom) compared with WT. This pathway was not enriched in J (FDR = 0.389). (F) Enrichment plot of G1-S transition (HJ vs J), which represents frequent upregulations of gene sets associated with cell cycle and metabolism in $\Delta Hmga2/JAK2^{V617F}$ LSKs (shown in supplemental Tables 3 and 4). (G) Signal activation/inactivation in HJ and JE mice relative to J shown by an upstream analysis. Pathways with z score > |2| in both HJ and JE are shown. (H) CD71⁺Ter119⁺ erythroblasts sorted from BM of 12-week-old WT, H, J, and HJ mice (3 for each) were mixed for RNA-seq. Enrichment of gene sets for regulation of apoptosis (HJ vs J) is shown. (I) GSEA of RNA-seq data for p53-dependent G1 DNA damage response pathway in erythroblasts (HJ vs J). H, $\Delta Hmga2$; J, $JAK2^{V617F}$; HJ, $\Delta Hmga2/JAK2^{V617F}$; JE, $JAK2^{V617F}/Ezh2^{\Delta\Delta}$; NES, normalized enrichment score.

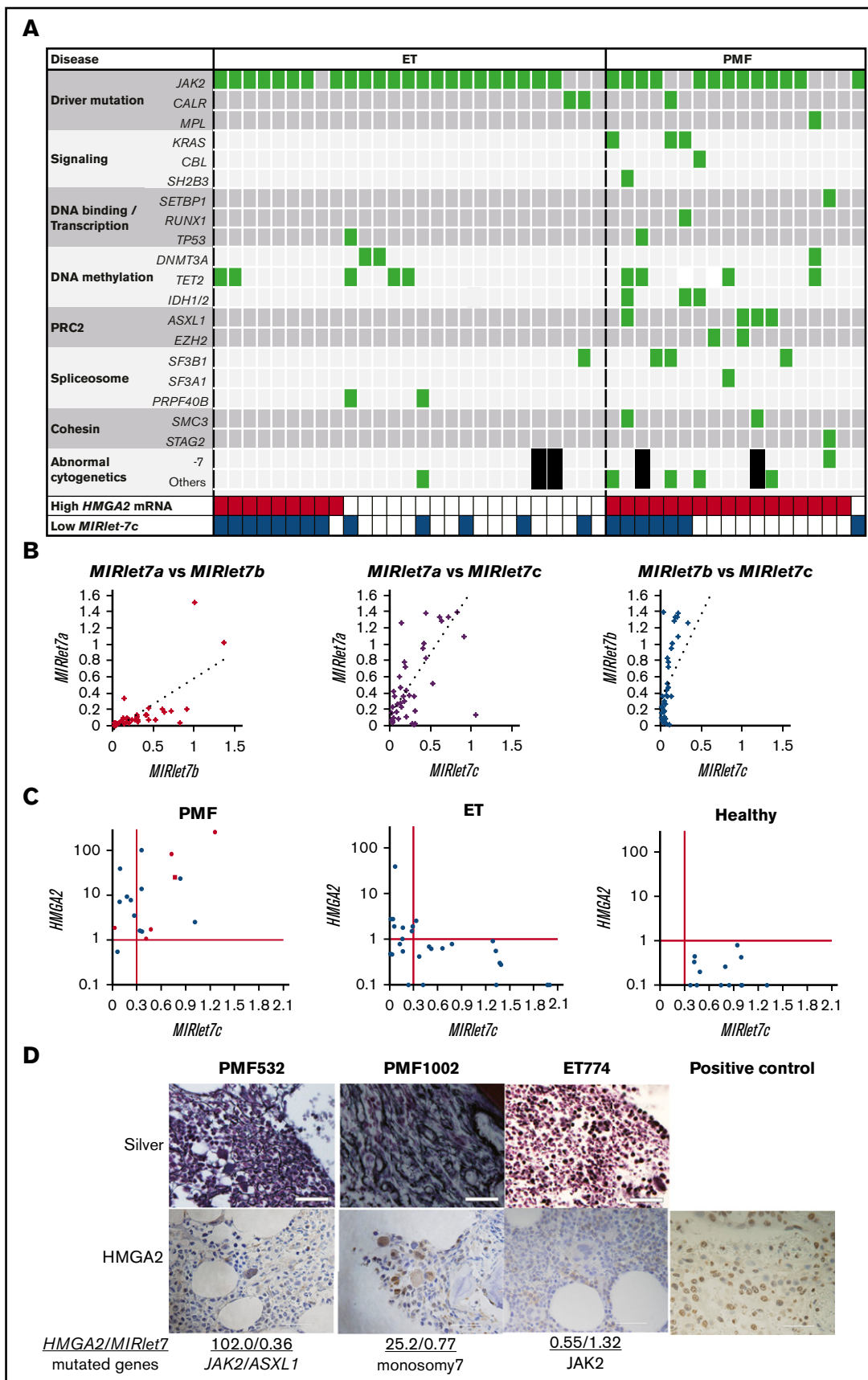


Figure 6.

sets associated with cell cycle and metabolism (Figure 5F; supplemental Tables 3 and 4). Pathway analysis with IPA also revealed the common activation of several pathways in LSKs of $\Delta Hmga2/JAK2^{V617F}$ and $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ mice compared with those of $JAK2^{V617F}$ mice, including chaperone-related *SCAP* and *SREBF1/2* pathways, whereas *TGFB* was upregulated only in those of $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ mice (Figure 5G).

We also investigated $CD71^+Ter119^+$ erythroblasts by RNA-seq to identify the mechanisms of severe anemia in $\Delta Hmga2/JAK2^{V617F}$ mice. Significantly affected groups (supplemental Tables 5 and 6) included gene sets of apoptosis regulation (Figure 5H) and p53 activation (Figure 5I), which is well known to induce anemia due to apoptosis of erythroid progenitors.⁴⁵⁻⁴⁷ Moreover, enriched gene sets in erythroblasts were different from those in LSKs. For example, interferon-related genes, involving the JAK-STAT pathway, were enriched in erythroblasts (FDR $q = 0.02$; supplemental Table 5), but not in LSKs (FDR $q > 0.5$), of $\Delta Hmga2/JAK2^{V617F}$ mice.

Most patients with PMF highly express *HMGA2* mRNA with the repression of *MIRlet-7* or mutations in PRC2 components

