

Mcl-1 and Bcl-xL regulate Bak/Bax-dependent apoptosis of the megakaryocytic lineage at multistages

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Anti-apoptotic Bcl-2 family proteins, which inhibit the mitochondrial pathway of apoptosis, are involved in the survival of various hematopoietic lineages and are often dysregulated in hematopoietic malignancies. However, their involvement in the megakaryocytic lineage is not well understood. In the present paper, we describe the crucial anti-apoptotic role of Mcl-1 and Bcl-xL in this lineage at multistages. The megakaryocytic lineage-specific deletion of both, in sharp contrast to only one of them, caused apoptotic loss of mature megakaryocytes in the fetal liver and systemic hemorrhage, leading to embryonic lethality. ABT-737, a Bcl-xL/Bcl-2/Bcl-w inhibitor, only caused thrombocytopenia in adult wild-type mice, but further induced massive mature megakaryocyte apoptosis in the Mcl-1 knockout mice, leading to severe hemorrhagic anemia. All these phenotypes were fully restored if Bak and Bax, downstream apoptosis executioners, were also deficient. *In-vitro* study revealed that the Jak pathway maintained Mcl-1 and Bcl-xL expression levels, preventing megakaryoblastic cell apoptosis. Similarly, both were involved in reticulated platelet survival, whereas platelet survival was dependent on Bcl-xL due to rapid proteasomal degradation of Mcl-1. In conclusion, Mcl-1 and Bcl-xL regulate the survival of the megakaryocytic lineage, which is critically important for preventing lethal or severe hemorrhage in both developing and adult mice.

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Anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1/A1, are known to have major roles in the inhibition of apoptosis via the mitochondrial pathway and thereby contribute to normal development and the survival of various tissues and organs.¹ During hematopoiesis, in particular, they are essential regulators of hematopoietic cell survival, maintaining an appropriate balance between protection of progenitors and elimination of damaged cells.²

Mx1-Cre-inducible deletion of the *mcl-1* gene has been reported to cause rapid, fatal, multi-lineage hematopoietic failure of HSCs (hematopoietic stem cells), CMPs (common myeloid progenitor cells), GMPs (granulocyte monocyte progenitor cells) and CLPs (common lymphoid progenitor cells),³ thus establishing the concept that Mcl-1 is important for the survival of hematopoietic cells in early differential stages of hematopoiesis.² On the other hand, recent studies have revealed that Mcl-1 is required for granulocyte development but not for the development of monocytes and macrophages,^{4,5} suggesting a selective role of Mcl-1 in the terminally differentiated stages of hematopoiesis. In addition, loss-of-function studies have demonstrated that Bcl-xL is an essential pro-survival molecule of the definitive erythrocytes,⁶

while Bcl-2 and A1 are involved in the survival of lymphocytes and neutrophils, respectively.^{7,8} These findings indicated that the significance of each anti-apoptotic Bcl-2 protein in terminally differentiated stages of hematopoiesis is different and dependent on the cellular context.

Regarding their involvement in the survival of the megakaryocytic lineage, Mcl-1 is reported to be important for the survival of the earlier differential stages including MPPs (multipotent progenitors) and CMPs.³ However, the relationship of Mcl-1 with the terminally differentiated megakaryocytes has not been well understood, with the exception of a report describing the existence of Mcl-1 protein in megakaryocytes.⁹ Bcl-xL is also continuously expressed in the megakaryocytic lineage during megakaryopoiesis¹⁰ and is required for platelet survival.¹¹ However, mature megakaryocytes increased in mice with genetic deletion of Bcl-xL.¹² Genetic studies deleting other anti-apoptotic Bcl-2 family genes have not reported any abnormality of the megakaryocytic lineage.^{7,8,13,14} Therefore, the essential anti-apoptotic actors regulating survival of the megakaryocytic lineage, especially megakaryocytes, have been unclear and disputed.

In the present study, among the five anti-apoptotic Bcl-2 family members, we focused on Mcl-1 and Bcl-xL and

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Abbreviations: APS, anti-platelet serum; BH3, Bcl-2 homology domain 3; BM, bone marrow; ED, embryonic day; ER, endoplasmic reticulum; ET, essential thrombocythemia; P14, platelet factor 4; TPO, thrombopoietin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; VWF, von Willebrand factor

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found them to be important regulators for the survival of mature megakaryocytes and reticulated platelets. We also found that their survival is critically important for preventing lethal or severe hemorrhage in both developing and adult mice.

