While erythropoietin (EPO) constitutes the major treatment for anemia, a range of anemic disorders remain resistant to EPO treatment. The need for alternative therapeutic strategies requires the identification of mechanisms that physiologically restrain erythropoiesis. Here we show that P38α restrains erythropoiesis in mouse and human erythroblasts independently of EPO by integrating apoptotic signals during recovery from anemia. P38α deficiency promotes JNK activation through increased expression of Map3k4 via a negative feedback mechanism. JNK prevents Cdk1-mediated phosphorylation and subsequent degradation by Smurf2 of the epigenetic silencer Ezh2. Stabilized Ezh2 silences Bim expression and protects erythroblasts from apoptosis. Thus, we identify P38α/JNK signaling as a molecular brake modulating erythropoiesis through epigenetic silencing of Bim. We propose that inhibition of P38α, by enhancing erythropoiesis in an EPO-independent fashion, may provide an alternative strategy for the treatment of anemia.
A remarkable feature of erythropoiesis is the coordination of proliferation, differentiation, and apoptosis of erythroid cells to precisely achieve erythropoietic homeostasis to avoid anemia and polycythemia. Anemia is a common disease arising from various causes, including Myelodysplastic syndromes, thalassemia, cancer chemotherapy, chronic kidney disease, and hemorrhage. The pro-erythropoietic factor erythropoietin (EPO) is often employed for anemia therapy. However, questions have been raised about the safety of EPO given its potential for tumor promotion in cancer-related anemia. Moreover, many acute and chronic anemias, including hemolysis, sepsis, and genetic bone marrow failure diseases such as Diamond-Blackfan anemia are untreatable with EPO. To overcome these hurdles, new molecular mechanisms need to be identified that physiologically restrain erythropoiesis by acting as molecular brakes to prevent over-active erythropoiesis caused by pro-erythropoietic signals. Inhibiting these restraining mechanisms could provide alternative approaches to treat anemia in an EPO-independent fashion.

P38 MAPK (Mitogen-activated protein kinase) is an important pathway involved in diverse biological processes. P38 modulates cell proliferation, controls cell survival and decides cell fate during differentiation. P38 pathway functions mainly by phosphorylating and activating important transcription factors in response to different stimuli, including ATF2, CREB, and MEF2. There are four members within the P38 MAPK family, including P38α, P38β, P38γ, and P38δ. These members are encoded by different genes and have different tissue expression patterns. Among them, P38α is ubiquitously expressed. P38α modulates the function of different cell types. There are two distinct developmental defects reported in global P38α knockout mice by two separate groups using different mouse strains. One displayed embryonic death with highly anemic appearance due to reduced EPO production and another showed even earlier embryonic lethality due to placental developmental defects. In a P38α conditional mice model in which Cre recombinase was expressed in the whole-mouse embryo but not in the placenta by crossing to MORE-Cre mice, no anemia or EPO defects were observed. However, the intrinsic and cell autonomous role of P38α in adult steady-state or stressful erythropoiesis has not been established. Loss of P38α causes activation of JNK in the liver. P38 inhibitors are in clinical trials and have the potential for the treatment of human disease. Therefore, it is important to understand the downstream targets and functional outcomes induced by P38α deficiency.

Using primary human erythroblasts derived from human CD34+ hematopoietic stem and progenitor cells (HSPCs) and P38α conditional knockout mice, we find that P38α acts as a molecular brake during anemia recovery through integrating apoptotic signals and by shortening the lifespan of erythroblasts to prevent potential over-active erythropoiesis caused by pro-erythropoietic signaling. Loss of P38α in erythroblasts activates JNK through augmented Map3k4 via a negative feedback circuit revealed by gene expression profiling. Functionally, JNK serves as a pro-survival signal independent of EPO by compromising Bim expression via stabilizing the epigenetic silencer EzH2 in erythroblasts. JNK-controlled Cdk1 activity modulates full interaction of EzH2 to the E3 ligase Smurf2 through multiple threonine phosphorylation sites within EzH2. Our findings identify a key signaling cascade involving P38α/JNK/Cdk1/Smurf2/EzH2/Bim in fine tuning stress erythropoiesis.

**Results**

**Self-restraint role of P38 in stress erythropoiesis.** Human CD34+ HSPCs were induced to undergo erythroid differentiation after stimulation with pro-erythropoietic factors erythropoietin (EPO) and stem cell factor (SCF), providing a valuable tool to study human erythropoiesis in vitro. The developmental characteristics of CD34+ HSPCs-derived erythroblasts were evaluated by examining the expression of cell surface markers CD71 and CD235a and by cell morphology (Supplementary Fig. 1a). We detected a more enduring and sustained activation of P38 in those cells stimulated by EPO than by SCF, suggesting a physiologic role for P38 in erythropoiesis (Fig. 1a and Supplementary Fig. 1b). To address the role of P38 in regulating erythroblasts, we found that SB203580, a specific inhibitor of P38, which inhibited the phosphorylation of ATF2 (a well-known P38 target), did not alter erythroid differentiation of HSPCs (Supplementary Fig. 1c, d). Cell cycle distribution was similar between control and P38-inhibited human erythroblasts using ki67 staining (Supplementary Fig. 1e). Given the association between elevated P38 activity and stress-induced apoptosis, we assessed whether suppression of P38 activity protected erythroblasts from oxidative stress or chemotherapy-triggered cell death, which is often observed in anemic patients. Buthionine sulfoximine (BSO) causes oxidative stress by depleting intracellular glutathione. Inhibition of P38 markedly elevated resistance of human erythroblasts to BSO-induced cell death without disturbing the cell cycle (Fig. 1b and Supplementary Fig. 1f). Inhibition of P38 also notably reduced cisplatin-induced apoptosis of human erythroblasts with no profound effects on cell cycle status (Supplementary Fig. 1g, h). We predicted that pro-survival signaling downstream of EPO and SCF would compromise pro-apoptosis P38 signaling in human erythroblasts under normal conditions. As expected, inhibition of Jak2, which inhibited the phosphorylation of stat5 (a well-known Jak2 target), uncovered the pro-apoptotic function of P38 (Fig. 1c and Supplementary Fig. 1i, j). Therefore, our finding that pro-apoptosis P38 pathway is activated by pro-erythropoietic signals such as EPO and SCF indicate that P38 potentially performs an intrinsic restraining function to limit over-active erythropoiesis by integrating apoptotic signals.