Subsequently, we studied the *HMGA2* mRNA and *MIRlet-7* levels, and mutations in the epigenetic modifiers in patients with MPNs. We focused on ET and PMF because the megakaryocyte lineage is dominant in those groups and an *Ezh2* deletion in megakaryocytes played an important role in the progression of $JAK2^{V617F}$ -induced MF.²⁷ High *HMGA2* mRNA levels were observed in granulocytes of some ET (9 of 27, 33.3%) and most PMF (17 of 18, 94.4%). Most patients had a driver mutation in *JAK2*, *CALR*, or *MPL* in both ET (25 of 27, 92.6%) and PMF (15 of 18, 83.3%). For other investigated genes, most patients with PMF harbored at least 1 additional mutation, whereas ET rarely had such a mutation, except for DNA methylation-related enzymes (Figure 6A; supplemental Table 7). Patients with PMF also carried chromosomal abnormalities; none of them showed 12q rearrangement involving the *HMGA2* locus, whereas 1 patient had monosomy 7, which causes *EZH2* loss.¹⁹

The expression levels of *MIRlet-7a*, *-7b*, and *-7c* were correlated with each other (Figure 6B), and the expression of *MIRlet-7c* represents those of *MIRlet-7* family members. Interestingly, the reduced *MIRlet-7c* levels were correlated with high *HMGA2* mRNA levels in most patients with ET, but in less than half of the PMF. Patients with MPNs who highly expressed *HMGA2* mRNA (6 of 26, 23.1%) more often harbored monosomy 7 or mutations in PRC2 members, *EZH2* and *ASXL1*, compared with patients without high *HMGA2* expression (0 per 19, 0%; $P = .032$), especially in patients without a reduction in *MIRlet-7* expression (5 of 11, 45.5%) (Figure 6A,C). In contrast, there were no differences in the frequencies

of mutations in other gene groups. Therefore, the loss of PRC2 members might be associated with the upregulation of *HMGA2* in patients as in mice.²⁷⁻²⁹ We then investigated the expression of *HMGA2* in biopsied BM. *HMGA2* is highly expressed primarily in hematopoietic cells, especially the nucleus of megakaryocytes (Figure 6D), suggesting the possibility that *HMGA2* correlates with proliferation of megakaryocytes in these patients as in $JAK2^{V617F}$ mice overexpressing *HMGA2*.