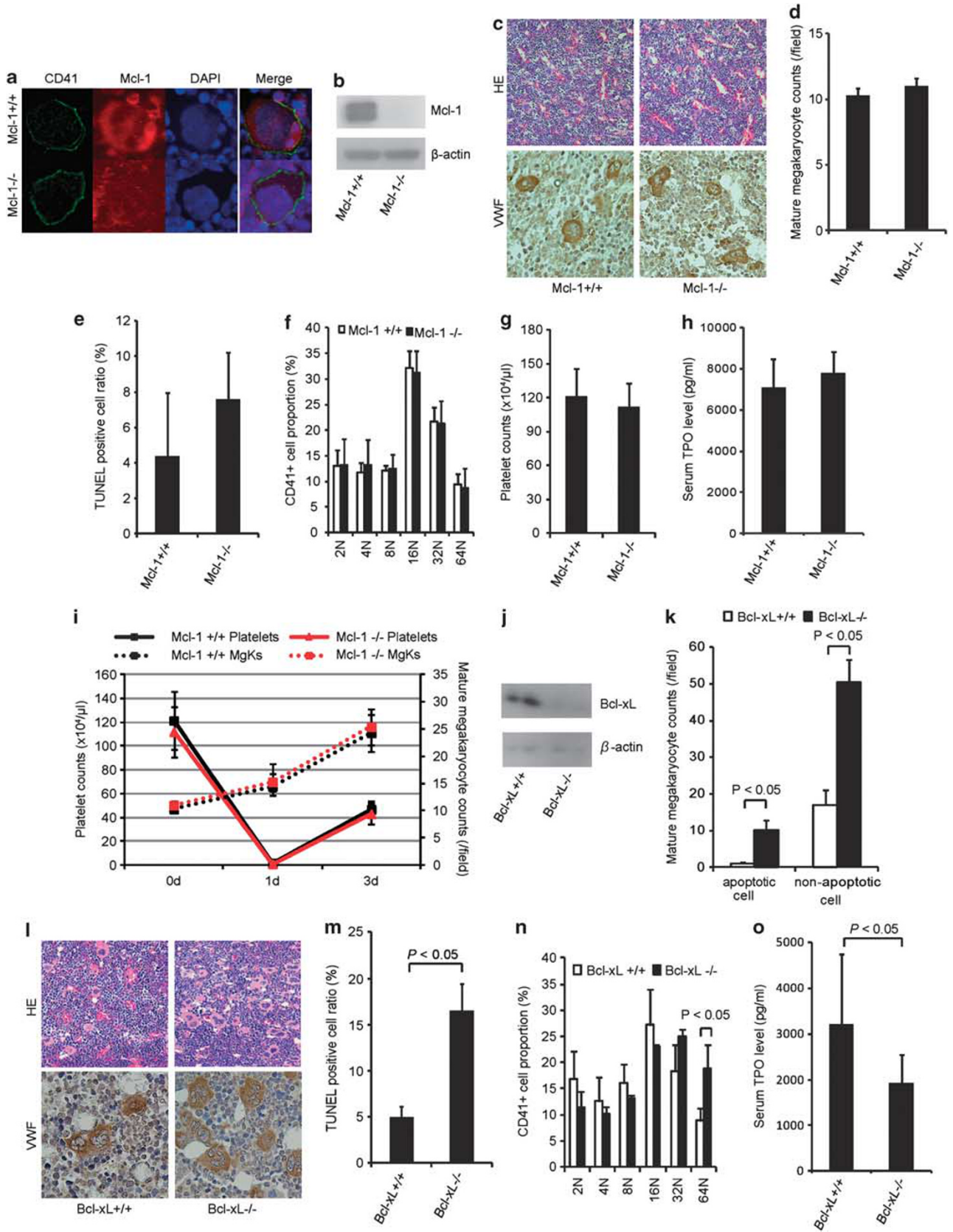
Results

Megakaryocyte development and survival is not impaired in megakaryocytic lineage-specific Mcl-1 knockout mice. To investigate the involvement of Mcl-1 in the development and survival of the megakaryocytic lineage, we generated megakaryocytic lineage-specific Mcl-1 knockout mice by mating Mcl-1 floxed mice (*mcl-1^{flox/flox}*) and platelet factor 4 (P4)-Cre transgenic mice (*pf4-Cre*). Megakaryocytic lineage-specific Mcl-1 knockout mice (*mcl-1^{flox/flox} pf4-Cre*) were born at the expected Mendelian frequency and grew up normally (data not shown). Mcl-1 protein was expressed in mature megakaryocytes of the control littermates but not in those of the knockout mice (Figures 1a and b), according to immunocytochemical study of primary megakaryocytes and western blot of cultured megakaryocytes. Hematoxylin–eosin (HE) and von Willebrand factor (VWF) staining of the bone marrow (BM) showed that mature megakaryocyte counts in the knockout mice were not different from those in the control littermates (Figures 1c and d). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of the BM showed that apoptosis of mature megakaryocytes did not increase in the knockout mice (Figure 1e). Mcl-1 deficiency did not affect the ploidy of the primary megakaryocytes in the BM (Figure 1f). Circulating platelet counts were normal in both knockout and control mice (Figure 1g). Serum thrombopoietin (TPO) levels did not differ between them (Figure 1h). To assess platelet production capacity *in vivo*, we examined platelet and mature megakaryocyte counts in response to anti-platelet serum (APS)-induced thrombocytopenia. Upon APS treatment, after the rapid disappearance of circulating platelets, megakaryocyte counts increased and platelet counts recovered in both mice to a similar extent (Figure 1i). Based on these findings, we concluded that Mcl-1 was not essential for the development and survival of the megakaryocytic lineage despite its presence in mature megakaryocytes.

Bcl-xL is involved in preventing mature megakaryocytes from apoptosis but is not essential for their growth and development. We next investigated the involvement of Bcl-xL, another anti-apoptotic Bcl-2 family protein known to exist in megakaryocytes,¹⁰ in the development and survival of the megakaryocytic lineage. We used previously generated megakaryocytic lineage-specific homozygote Bcl-xL knockout mice (*bcl-x^{flox/flox} pf4-cre*),¹⁵ which presented with severe thrombocytopenia due to massive platelet apoptosis.^{11,12} As previously reported, despite severe thrombocytopenia, Bcl-xL knockout mice were born at the expected Mendelian frequency and did not show any gross abnormality;¹⁵ analysis at embryonic day (ED) 13.5 also revealed that Bcl-xL knockout embryos grew up normally at the