To further explore the role of P38 in erythropoiesis in vivo, we generated a conditional knockout mouse model in which P38α was deleted in hematopoietic cells by crossing P38αflox/flox mice with Mx-Cre mice. P38α locus was excised and P38α protein was completely abolished in sorted erythroblasts from P38α−/− mice after poly IC injections compared to littermate controls (P38α+/− mice) (Supplementary Fig. 2a). In steady-state erythropoiesis, loss of P38α showed normal peripheral blood erythroid parameters, including hematocrits (HCT) and RBC counts (Fig. 1d, 0 time point). Differentiation of mouse erythroblasts can be monitored by assessing the expression different cell surface proteins. We next examined erythroblast differentiation characteristics by monitoring cell surface expression of CD71 and Ter119 combined with forward scatter (FSC) using flow cytometry. A modest increase in pro-erythroblasts (Pro-E) associated with reduced apoptosis was observed in P38α−/− bone marrow (BM) cells relative to controls; whereas, the frequency, viability and proliferation of basophilic erythroblasts (Ery-A), late basophilic and polychromatic erythroblasts (Ery-B) and orthochromatic erythroblasts (Ery-C) was comparable between P38α−/− and P38α+/− mice (Supplementary Fig. 2b-d). No obvious difference was observed in the composition of splenic erythroblast subsets (Supplementary Fig. 2e-g). These results demonstrate that P38α may modulate survival of early erythroblasts in BM under steady-state conditions, but loss of P38α is not sufficient to perturb erythropoietic homeostasis.

To investigate whether P38α modulates the response to anemia, we subjected P38α+/− and P38α−/− mice to phenylhydrazine (PHZ) treatment to induce hemolytic anemia. In response to PHZ challenge, P38α−/− mice showed accelerated recovery of HCTs and RBC counts in peripheral blood compared to P38α+/− controls, suggesting a role for P38α in erythropoiesis under stress conditions.
to P38α+/− mice (Fig. 1d). To further evaluate cell autonomous role of P38α in stress erythropoiesis, we transplanted BM cells from P38α+/− and P38α−/− mice into lethally irradiated recipient C57BL/6-CD45.1 mice. After donor chimerism stabilized (~90%), transplanted mice were subjected to PHZ challenge. An accelerated erythropoietic recovery from anemia was observed in recipient mice transplanted with P38α−/− BMs, suggesting the function of P38α in stress erythropoiesis is cell intrinsic (Supplementary Fig 3a and Fig. 1e).

EPO concentration in the serum and expression of EPOR and TFR2 in erythroblasts was comparable between P38α+/− and P38α−/− mice after PHZ challenge, (Fig. 1f, g). During recovery...
from anemia, a significant increase in phosphorylated P38 was unexpectedly observed in erythroblasts (Fig. 1h) and P38α−/− mice exhibited increase in the number of Pro-E, Ery-A and Ery-B cells in the BM and in the spleen compared to controls (Fig. 1i and Supplementary Fig. 3b). Although the frequency of Pro-E, Ery-A, and Ery-B was greater in P38α+/− BM compared to P38α−/− BM, the frequency of Ery-A, Ery-B, and Ery-C within the Ter119+ population was comparable between P38α−/− and P38α+/− mice, suggesting P38α may not be involved in modulating the differentiation of these subsets during stress erythropoiesis (Fig. 1i). PHZ-challenged mice showed enhanced cell cycle progression in erythroblasts in both P38α−/− and P38α+/− mice, demonstrating similar compensatory proliferation in erythroblasts during anemia recovery (Supplementary Fig. 3c, d). However, deletion of P38α led to reduced apoptosis in Pro-E, Ery-A, and Ery-B subsets both in the BM and in the spleen during recovery from anemia (Fig. 1j and Supplementary Fig. 3o). Administration of SB203580 also prevents apoptosis of BM erythroblasts without affecting cell cycle distribution in PHZ-challenged wild-type mice (Fig. 1k and Supplementary Fig. 3m). These results suggest that P38 integrates apoptotic cues in erythroblasts during the recovery from anemia to potentially limit over-active erythropoiesis.

Next, we challenged the mice with 5-fluorouracil (5-FU) (Supplementary Fig. 4a). During the recovery of this central anemia model, which induces anemia by depletion of proliferating progenitor cells, an enhanced P38 activity was observed (Supplementary Fig. 4b) and P38α−/− mice exhibited accelerated erythropoietic recovery (Supplementary Fig. 4c), associated with increase in the numbers of Pro-E, Ery-A, and Ery-B and reduced apoptosis relative to P38α+/− mice (Supplementary Fig. 4d, e). Thus, activation of P38 represents a physiologic molecular brake during the recovery phase of anemia by regulating erythroblast apoptosis; suppression of P38 activity relieves this restriction and promotes stress erythropoiesis.

**Loss-of-P38α drives JNK activation via augmented Map3k4.** To understand how P38α deficiency exerts a pro-survival function in erythroblasts, we performed gene expression profiling in P38α+/− versus P38α−/− BM erythroblasts during the recovery of anemia in response to PHZ challenge. A total of 1086 genes were identified to be differentially expressed (DE) between P38α−/− and P38α+/− erythroblasts (q-value ≤0.05; fold change ≥2). Ingenuity pathway analysis (IPA) revealed “cell death and survival” as the most enriched molecular and cellular functional category from DE genes (q-value ≤0.05; fold change ≥1.5) (Supplementary Fig. 5a). Gene set enrichment analysis (GSEA) identified enriched Molecular Signature database gene sets in P38α−/− erythroblasts, including suppression in the “inflammatory response pathway” (Supplementary Fig. 5b). Consistently, we detected reduced expression of TNF-α in P38α−/− erythroblasts compared with P38α+/− erythroblasts (Supplementary Fig. 5c).

Gene expression analysis revealed elevated expression of JunD, a downstream target of JNK, in P38α−/− erythroblasts. We wondered whether JNK is activated in erythroblasts in response to loss of P38α as reported in liver16, P38α−/− erythroblasts showed similar JNK activation under steady-state and stress conditions compared to P38α+/− erythroblasts (Fig. 2a). Unexpectedly, GSEA revealed upregulation of the “P38 MKK3/6 pathway” in P38α−/− erythroblasts, implying that a negative feedback circuit may occur in P38α−/− erythroblasts in order to restore P38α signaling (Fig. 2b, c). q-PCR confirmed enhanced expression of MKK6, MKK3, and GADD45g (Fig. 2d), two direct upstream kinases and a protein responsible for promoting P38 activation5,16, but not MKK4 and MKK7, the direct upstream kinases of JNK, in P38α−/− erythroblasts (Supplementary Fig. 5d). Enhanced expression of other P38 upstream kinases like Tau kinase 316 and RIPK118 were also noted in our gene expression profile (Fig. 2c). Remarkably, gene expression profiling also revealed upregulation in the expression of Map3k4 and Map3k10, which are upstream kinases of MKKs and mediate activation of P3819,20 in P38α−/− erythroblasts (Fig. 2c). Increased Map3k4 expression was confirmed in P38α−/− erythroblasts, whereas expression of Map3k11 was unaltered, with Map3k10 mRNA level too low to detect (Fig. 2d, e). These findings highlight the importance of P38α in stress erythropoiesis and demonstrate that negative feedback-induced augmentation of the upstream kinase of P38α is not limited to MKKs but acts upstream to the level of Mapkks, in an attempt to restore P38α signaling.