Depletion of endogenous *Hmga2* ameliorates MPN with anemia in $JAK2^{V617F}$ mice with the loss of *Ezh2*

To confirm the importance of *Hmga2* in MPNs with $JAK2^{V617F}$ and the loss of *Ezh2*, we crossed *Hmga2*-KO (*Hmga2*^{-/-}) mice³⁶ with $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ mice, and obtained $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{-/-}$ and $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{WT/WT}$ mice after the second generation. Mice transplanted with $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ cells and those with $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{WT/WT}$ cells showed a similar MPN-like phenotype with more severe anemia, leukocytosis, and splenomegaly compared with mice transplanted with $JAK2^{V617F}$ cells,²⁷ and mimic patients with *EZH2* mutations because mutations in *EZH2* rarely acquire homozygosity in patients.²⁸ Thus, we performed BMT from $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{-/-}$ and $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{WT/WT}$ mice (Figure 7A). Strikingly, $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{-/-}$ recipients, whose *Hmga2* mRNA was suppressed to the basal level (0.9-fold) of $JAK2^{V617F}$ mice, revealed significantly reduced splenomegaly, leukocytosis, thrombocytopenia, and anemia, compared with $JAK2^{V617F}/Ezh2^{\Delta/\Delta}/Hmga2^{WT/WT}$ recipients with a twofold higher expression of *Hmga2* mRNA compared with $JAK2^{V617F}$ mice (Figure 7B-C).

Discussion

HMGA2 is overexpressed in solid cancers, and is associated with their poor prognosis,³² but little is known about its role in hematologic diseases. In $\Delta Hmga2$ mice, *Hmga2* is overexpressed by deleting its 3'UTR containing *MIRlet-7*-binding sites; such deletions have been found in a variety of cancers,²⁴ but are rare in MPNs.^{21,33} The truncated *HMGA2* make fusion genes, which may affect the disease phenotype, although *MIRlet-7* is more important for regulation of gene expression than the fusions in vitro.²⁴ In our former study, none of the 38 patients with high *HMGA2* mRNA levels carried a rearrangement of the *HMGA2* locus, and *MIRlet-7* was low in the majority, but not in all of them.²¹ The present study revealed that about half of PMF patients with high *HMGA2* mRNA levels, without the repression of *MIRlet-7*, harbor mutations of PRC2-related *EZH2* and *ASXL1* (Figure 6C). This might be in line with a previous report describing upregulation of *Hmga2* by loss of PRC2 through H3K27 trimethylation.²⁷ Here, our murine data may imply that *Hmga2* is a key player in the progression of MPNs with loss of PRC2 or *MIRlet-7*. First, the addition of 3'UTR-deleted *Hmga2* to

Figure 6. High *HMGA2* mRNA levels in patients with PMF are correlated with either repressed *MIRlet-7* expressions or mutations in PRC2 components. (A) Mutations in the patients with ET (N = 27) and PMF (N = 18). Black squares indicate no metaphase cells in the chromosomal analysis. (B) Correlations in the expression of *MIRlet-7a*, *-7b*, and *-7c* in granulocytes of the patients with ET and PMF. *MIRlet-7a* vs *-7b*, $R = 0.70$ (left); *MIRlet-7a* vs *-7c*, $R = 0.74$ (center); *MIRlet-7b* vs *-7c*, $R = 0.85$ (right) (R indicates correlation coefficients). (C) Correlations in the expression levels of *MIRlet-7c* and *HMGA2* mRNA in the granulocytes of individual patients. The red lines indicate the lower (*MIRlet-7c*) or upper (*HMGA2*) limit of the normal range determined according to the data of the healthy controls (see "Materials and methods"). Red circles indicate the patients with mutations in PRC2-related *EZH2* and *ASXL1*, and a rhomboid denotes a patient with monosomy 7 involving *EZH2*. (D) Immunostaining of biopsied BM. The nuclei of megakaryocytes from 2 PMF patients with high expression of *HMGA2* were positively stained by anti-*HMGA2* antibody; those of the ET patient with low expression of *HMGA2* were not stained. The tissue from breast cancer, in which the nuclei of neoplastic cells were positively stained, was used as a positive control. Scale bars indicate 50 μ m.