expected Mendelian frequency (Supplementary Table 1). We confirmed that Bcl-xL expression was efficiently diminished in the cultured megakaryocytes of the knockout mice (Figure 1j) as well as in the platelets.¹⁵ While both apoptotic and non-apoptotic megakaryocyte counts were significantly higher in the BM of the knockout mice than the control littermates (*bcl-x^{flox/flox}*) (Figures 1k and l), the apoptotic cell ratio significantly increased in the knockout mice (Figure 1m), indicating enhanced apoptosis of mature megakaryocytes in the knockout mice. Ploidy analysis revealed an increase in large polyploid megakaryocytes (32N, 64N) of the knockout mice (Figure 1n) and their serum TPO levels were significantly lower than those of the control littermates (Figure 1o); both findings were consistent with previous reports.¹⁶ These findings suggested that Bcl-xL was involved in preventing mature megakaryocytes from apoptosis but was not essential for their growth and development.

Megakaryocytic lineage-specific Mcl-1 and Bcl-xL knockout mice are embryonically lethal in association with apoptotic loss of mature megakaryocytes and systemic hemorrhage. To investigate the redundancy of these anti-apoptotic proteins in the growth and development of megakaryocytic lineage, we generated mice without both genes by crossing *mcl-1^{flox/flox} bcl-x^{flox/+} pf4-cre* mice and *mcl-1^{flox/flox} bcl-x^{flox/flox}* mice. No offspring resulted from megakaryocytic lineage-specific homozygote Mcl-1 and Bcl-xL double knockout mice (*mcl-1^{flox/flox} bcl-x^{flox/flox} pf4-cre*) (Table 1). To investigate the mechanism of their embryonic lethality, we performed embryonic analysis of all the littermates crossing *mcl-1^{flox/flox} bcl-x^{flox/+} pf4-cre* mice with *mcl-1^{flox/flox} bcl-x^{flox/flox}* mice, and *bcl-x^{flox/flox} pf4-cre* mice with *bcl-x^{flox/flox}* mice, respectively. On ED 13.5, genotyping results of the offspring showed that homozygote Mcl-1 and Bcl-xL double knockout embryos resulted at the expected Mendelian frequency (Table 1). However, morphological analyses revealed that all of the double knockout embryos were enlarged and suffered from massive internal hemorrhage throughout their bodies (Figure 2a), while these abnormalities were not apparent in any of the other embryos studied, that is, *mcl-1^{flox/flox} bcl-x^{flox/+} pf4-cre* and *bcl-x^{flox/flox} pf4-cre* (Figures 2a and b). Histological analyses of the fetal livers showed that mature megakaryocyte counts increased in the Bcl-xL knockout embryos (Figures 2c and d), which agreed with those of the adult BM of the knockout mice. In sharp contrast, they were greatly diminished in the double knockout embryos (Figures 2e and f), and apoptosis morphology, such as nuclear condensation and fragmentation, was observed in the residual mature megakaryocytes (Figure 2g), suggesting apoptotic loss in the hematopoietic liver. On ED 18.5, genotyping results showed that Mcl-1 and Bcl-xL double knockout embryos existed even at a lower rate than the expected Mendelian frequency but all had stopped developing and were not alive (Table 1; Supplementary Figure 1). These results clearly indicated that the presence of either Mcl-1 or Bcl-xL in the megakaryocytic lineage was required for the survival of mature megakaryocytes in the fetal liver and was indispensable for normal embryonic development.