An important feature of Map3k4 is to activate both P38 and JNK20. We reasoned that enhanced Map3k4 expression in P38α−/− cells exclusively activates JNK due to lack of competition from P38α. Interestingly, elevated GADD45g can collaborate with Map3k16,21. As expected, silencing Map3k4 by delivering a retrovirus containing shRNA against Map3k4 into P38α−/− and P38α+/− cells effectively ablated P38α deficiency-induced activation of JNK (Supplementary Fig. 5e and 5f). In human erythroblasts, inhibition of P38 by SB203580 activated JNK and this was associated with elevated expression of Map3k4 (Fig. 2g). This reveals a conserved mechanism in mice and humans which involves rewiring of JNK activation in the absence of P38α (Fig. 2h).

**JNK signaling protects erythroblasts independent of EPO.** Since loss of Map3k4 leads to apoptosis in certain tissues20 and JNK activity supports the survival of acute myeloid leukemia cells22, we questioned if JNK plays a role in modulating erythroblast function. We first examined whether JNK can be activated by EPO in human erythroblasts. Unlike P38α, a relatively high basal JNK activity was observed in human erythroblasts, and EPO
phosphorylated JNK (46 and 54 kDa) was detected by immunoblotting.

Transduced with control or Map3k4-specific shRNA. 

Gene set enrichment analysis (GSEA) of the P38 pathway based on gene expression profiling of sorted CD71\textsuperscript{hi}Ter119\textsuperscript{+} erythroblasts from P38\textsuperscript{a+/–} and P38\textsuperscript{a−/−} mice during recovery from PHZ challenge. NES normalized enrichment score; FDR false discovery rate.

P38\textsuperscript{a−/−} and P38\textsuperscript{a+/–} CD71\textsuperscript{hi}Ter119\textsuperscript{+} erythroblasts by q-PCR. 

Heat map of P38x-related genes. 

mRNA levels of key P38 and JNK regulatory genes in sorted P38\textsuperscript{a+/–} and P38\textsuperscript{a−/−} CD71\textsuperscript{hi}Ter119\textsuperscript{+} erythroblasts. 

Data are shown as mean ± s.e.m from three separate experiments.

Expression of phosphorylated JNK and Map3k4 as assessed by immunoblotting. 

Expression of phosphorylated JNK and Map3k4 in human erythroblasts cultured with DMSO or SB203580. 

Schematic diagram demonstrating mechanism by which loss of P38x rewire increased Map3k4 to exclusively activate JNK. Blots are representative of three independent experiments. Data are shown as mean ± s.e.m. *P < 0.05 (two-tailed unpaired Student’s t-test).

Since activated JNK contributed to the enhanced survival seen in P38\textsuperscript{a−/−} mouse erythroblasts similar to human erythroblasts, we predicted that P38\textsuperscript{a−/−} erythroblasts would be more vulnerable to JNK inhibition. Indeed, SP600125 administration in P38\textsuperscript{a−/−} mice resulted in an early and more severe decline in HCTs and RBCs, and reduction in the numbers of BM Pro-E, Ery-A, and Ery-B subsets, which was accompanied by enhanced apoptosis (Supplementary Fig. 5j and Fig. 3d, e). The fact that dependence on high JNK activity sensitizes mouse erythroblasts similar to human erythroblasts, 

Elevated expression of JunD mRNA and protein were observed in P38\textsuperscript{a−/−} erythroblasts with high JNK activity compared to P38\textsuperscript{a+/–} erythroblasts (Fig. 3f, g). In human erythroblasts, 

Fig. 2 Upregulated Map3k4 due to P38x deficiency mediates JNK activation in erythroblasts. 

a) In sorted erythroblasts from P38\textsuperscript{a+/–} and P38\textsuperscript{a−/−} mice, phosphorylated JNK (46 and 54 kDa) was detected by immunoblotting.

b) Gene set enrichment analysis (GSEA) of the P38 pathway based on gene expression profiling of sorted CD71\textsuperscript{hi}Ter119\textsuperscript{+} erythroblasts from P38\textsuperscript{a+/–} and P38\textsuperscript{a−/−} mice during recovery from PHZ challenge. NES normalized enrichment score; FDR false discovery rate.

c) Western blot (left) and densitometry analysis (right) of Map3k4 protein level in sorted GFP\textsuperscript{+} Map3k4\textsuperscript{+/–} (left) and Map3k4\textsuperscript{−/–} (right) mouse erythroblasts by q-PCR.

Expression of phosphorylated JNK and Map3k4 as assessed by immunoblotting.

Expression of phosphorylated JNK and Map3k4 in human erythroblasts cultured with DMSO or SB203580. 

Schematic diagram demonstrating mechanism by which loss of P38x rewire increased Map3k4 to exclusively activate JNK. Blots are representative of three independent experiments. Data are shown as mean ± s.e.m. *P < 0.05 (two-tailed unpaired Student’s t-test).
inhibition of JNK substantially ablated expression of JunD (Fig. 3h). Functionally, knockdown of JunD resulted in apoptosis of human erythroblasts more moderately than JNK1 silencing (Supplementary Fig. 5k and Fig. 3i), suggesting there might be other targets of JNK, in addition to JunD, involved in protecting erythroblasts.

JNK protects erythroblasts by compromising Bim expression. Since the tumor suppressor P53 is a master regulator of apoptosis in many cell types, including erythroblasts, and mutations in the ribosomal protein genes cause erythroid cell death in patients with Diamond-Blackfan anemia via P53 activation, we examined whether P53 is involved in JNK inhibition-induced apoptosis.
in human erythroblasts. We evaluated the impact of JNK inhibition on expression of P53, and surprisingly found that protein levels of P53 and Noxa, a well-known P53 target, were markedly reduced upon inhibition of JNK in human erythroblasts (Fig. 4a). Cycloheximide (CHX)-chase assay revealed that JNK inactivation triggers accelerated degradation of P53 compared to controls (Fig. 4b), suggesting that JNK activity is essential for maintaining stability of P53 in human erythroblasts. Moreover, P38α−/− erythroblasts, with high JNK activity, exhibited increased expression of P53 protein, but not P53 mRNA, compared to P38α+/− erythroblasts (Fig. 4c). This is consistent with GSEA analysis that displayed enhanced enrichment of the P53 pathway in P38α−/− erythroblasts (Fig. 4d). These data suggest that P38α is a physiological negative regulator of P53 via JNK and that P53 might not contribute to JNK inhibition-induced apoptosis in erythroblasts.