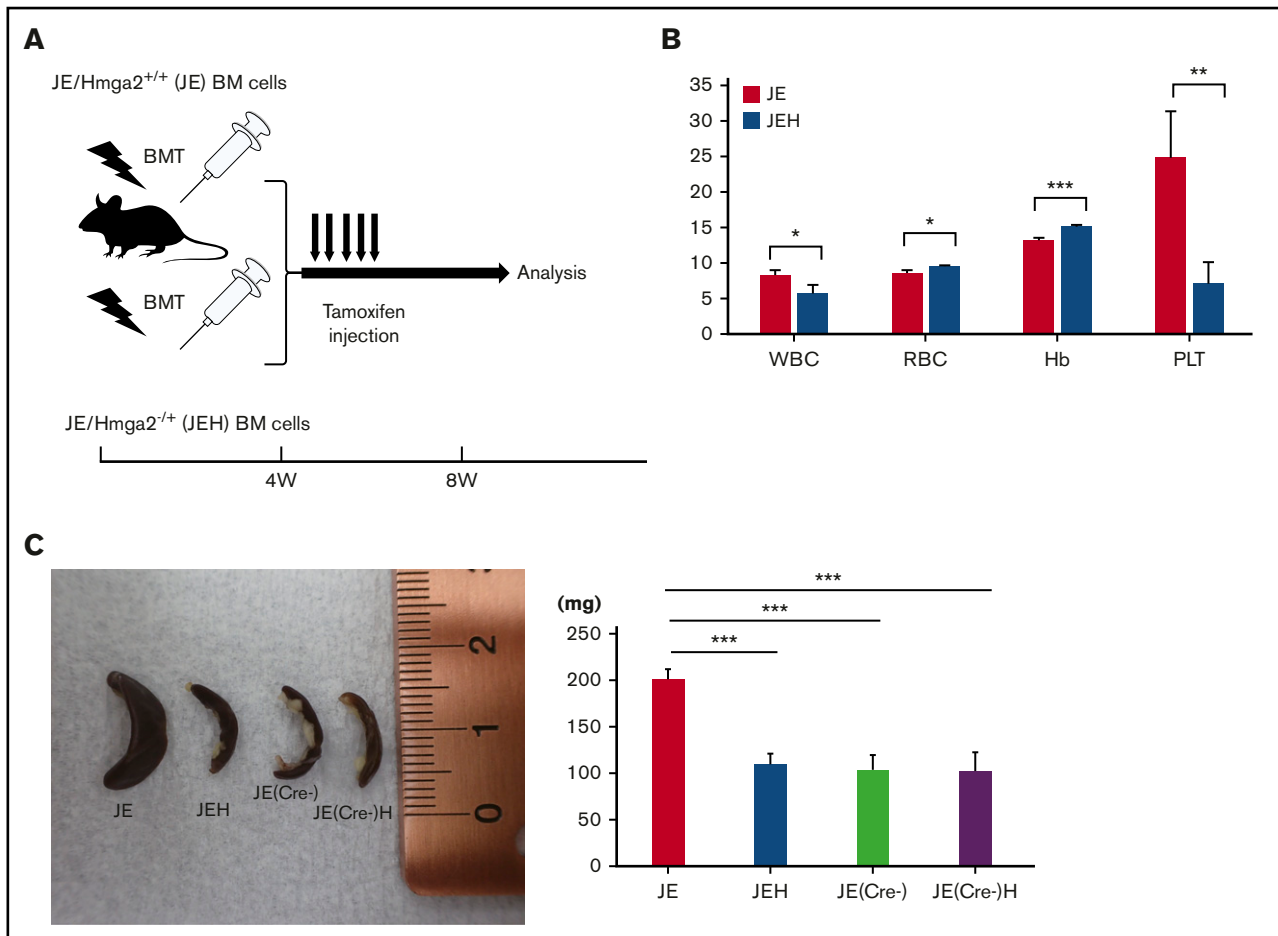


Figure 7. *Hmga2* inhibition ameliorates MPN in *JAK2*^{V617F} mice with *Ezh2* KO. (A) Schematic diagram of *Hmga2* KO (*Hmga2*^{-/-}) in *JAK2*^{V617F}/*EZH2*^{flex/WT}/*CRE-ERT* (JE). BM cells were collected from JE with *HMGA2*^{-/-} (JEH) or *HMGA2*^{+/+} (JE) mice and transplanted. Conditional *Ezh2* KO was initiated by tamoxifen 4 weeks after transplantation. (B) Complete blood cell counts (means ± SEM). N = 5 for each. **P* < .05; ***P* < .01; ****P* < .001 (Student *t* test). (C) Splens at 8 weeks after transplantation with JE, JEH, JE without Cre [JE(Cre-)], and JEH without Cre [JE(Cre-)H] cells. Left, Representative figure. Right, Mean spleen weight. N = 3 for each; ****P* < .001 (Turkey-HSD test).

JAK2^{V617F} ($\Delta Hmga2/JAK2^{V617F}$) mice, which represents the disruption of the *MIRlet-7/HMGA2* axis in patients with MPNs, markedly exacerbated anemia, leukocytosis, and splenomegaly. These were similarly seen in *JAK2*^{V617F}/*Ezh2* Δ/Δ mice that represent loss of PRC2.²⁷ Correspondingly, anemia, leukocytosis, and splenomegaly were effectively ameliorated by KO of *Hmga2* in *JAK2*^{V617F}/*Ezh2* Δ/WT mice. Moreover, LSKs of $\Delta Hmga2/JAK2^{V617F}$ mice showed overlapping gene upregulations with *JAK2*^{V617F}/*Ezh2* Δ/Δ mice.

Importantly, cells from $\Delta Hmga2/JAK2^{V617F}$ mice could reconstitute the disease phenotype following the second BMT. The influence of *JAK2*^{V617F} on HSC ability is controversial; some researchers reported that *JAK2*^{V617F} attenuates HSC ability,^{9,10,34,48} whereas others showed that *JAK2*^{V617F} increases LSKs and repopulating ability.