Disruption of Bak and Bax prevents apoptotic loss of mature megakaryocytes and the embryonic lethality caused by Mcl-1 and Bcl-xL deficiency *in vivo*.

Both Mcl-1 and Bcl-xL inhibit the mitochondrial pathway of apoptosis but also may have additional functions such as regulating cell-cycle inhibition, DNA repair in the nucleus and autophagy inhibition in the endoplasmic reticulum (ER).^{17,18} To examine whether the lethality of megakaryocytic lineage-specific Mcl-1/Bcl-xL-deficient embryos is ascribable to their effect on megakaryocyte apoptosis, we further knocked out Bak and Bax, their downstream effector molecules toward apoptosis. The quadruple knockout mice (*mcl-1^{flox/flox} bcl-x^{flox/flox} bak -/- bax^{flox/flox} pf4-cre*) were born at the expected Mendelian frequency and grew up normally (data not shown). Western blot of cultured megakaryocytes confirmed that protein expression of Mcl-1, Bcl-xL, Bak and Bax was absent from these mice (Figure 3a). We thus crossed *mcl-1^{flox/flox} bcl-x^{flox/flox} bak -/- bax^{flox/flox} pf4-cre* mice with *mcl-1^{flox/flox} bcl-x^{flox/flox} bak -/- bax^{flox/flox}* mice. In the following experiments, we used *mcl-1^{flox/flox} bcl-x^{flox/flox} bak -/- bax^{flox/flox} pf4-cre* mice as the quadruple knockout mice and *mcl-1^{flox/flox} bcl-x^{flox/flox} bak -/- bax^{flox/flox}* mice as the control Bak knockout littermates. It should be noted that Bak deficiency caused modest thrombocytosis due to the prolonged lifespan of their platelets.^{11,12} Mature megakaryocyte and platelet counts of the quadruple knockout mice were not significantly different from the control Bak knockout littermates (Figures 3b–d). The TUNEL-positive cell ratio of mature megakaryocytes in the quadruple knockout mice was very low and not different from their littermates

(Figure 3e), suggesting that mature megakaryocyte apoptosis in the absence of both Bcl-xL and Mcl-1 could be prevented in a Bak and Bax knockout background. In addition, megakaryocyte ploidy and serum TPO levels did not differ much between these mice (Figures 3f and g). We next performed embryo analysis of the quadruple knockout mice. The quadruple knockout embryos also existed at the expected Mendelian frequency without abnormal hemorrhage in their bodies at ED 13.5 (Table 2; Figure 3h), and histological analysis revealed that mature megakaryocytes similarly existed in the fetal liver of the quadruple knockout mice and control Bak knockout littermates (Figures 3i and j). These findings indicated that mature megakaryocyte apoptosis in the fetal liver via the Bak/Bax-dependent mitochondrial pathway was responsible for the massive systemic hemorrhage and embryonic lethality of the megakaryocytic lineage-specific Mcl-1 and Bcl-xL knockout mice.

ABT-737 induces Bak/Bax-dependent apoptotic loss of mature megakaryocytes and circulating platelets with hemorrhagic anemia in megakaryocytic lineage-specific Mcl-1 knockout mice.