To address whether cell death receptor pathways which play a critical role in controlling erythroblast survival are downstream targets of JNK and responsible for mediating erythroblast cell death, we performed q-PCR analysis. As seen in Supplementary Fig. 6a, JNK inactivation did not significantly alter the expression of Fas, DR3, and DR4 but decreased the expression of Fasl. Bcl-2 family proteins play a central role in regulating erythroblast cell death.25,26 Among the Bcl-2 family members, we found the expression of Bim, but not bcl-XI, Mcl-1, and Bik, to be significantly elevated by SP600125 (Fig. 4e and Supplementary Fig. 6b). Knockdown of JNK1 by shRNA also activated Bim expression in these cells (Fig. 4f). Importantly, knockdown of Bim by lentivirus carrying Bim-specific shRNA enabled erythroblasts to resist JNK inactivation-induced cell death (Supplementary Fig. 6c and Fig. 4g). Thus, the JNK pathway supports the survival of erythroblasts by regulating the expression of Bim.

Smurf2-mediated degradation of Ezh2 triggers Bim expression. Elevated Bim mRNA levels imply that JNK perhaps modulates Bim transcription. Therefore, we focused on transcription factors Foxo3a and LRF, both of which are involved in regulating Bim expression and erythropoiesis.25,27 Phosphorylation of Foxo3a inhibits its function.28 We found Foxo3a phosphorylation to be unaltered in response to SP600125. Expression of LRF protein remained unchanged by JNK inactivation (Supplementary Fig. 7a). These results suggest that both Foxo3a and LRF might not be involved in regulating JNK-mediated Bim expression. This prompted us to seek alternative targets.

Ezh2, a component of the polycomb repressive complex 2 (PRC2), has been reported to negatively regulate Bim expression.29,30 Ezh2 regulates erythropoiesis.31,32 We wondered whether Ezh2 is a downstream target of JNK in regulating Bim expression. In human erythroblasts, Ezh2 mRNA levels were modestly elevated, whereas Ezh2 protein was markedly reduced upon inhibiting the activity of JNK (Fig. 5a). Silencing JNK1 also significantly attenuated Ezh2 expression (Fig. 5b). Cycloheximide chase assay performed in human erythroblasts revealed that inactivation of JNK notably accelerated degradation of Ezh2 protein, suggesting that JNK might control Ezh2 protein stability (Fig. 5c). Importantly, degradation of Ezh2 by JNK inactivation could be blocked by proteasome inhibitor MG132, suggesting the involvement of the ubiquitin-proteasome pathway in Ezh2 regulation (Fig. 5d). Consistently, elevated HOXA9 mRNA level, a well-known target of PRC2, was observed in human erythroblasts when JNK activity was compromised by SP600125 or by JNK1 silencing (Fig. 5e). In agreement with these results, we found reduced binding of Ezh2 and H3K27 trimethylation on the Bim promoter, as measured by a quantitative chromatin immunoprecipitation assay (Fig. 5f).

We then tested the role of Ezh2 in modulating erythroblast survival. In human erythroblasts, GSK126, an Ezh2 inhibitor, induced Bim expression while compromising global H3K27me3 and initiated cell death in a concentration dependent manner (Fig. 5g, h and Supplementary Fig. 7b). Similar results were observed when Ezh2 was silenced by shRNA (Supplementary Fig. 7c and Fig. 5i, j). Ezh2 activity was also required for the survival of TF1 cells (Supplementary Fig. 7d). These results indicate that Ezh2 is required for protecting human erythroblasts by inducing epigenetic silencing at Bim locus. In accordance with findings in human erythroblasts, P38α−/− erythroblasts, which show increased JNK activation, also displayed elevated Ezh2 protein compared with controls (Fig. 5k). Consistently, reduced expression of HOXA9 was observed in P38α−/− erythroblasts compared to P38α+/− erythroblasts (Fig. 5l). Moreover, P38α−/− erythroblasts showed greater sensitivity to Ezh2 inhibition-induced apoptosis than P38α+/− erythroblasts, suggesting dependence of P38α−/− erythroblasts on higher level of Ezh2 (Fig. 5m).

Together, our findings demonstrate that Ezh2 is a target of the P38α/JNK pathway and is required for protecting erythroblasts by silencing Bim expression. Thus, our results link Ezh2, a major epigenetic regulator of gene expression, to major MAPK signaling pathway to define a mechanism for maintenance of erythropoietic homeostasis.

Smurf2, a ubiquitin E3 ligase, can degrade Ezh2.33 To investigate the role of smurf2 in JNK-mediated Ezh2 protein stability in human erythroblasts, we silenced smurf2 by delivering lentivirus containing shRNA against smurf2 into human erythroblasts. This treatment enhanced the expression of Ezh2 under steady-state and JNK inactivation conditions compared to control shRNA and compromised the expression of Bim upon JNK inactivation (Supplementary Fig. 7e and Fig. 5n). Importantly, erythroblasts lacking Smurf2 showed enhanced resistance to apoptosis in response to JNK inhibition (Fig. 5o). These results support a key signaling cascade involving JNK/Smurf2/Ezh2 in regulating survival of erythroblasts.

Cdk1 phosphorylates Ezh2 to mediate its binding to Smurf2. To further explore the mechanisms underlying Ezh2 regulation by Smurf2, we mapped the binding domain between Ezh2 and Smurf2. Smurf2 contains an N-terminal C2 domain and a C-terminal HECT domain which has E3 ligase activity with three WW domains in the middle.34 Co-immunoprecipitation showed...
strong interaction between full-length Ezh2 and the HECT domain of Smurf2, but not the C2 or WW domains (Fig. 6a).

Next, we mapped the region within Ezh2 that mediates interaction with the HECT domain of Smurf2. We designed a series of Ezh2 truncated constructs, including an N-terminal fragment (amino acid residues 1–348), referred to as Ezh2-N, middle region amino acid residues 330–530 which consist of multiple threonine phosphorylation sites referred to as Ezh2-M and a C-terminal domain containing amino acid residues 523–751 referred to as Ezh2-C. We observed strong binding of the HECT domain to the Ezh2-M fragment (Fig. 6b). These results demonstrate that the middle region of Ezh2 interacts with the C-terminal HECT domain of Smurf2.