^{11,49} Such differences may come from variations in the strategies of introduction and the expression level of *JAK2*^{V617F}.⁴⁸ However, *Hmga2* expression conferred a further proliferative capacity to already proliferative *JAK2*^{V617F} cells and improved dominance in serial BMT, although we used the tg-*JAK2*^{V617F} system in which HSC ability may be more impaired than in other systems.⁵⁰ Furthermore, LSKs were decreased in *JAK2*^{V617F} BM

compared with WT or $\Delta Hmga2$ BM, and *Hmga2* expression restored LSKs to *JAK2*^{V617F} BM (Figure 2B). Thus, *Hmga2* may contribute to both proliferation and expansion of an MPN clone at the HSC level, and compensate number and/or function of HSCs impaired by *JAK2*^{V617F}. We also found that BMT recipients from HJ mice showed a similarly shortened survival period (97 days of median survival after the BMT; Figure 4D) as individual tg HJ mice (92 days after the birth, Figure 1E), suggesting that their cause of death was associated with hematologic abnormality. In the recipients of HJ BM, splenomegaly was more severe than in recipients of J BM, while less severe compared with individual HJ mice. On the other hand, moribund recipients of HJ BM consistently showed severe anemia (<5 g/dL hemoglobin) as seen in individual HJ mice, implying that anemia may be a possible cause of death.

In BM MNCs of $\Delta Hmga2/JAK2^{V617F}$ mice, Stat3 expression was higher than in other strains, and some genes involved in the JAK-STAT pathway were upregulated in erythroblasts but not LSKs of $\Delta Hmga2/JAK2^{V617F}$ mice, indicating that differentiated cells are more responsible to proliferative phenotype than HSPC level.