There are four threonine phosphorylation sites within Ezh2-M, including Ter350, Ter372, Ter419, and Ter492. Three of them have been reported to be phosphorylated by Cdk1/2, and two of them (T350 and T492) regulate Ezh2 stability35,36. Although a previous report showed that T372 can be phosphorylated by P388, motif analysis strongly predicts T372 as a CDK consensus site40. To this end, we generated one quadruple-point mutant with four threonine residues mutated to alanine (A) (referred to as Ezh2-M 4A), and one triple-point mutant with a T372 residue remaining intact (referred to as Ezh2-M 3A372T). Immunoprecipitation showed enhanced phosphorylation of the 3A372T mutant as revealed by a phosphorylated CDK substrate antibody (pT-P Ab) compared to a 4A mutant. Notably, Roscovitine, a CDK inhibitor, significantly ablated the phosphorylation of Ezh2-M 3A372T mutant (Fig. 6c).

Since we noticed that Lysine-42133, which is critical for smurf2-mediated Ezh2 degradation, is surrounded by four threonine sites, we assessed if phosphorylation of these threonine sites mediates the interaction between Ezh2 and smurf2. Wild type (Ezh2-M) and a series of Ezh2 mutants including a quadruple threonine mutant (Ezh2-M 4A), two double threonine mutants (Ezh2-M T350A/T492A and Ezh2-M T372A/T419A) were co-expressed with the HECT domain of smurf2.
Co-immunoprecipitation showed that the double threonine mutation in Ezh2-M significantly weakened the binding, compared to wild type, and the quadruple threonine mutation abolished the interaction completely (Fig. 6c). In support of these findings, in vivo ubiquitination assay showed Smurf2 caused dramatically reduced poly-ubiquitination of the 4A mutant of Ezh2 compared to wild-type Ezh2 (Fig. 6d). To further address the functional significance of the threonine phosphorylation sites in Ezh2, we overexpressed the full length wild type or 4A mutant of Ezh2 in TF1 cells. Compared to wild type, the 4A mutant of Ezh2 displayed enhanced stability in a CHX chase assay and showed resistance to degradation induced by JNK inactivation (Fig. 6e, f). It has been proposed that individual threonine sites may have distinct impact on Ezh2 activity35,36. Expression of the Ezh2 4A mutant significantly increased global H3K27 trimethylation compared with wild-type Ezh2 in sorted GFP+ TF1 cells (Fig. 6g). Importantly, expression of the Ezh2 4A mutant compromised the expression of Bim and protected TF1 cells

**Figure Legends**

- **Panel a**: Relative Ezh2 mRNA expression in TF1 cells treated with DMSO or SP600125.
- **Panel b**: Western blot analysis of Ezh2 and JNK1 in TF1 cells treated with Vehicle or SP600125.
- **Panel c**: Immunoprecipitation of H3K27me3 and Ezh2 in TF1 cells treated with Vehicle or SP600125.
- **Panel d**: Western blot analysis of Ezh2 and Actin in TF1 cells treated with Vehicle or SP600125.
- **Panel e**: Relative HOXA9 mRNA expression in TF1 cells treated with DMSO, SP600125, Ctrl shRNA, and JNK1 shRNA.
- **Panel f**: Percentage of input of IgG and Ezh2 in TF1 cells treated with Vehicle or SP600125.
- **Panel g**: Relative Bim mRNA expression in TF1 cells treated with DMSO, GSK126, and Ezh2 shRNA.
- **Panel h**: Annexin V+ erythroblasts in TF1 cells treated with GSK126 and Ezh2 shRNA.
- **Panel i**: Relative HOXA9 mRNA expression in TF1 cells treated with SP600125 and Ezh2 shRNA.
- **Panel j**: Gated GFP+CD71+ cells in TF1 cells treated with SP600125 and Ezh2 shRNA.
- **Panel k**: Percentage of input of IgG and H3K27me3 in TF1 cells treated with Vehicle or GSK126.
- **Panel l**: Relative HOXA9 mRNA expression in TF1 cells treated with Vehicle or GSK126.
- **Panel m**: Apoptotic ter119+ cells in TF1 cells treated with GSK126 and P38α+/−/−.
- **Panel n**: Relative HOXA9 mRNA expression in TF1 cells treated with SP600125 and Smurfl2 shRNA.
- **Panel o**: GFP+CD71+ gated annexin V+ cells in TF1 cells treated with Vehicle or Smurfl2 shRNA.
from JNK inhibition-induced cell death compared to wild-type Ezh2 expressing cells (Fig. 6h, i). Our findings demonstrate that four threonine phosphorylation sites negatively regulate Ezh2 activity and are required for full interaction between Ezh2 and smurf2 for its degradation. Thus, regulation of Cdk1 activity is a key step in JNK-mediated modulation of Ezh2 function.

Cdk1 modulates Ezh2 and survival of erythroblasts. Next, we questioned how JNK controls the activity of Cdk1. Dephosphorylation of Tyr-15 is required for Cdk1 activation\(^1\). Suppression of JNK by SP600125 or JNK1 knockdown significantly reduced phosphorylation of Tyr-15 of Cdk1 in human erythroblasts (Fig. 7a, b). Conversely, elevated Cdk1 Tyr-15 phosphorylation was observed in P38α\(^{-/-}\) erythroblasts which bear enhanced JNK activity compared with P38α\(^{+/+}\) erythroblasts, implying that JNK modulates Cdk1 via Tyr-15 (Fig. 7c). To address the role of Cdk1 in the regulation of erythroblast survival, we silenced Cdk1 expression by a Cdk1-specific shRNA. This resulted in increased expression of Ezh2 under both basal and JNK inactivation conditions (Supplementary Fig. 8c and Fig. 7d). Importantly, lack of Cdk1 compromised the transcription of Bim and protected erythroblasts under JNK inactivation conditions (Fig. 7e, f). These findings demonstrate that Cdk1 functions downstream of JNK and bridges JNK activity to Ezh2 modulation. Our results thus establish a critical signaling cascade involving P38/JNK/Cdk1/Smurf2/Ezh2/Bim in controlling survival of erythroblasts.

Discussion
Our work has uncovered a self-restraining function of P38α in stress erythropoiesis by integrating apoptotic signals in erythroblasts during the recovery from anemia. Therefore, P38α acts as a molecular brake to limit over-active erythropoiesis in response to stress. Relief of this molecular brake by inhibiting P38 enhances stress erythropoiesis and accelerates recovery from anemia, thus providing a potential therapeutic strategy for treating patients with anemia (Fig. 7g).

Both P38 and JNK belong to MAPK family. Our findings show that in human primary erythroblasts, EPO-induced activation of JNK only occurs briefly, whereas activation of P38 persists. The intensity, duration, and fluctuation of stimulation of signaling pathways are all critical in determining the eventual output to affect cell fate decisions\(^4\). Endurance of activated signaling pathways by EPO/EPOR is regulated by various mechanisms such as availability of adaptor proteins, endocytosis of EPO/EPOR\(^4\), expression of specific phosphatases, and cross-talk between multiple downstream pathways of EPO/EPOR\(^4\). Adding to the complexity, recent findings demonstrate that extracellular binding changes between EPO and EPOR due to EPO variant result in selectively altered downstream signaling responses, which reveals a mechanism in EPO/EPOR signaling modulation\(^5\).

Gene expression profiling did not reveal elevated expression of other members of the P38 kinase family (P38β/γ/δ/β) in P38α\(^{-/-}\) erythroblasts. Since P38β can also be activated by MKK6\(^6\), we have performed q-PCR experiments and found that expression of P38β in mouse erythroblasts was very low, which is in agreement with previous findings in human erythroblasts\(^7\), suggesting that P38β may not play an important role in regulation of erythropoiesis. The expression of P38γ was even lower than P38β in mice erythroblasts, although P38γ was found to express in human erythroblasts\(^8\). Hence, other members of P38 family may not compensate for the loss of P38α in mice erythroblasts, highlighting the importance of P38α in erythropoiesis.

We propose that the JNK/Ezh2 signaling serves as a pro-survival mechanism in erythroblasts independent of EPO. Data from us and others suggests that JNK activity is required for maintaining P53 protein. Loss-of-MKK7 inhibits JNK activity, accelerates P53 degradation, and promotes lung cancer development\(^9\). In contrast, in hematopoietic system-derived malignancies, like leukemia, which rarely bear mutations in P53 compared to solid tumors, JNK activity is required for leukemic cells to survive\(^10\). Our findings that inhibition of JNK causes apoptosis while promotes degradation of P53 in human erythroblasts, indicate that the consequence of JNK activation may depend on whether P53 plays an essential role in regulating apoptosis in specific cell types. Indeed, there is evidence to suggest that P53-independent regulation of apoptosis via LRF/Bim, Fas/Fasl, and TRAIL exist in erythroid cells\(^11\). Our findings show that Ezh2 is a downstream target of P38α/JNK pathways, establishing a connection between critical P38α/JNK signals and PRC2, an essential epigenetic regulator. Our results reveal a signal transduction mechanism for sensing extracellular/intracellular changes to chromatin re-modeling, which enables erythroblasts to adapt to changing environment to maintain homeostasis.

Our findings demonstrate that Cdk1 functions downstream of JNK to regulate Ezh2 stability and its activity. Phosphorylation of four threonine sites in the middle domain of Ezh2 by Cdk1 mediates full interaction to bind to Smurf2 for its degradation. We propose a two-step mechanism by which JNK regulates Ezh2 via Cdk1 in erythroblasts. If JNK inhibition is short-term, activated Cdk1 phosphorylates and decreases Ezh2 activity to trigger target gene expression. However, if JNK inactivation persists, endured activated Cdk1 mediates phosphorylation and degradation of Ezh2 via Smurf2 for a longer duration compromising Ezh2 function.

P38 has been reported to positively regulate P53\(^10\). However, those results are mostly from cancer cell lines in which signal transduction usually has been dysregulated. In contrast, we observed enhanced P53 protein in P38α\(^{-/-}\) erythroblasts, as availability of adaptor proteins, endocytosis of EPO/EPOR\(^4\), and protected erythroblasts under JNK inactivation conditions. Data are shown as mean ± s.e.m. *P < 0.05 (two-tailed unpaired Student’s t-test).
**Fig. 6** Multiple cdk1 threonine phosphorylation sites in Ezh2 modulate its full interaction with Smurf2.  

**a** HECT domain of Smurf2 interacting with Ezh2 by co-immunoprecipitation.  

**b** Binding of middle domain of Ezh2 (Ezh2-M) to Smurf2 by co-immunoprecipitation.  

**c** Ezh2-M wild type and mutants were co-transfected with HECT domain of Smurf2 and cell lysates were subjected to co-immunoprecipitation after pre-treatment with MG132 for 6 h.  

**d** Ubiquitination of wild-type Ezh2 and Ezh2 4A mutants in cells co-expressing HA-tagged ubiquitin with or without smurf2 in the presence of MG132.  

**e, f** Sorted GFP$^+$ TF1 cells expressing Flag tagged wild type or 4A mutant Ezh2 were subjected to CHX treatment (**e**) or SP600125 (**f**) and Ezh2 protein abundance was measured by immunoblotting using anti-Flag antibody.  

**g** In sorted GFP$^+$ TF1 cells expressing control or Flag tagged wild type or 4A mutant Ezh2, protein levels of H3K27me3 and total H3 levels measured by immunoblotting (**g**) mRNA expression of Bim by q-PCR (**h**) and cell death by MTT assay (n = 4) (**i**) after SP600125 treatment. Blots are representative of two independent experiments. Data are shown as mean ± s.e.m. *P < 0.05 (two-tailed unpaired Student’s t-test).
suggesting that P38a physiologically negatively modulates P33. Recent studies show that P33 is a potential target for certain cancer therapies. Our findings that P33 regulates P33 and Ezh2 through JNK further provides insight into the role of P33 in tumorigenesis (Fig. 7g). Given the fact that many anemias are caused by chemotherapy, we propose an alternative therapeutic strategy for improving the outlook in anemia patients through inhibition of P33. Inhibition of P33 not only promotes erythropoiesis by relief of self-restraint in erythroblasts but also potentially maintains a higher level of P33 in cancer cells which is doubly beneficial for cancer patients who bear wild-type P33.

Methods

Reagents and cell culture. P33 inhibitor SB203580, JNK inhibitor SP600125, Jak2 inhibitor II, and MG132 were purchased from EMD Bioscience. Cdk inhibitor Roscovitine was purchased by Cell Signaling technology. Cycloheximide was purchased from Sigma-Aldrich. Human Stem cell factor (SCF), thrombopoietin (TPO), and FLT3-ligand (FLTL3), human GM-CSF, murine IL-3 and SCF were purchased from Sigma-Aldrich. Human Stem cell factor (SCF), thrombopoietin (TPO), and FLT3-ligand (FLT3L), human GM-CSF, murine IL-3 and SCF were purchased from Peprotech. EPO was manufactured by Amgen. Fetal bovine serum (FBS) was purchased from Hyclone. TF1 human erythroid cells (American Type Culture Collection (ATCC)) were cultured in RPMI 1640 medium supplemented with 10% FBS and GM-CSF. Tf1 human erythroid cells were tested for mycoplasma contamination. All cell lines were authenticated by supplier. All genetic background. Mice were randomized based on weight. The investigators were not blinded for group allocations. All mice were maintained in specific pathogen-free conditions, and experiments were performed by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of medicine. Mice were injected intraperitoneally with SB203580 (15 mg per kg body weight) once to induce hemolytic anemia. For induction of central anemia, a single intraperitoneal injection of 5-fluorouracil (150 mg per kg body weight) was utilized. To inhibit JNK, P33a−/− and P33a−/− mice were injected daily intraperitoneally with SB600125 (40 mg per kg body weight). Peripheral blood was collected from tail vein at indicated times and hematological parameters were analyzed using a HEMAVET 950FS analyzer.