Despite more severe anemia, the severity of MF was similar and BM cell number was not decreased in $\Delta Hmga2/JAK2^{V617F}$ mice compared with $JAK2^{V617F}$ mice. Considering that the apoptosis of erythroblasts was increased in $\Delta Hmga2/JAK2^{V617F}$ mice with enrichment of p53 activation (Figure 5H), ineffective erythropoiesis associated with p53-mediated apoptosis⁴⁵⁻⁴⁷ might be a factor that increases anemia. In contrast to $\Delta Hmga2/JAK2^{V617F}$, $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ mice presented more severe MF with decreased BM cellularity, compared with $JAK2^{V617F}$ mice.²⁷ In the RNA-seq of LSKs, the transforming growth factor (TGF)-related pathway was upregulated in $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ rather than $\Delta Hmga2/JAK2^{V617F}$ mice, relative to $JAK2^{V617F}$ mice. Notably, *TGFBR1* is a direct target of EZH2,²⁹ and activated TGF β signaling is important to evoke MF.⁵¹⁻⁵³ Therefore, targets of *Ezh2* other than *Hmga2* may play a greater role in fibrosis in this context. RNA-seq also revealed that an *Ezh2* deletion and overexpression of *Hmga2* share deregulated oncogenic targets such as *Lmo1*⁵⁴⁻⁵⁶ in the presence of $JAK2^{V617F}$. These observations give rise to the hypothesis that when the loss of *Ezh2* and/or *MIRlet-7* block the inhibition of some oncogenes, *Hmga2* may synergistically accelerate such upregulation in the MPN stem cells. Moreover, chaperone-related pathways were affected in both $JAK2^{V617F}/Ezh2^{\Delta/\Delta}$ and $\Delta Hmga2/JAK2^{V617F}$ mice (Figure 5E). This may be consistent with the finding that HMGA2 recruits chaperones to alter chromatin structures that contribute to the oncogenic activity of HMGA2.^{43,57}

Apart from PMF, our patients with ET did not have a mutation in *EZH2* or *ASXL1*, in agreement with a previous report,⁵⁸ and the expression levels of *HMGA2* were inversely correlated with those of *MIRlet-7* (Figure 6C). Thus, the cause of high expression of *HMGA2* in these patients may be repression of *MIRlet-7*, but it is unclear how *MIRlet-7* is downregulated. *Lin28b* is a well-known negative regulator of *MIRlet-7*.^{59,60} Although the *Lin28b/MIRlet-7/Hmga2* axis regulates the self-renewal of fetal HSCs, *Lin28b* is silenced after birth.^{30,61} In agreement, most of our patients did not express *LIN28B* at detectable levels (data not shown). At present, low-level *LIN28B* or other alterations, for example, DNA methylation,^{62,63} and NF45/90⁶⁴ may be candidates for future investigation.

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In conclusion, our study suggests a crucial role of *Hmga2* in the progression of MPNs. Amelioration of the severe phenotype in $JAK2^{V617F}$ mice with *Ezh2* KO, by inhibition of *Hmga2*, indicates that *HMGA2* may be one of the most important targets upregulated by mutations of PRC2 components and encourages the development of HMGA2-targeted therapy for patients with high-risk MPNs.

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Authorship

Contribution: K.U. designed the research, performed experiments, analyzed data, and wrote the manuscript; K.I. designed and supervised the research, performed experiments, analyzed data, and wrote the manuscript; T.I., K.O., Y.S., P.J.M., and M.B. supervised the study; K.H.-S. performed experiments and analyzed data; Y.H. performed histologic study; T.S., H.O., S. Kimura, and A.S.-N. collected and preserved patients' samples and analyzed data; Y.N. performed experiments; T.M. analyzed data; S.M. and N.K. examined mutations; K. Shide and K. Shimoda provided mice and interpreted results; S. Koide, K.A., and M.O. performed RNA-seq; A.I. performed RNA-seq and supervised the study; and Y.T. supervised the study and approved the manuscript.

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