Administration of P33 inhibitor SB203580 to mice was performed in brief, Mice were injected intraperitoneally with SB203580 (15 mg per kg body weight) or vehicle every other day before (2 injections) and during PHZ challenge. Apoptosis and cell cycle of BM erythroblast subsets was measured by flow cytometry. For the transplantation experiment, 1 × 10⁶ bone marrow cells from P33a−/− and P33a−/− mice (CD45.2−) were injected and isolated into lethally irradiated C57BL/6-Cd45.1 recipient mice (CD45.1−CD45.2+) through tail vein. The donor chimerism were monitored by flow cytometry analysis of peripheral blood samples with anti-mouse CD45.1-PE (553776, BD Bioscience) and CD45.2-FITC antibodies (553772, BD Bioscience) at a 1:5 dilution. After donor chimerism was stable over 90%, chimeric mice were subject to PHZ challenge.

Lentiviral transduction and erythroblast differentiation. Purified human cord blood CD34+ HSPCs were purchased from the Angio Biocore, IUL Melvin and Biren Simon Cancer center and cultured in SFEM serum-free expansion medium. (StemCell Technologies) supplemented with stem cell factor (SCF) (100 ng ml⁻¹), thrombopoietin (TPO) (100 ng ml⁻¹), and FLT3-ligand (FLTL3) (100 ng ml⁻¹). For erythroblast differentiation, CD34+ HSPCs were cultured in SFEM serum-free medium containing SCF (40 ng ml⁻¹) and erythropoietin (EPO) (0.5 U ml⁻¹) for around 5 days for further experiments. For generation of lentivirus, HEK293T cells were transfected with the lentiviral vectors described below along with packaging vectors using Profection Mammalian Transfection System (E1200, Promega) according to the manufacturer’s protocol. Supernatants containing viral particles rested for 6 weeks prior to initiation of experiments, including gene analysis. All studies were performed in a C57Bl/6 genetic background. Mice were randomized based on weight. The investigators were not blinded for group allocations. All mice were maintained in specific pathogen-free conditions, and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of medicine.
were collected and filtered with 45 µm filter after 48 h of transfection. For lentiviral transduction, CD34- cells were infected with lentivirus after culturing for 24 h in the presence of 4 µg/ml polybrene (Sigma-Aldrich) and spun at 800 x g for 90 min at room temperature.

**Cytosin preparation.** A total of 1–2 × 10^5 cells in 200 µl were used for cytospin using the Thermo Scientific Shandon Cytospin 3 cytocentrifuge. The slides were stained with May-Grünwald solution (Sigma-Aldrich) for 5 min, rinsed in deionized water four times for each 30 s, and subsequently stained with Giemsa solution (Sigma-Aldrich) for 15 min. The images were taken using a Leica inverted microscope.

** Constructs. **pRK-MyeSmurf2 (13678) was provided by Dr Ying Zhang, pCS2-Mye-CyclinB1 (121176) by Dr Marc Kirschner, and pUHD-HA-CDK1(2#7652) by Greg Enders and purchased from Addgene (Cambridge, MA). To generate truncated knockdown constructs, C2, WW2, and HDCT domains of Smurf2 were cloned by PCR from full-length Smurf2 and inserted into CMV-Myc vectors with the following primers (5′–3′): C2 domain, forward, GAGTCTCGAGTACGAGTTTGGGTTG; reverse, GAAGCTTGTTTGGCCGGGGACATGCTC. WW2 domain, forward, GAGTTCTAGGCGATCCGCTGCCTG; reverse, TAATGGTGGGGGCGCTGGGCCTGCT; HDCT domain, forward, GAGGTTTACCGATCGAGTTTGGGTTG; reverse, GAAGCTTGTTTGGCCGGGGACATGCTC. The Tag flag Ezh2 truncations were generated with the following primers (5′–3′): Ezh2 N (1–1348), forward, GGAGGTTTACCGATCGAGTTTGGGTTG; reverse, GAAGCTTGTTTGGCCGGGGACATGCTC; Ezh2 M (1349–530), forward, GGAGGTTTACCGATCGAGTTTGGGTTG; reverse, GAAGCTTGTTTGGCCGGGGACATGCTC; Ezh2 S (531–930), forward, GGAGGTTTACCGATCGAGTTTGGGTTG; reverse, GAAGCTTGTTTGGCCGGGGACATGCTC.

For the construction of shRNA sequence targeting human JunD (5′-GGAAGTGATCTCTGGACTAAT-3′), the C2 domain, forward, GAATTCGTCAAGCTGCGCCTGACAGTACTC, reverse, TACGTTATACATACCCTGAGAATGTTTCTC; Ezh2-C (5′-GGAAGTGATCTCTGGACTAAT-3′), forward, GAATTCGTCAAGCTGCGCCTGACAGTACTC, reverse, TACGTTATACATACCCTGAGAATGTTTCTC; Ezh2-F (5′-GGAAGTGATCTCTGGACTAAT-3′), forward, GAATTCGTCAAGCTGCGCCTGACAGTACTC, reverse, TACGTTATACATACCCTGAGAATGTTTCTC; Ezh2-M (5′-GGAAGTGATCTCTGGACTAAT-3′), forward, GAATTCGTCAAGCTGCGCCTGACAGTACTC, reverse, TACGTTATACATACCCTGAGAATGTTTCTC; Ezh2-S (5′-GGAAGTGATCTCTGGACTAAT-3′), forward, GAATTCGTCAAGCTGCGCCTGACAGTACTC, reverse, TACGTTATACATACCCTGAGAATGTTTCTC. The 3′ end of the shRNA sequence was generated with the following primers (5′-end): T350A, forward, TCTCGGTGAGAGCGGATAAAGGCCCCACCAAAAC, reverse, GTTTTGGTGGGGTATGAGGACAGA; T419A, forward, TCTCGGTGAGAGCGGATAAAGGCCCCACCAAAAC, reverse, GTTTTGGTGGGGTATGAGGACAGA; and T427A, forward, TCTCGGTGAGAGCGGATAAAGGCCCCACCAAAAC, reverse, GTTTTGGTGGGGTATGAGGACAGA. The wild full length Ezh2 and quadruple 4A mutant Ezh2 were cloned into puc2CL6

### Fluorescence-activated cell sorting

Briefly, bone marrow cell suspensions from P38α−/− and P38α−/− mice were pre-treated with rat anti-mouse CD16/CD32 antibody (2.4G2, BD Biosciences), and subsequently stained with APC-conjugated anti-mouse Ter119 (BD Biosciences,1,50) and FITC-conjugated anti-mouse CD71 (BD Biosciences,1,50) for 30 min in the dark at 4 °C. After wash, cells were stained with Propidium iodide before sorted on BD SORP Aria (BD Biosciences).

**Gene expression profiling analysis and bioinformatics analysis.** P38α−/− and P38α−/− mice were challenged with PHZ to induce anaemia, on day 4 of recovery, erythroblasts (CD71[+]/Ter119+) from three mice of each genotype were sorted and tested to Miltenyi Biotech Inc (Auburn, CA). RNA was extracted, and gene expression profiling was performed using Agilent Whole-Genome Mouse Oligo Microarrays. Data preprocessing and discriminatory gene analysis was conducted by Miltenyi Biotech Inc (Auburn, CA). Based on the log2-transformed normalized intensity values, unpaired Student’s t-tests (two-tailed, equal variance) was applied to evaluate the differences between the means of P38α−/− and P38α−/− samples. A correction for multiple testing of the t-test p-values was conducted using the method of Benjamini and Hochberg. A p-value ≤0.05 was used as cutoff. The statistical tests were complemented by a non-parametric statistical quantification of the median expression difference between the two groups. An effect size of 2 (i.e., a fold change of ±2x) was chosen for the selection of candidate reporter.

The identified differentially expressed (DE) genes (fold change ≥1.5) were used to conduct pathway analysis by Qiagen Ingenuity Pathway Analysis (IPA) (Ingenuity Systems). To perform Gene set enrichment analysis (GSEA), pre-ranked gene list was analyzed by GSEA software (Broad Institute of MIT and Harvard) using the MsigDb gene sets.

**Cell viability assays.** Briefly, TF1 cells grown in 96-well plates were treated as described. Each 10 µl of MT (5mg/ml) was added to each well. The plate was incubated at 37 °C for 2 h. Then, DMSO was added to the control group thoroughly with the pipette to dissolve the formazan. Absorbance of each sample was read at 570 nm using a microplate reader ( Molecular Devices). After substrate of background control, cell viability was expressed as a ratio of absorbance relative to that of control.

**Immunoblotting.** Collected cells were lysed in a lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM glycerolphosphate, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1% Na3VO4, 1 µg ml−1 leupeptin) and phosphatase inhibitors. Freshly isolated BM cells were incubated in IMDM containing 20% FBS. Then, cells were washed and resuspended in staining buffer supplied with DAPI (Thermo Fisher Scientific) or Propidium iodide (PI) (Sigma-Aldrich). All samples were measured by a BD LSR-II or FACS Calibur (BD Biosciences) and analyzed with Flowjo (Treestar, Ashland, OR, USA).

**Apoptosis and cell cycle analysis.** Treated human erythroblasts or mice erythroblast subsets were stained with propidium iodide against surface markers as described above. To evaluate apoptosis, cells were incubated with APC-conjugated Annexin V (550475, BD Biosciences, 1,50) or PE-conjugated Annexin V (556422, BD Biosciences, 1,50) for 10 min at room temperature in the dark. Then, DAPI or Propidium iodide (PI) was added according to the instructions of the manufacturer. The samples were measured on a BD LSR-II (BD Biosciences) and analyzed with Flowjo. For cell cycle analysis, human erythroblasts or mice erythroblast subsets were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit according to the manufacturer’s protocol. Cells were then stained with Ki67 antibody (556027, BD Biosciences, 1,200). DAPI was then added before analysis on a BD LSR-II.

**Fluorescence-activated cell sorting.** Briefly, bone marrow cell suspensions from P38α−/− and P38α−/− mice were pre-treated with rat anti-mouse CD16/CD32 antibody (2.4G2, BD Biosciences), and subsequently stained with APC-conjugated anti-mouse Ter119 (BD Biosciences,1,50) and FITC-conjugated anti-mouse CD71 (BD Biosciences,1,50) for 30 min in the dark at 4 °C. After wash, cells were stained with Propidium iodide before sorted on BD SORP Aria (BD Biosciences).
Immunoprecipitation and co-immunoprecipitation. Cultured HEK293T cells were transfected or co-transfected with indicated plasmids for 48 h. After wash with cold PBS, ice-cold cell lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, protease inhibitor mixture (Sigma-Aldrich) and PMSF) were added to the cells for 30 min. After centrifuge, the supernatants were collected. Pre-cleared lysates were incubated with anti-Flag beads (Sigma-Aldrich) with gentle rocking overnight at 4 °C. Beads were washed three times with cell lysis buffer and suspended in SDS protein loading buffer. The samples were heated to 95–100 °C for 5 min. Immunoprecipitation lysates were analyzed by western blotting with indicated antibodies.

Chromatin Immunoprecipitation (ChIP) assays. After treated with or without Sp600125 (20 μM), human erythroblasts were subjected to the ChIP assay using the EZ-ChIP kit (EMD Millipore) according to the manufacturer’s instructions. Briefly, the cells were cross-linked for 10 min by addition of 37% formaldehyde to a final concentration of 1% at room temperature. 20X glycine were added to quench cross-link. After wash, cell pellets were suspended in SDS Lysis Buffer and sonicated to shear DNA. Collected supernatants were incubated with antibody overnight at 4 °C with rotation. After elution of protein/DNA complexes and reversal of cross-link, the DNA was purified using spin columns and further analyzed by quantitative PCR. The Primer sequences are showed in supplementary table 3. The antibodies used for ChIP experiments are anti-H3K27me3 (07-449, Millipore,1:500), anti-Ezh2 (5246, Cell Signaling, 1:100).

Statistical analysis. The animal sample sizes were estimated according to previous studies performed in the similar experiments and the known variability of the assays. The data distribution generally met the assumptions of the tests. All quantitative data are presented as mean ± s.e.m. Two-tailed, unpaired Student’s t-tests were performed. Result was considered significant if the P-value was below 0.05.

Data availability. Gene expression data have been deposited in the Gene Expression Omnibus (GEO) database under the accession code GSE111751. The authors declare that all the other data supporting the findings of this study are available within the article and its Supplementary Information, or from the corresponding author upon reasonable request.

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**Author contributions**

P.H. designed and performed experiments, analyzed data, and wrote the manuscript. A.R.N provided the P38α conditional knockout mice. H.H provided Lentiviral GFP shRNA vector and advised on lentivirus production. M.I and G.H.K. advised GSEA analysis. M.-D.F., M.C.Y. and H.E.B. advised project. R.K. conceived the project, supervised the study, analyzed data, and wrote the manuscript.

**Additional information**

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