To examine the involvement of these anti-apoptotic proteins in the survival of the megakaryocytic lineage in adult mice, we subjected the Mcl-1 knockout mice and the control wild-type littermates to intraperitoneal injection of ABT-737, which could inhibit Bcl-xL. While mature megakaryocyte counts were not affected in the BM of the wild-type mice until 24 h after the ABT-737 treatment (Figures 4a and b), the Mcl-1 knockout mice showed rapid decreases in their counts starting at 2 h after ABT-737 administration, with almost complete disappearance from the BM within 24 h (Figures 4a and b). The TUNEL-positive cell ratio in the mature megakaryocytes increased and was significantly higher in the knockout mice than the control littermates at 4.5 h after ABT-737 treatment (Figures 4c and d). These findings indicated that ABT-737 treatment caused apoptotic loss of the mature megakaryocytes in BM in the absence of Mcl-1, suggesting that either existence of Mcl-1 or Bcl-xL may also be required for the survival of mature megakaryocytes in the adult BM. Upon ABT-737 treatment, the circulating platelet count decreased in the wild-type mice (Figure 4e), which is consistent with previous findings.¹² However, platelet counts were more rapidly and greatly reduced in the Mcl-1 knockout mice compared with

Table 1 Genotyping of offspring obtained by crossing *mcl-1^{flox/flox} bcl-x^{flox/+} pf4-cre* mice and *mcl-1^{flox/flox} bcl-x^{flox/flox}* mice

	ED 13.5	ED 18.5	3 Weeks
<i>mcl-1^{flox/flox} bcl-x^{flox/flox} pf4-cre</i>	10	3*	0**
<i>mcl-1^{flox/flox} bcl-x^{flox/+} pf4-cre</i>	13	16	16
<i>mcl-1^{flox/flox} bcl-x^{flox/flox}</i>	10	12	17
<i>mcl-1^{flox/flox} bcl-x^{flox/+}</i>	13	10	24
Total	46	41	57

Abbreviation: ED, embryonic day

*, ***P* < 0.05 versus all

Note that each genotype is expected to account for one-fourth of the offspring from this mating

Figure 1 Megakaryocytic lineage-specific knockout of Mcl-1 or Bcl-xL. (a–i) Offspring from mating of *mcl-1^{flox/flox} pf4-Cre* mice and *mcl-1^{flox/flox}* mice were analyzed. Mcl-1 +/+ and Mcl-1 -/- stand for *mcl-1^{flox/flox}* and *mcl-1^{flox/flox} pf4-Cre* mice, respectively. Cytospins of bone marrow (BM) cells were stained with CD41 (green) and Mcl-1 (red) and representative images are shown (a). Western blot for Mcl-1 and β-actin protein of cultured megakaryocytes derived from BM (b). Sections of BM were stained with Hematoxylin and Eosin (upper) or von Willebrand factor (bottom) to identify megakaryocytes (original magnification: upper × 200, lower × 400) and representative images are shown (c). VWF-positive and morphologically recognizable megakaryocytes in the BM were counted per field of view; five mice per group (d). TUNEL-positive cell ratio of morphologically recognizable megakaryocytes in the BM; five mice per group (e). Ploidy distribution of primary megakaryocyte of the BM; data are presented as the proportion among CD41-positive cells of the BM; three mice per group (f). Circulating platelet counts; eight mice per group (g). Serum thrombopoietin levels; five mice per group (h). Circulating platelet counts and morphologically recognizable megakaryocyte counts of the BM in response to anti-platelet serum treatment; three mice per group, MgKs stands for megakaryocytes (i). (j–o) Offspring from mating of *bcl-x^{flox/flox} pf4-Cre* mice and *bcl-x^{flox/flox}* mice were analyzed. Bcl-xL +/+ and Bcl-xL -/- stand for *bcl-x^{flox/flox}* and *bcl-x^{flox/flox} pf4-Cre* mice, respectively. Western blot for Bcl-xL and β-actin protein of cultured megakaryocytes derived from BM (j). VWF-positive and morphologically recognizable megakaryocytes and TUNEL-positive megakaryocytes in the BM were counted per field of view; 4–5 mice per group, TUNEL-positive cell counts are presented as apoptotic cell counts and TUNEL-positive cell counts subtracted from total megakaryocyte counts are presented as non-apoptotic cell counts (k). Representative images of HE (upper) and VWF (bottom) staining of the BM (original magnification: upper × 200, lower × 400) (l). TUNEL-positive cell ratio of morphologically recognizable megakaryocytes in the BM; six mice per group (m). Ploidy distribution of primary megakaryocyte of the BM; data are presented as the proportion among CD41-positive cells of the BM; three mice per group (n). Serum thrombopoietin levels; five mice per group (o). Statistical analysis was performed using Mann–Whitney’s *U*-test

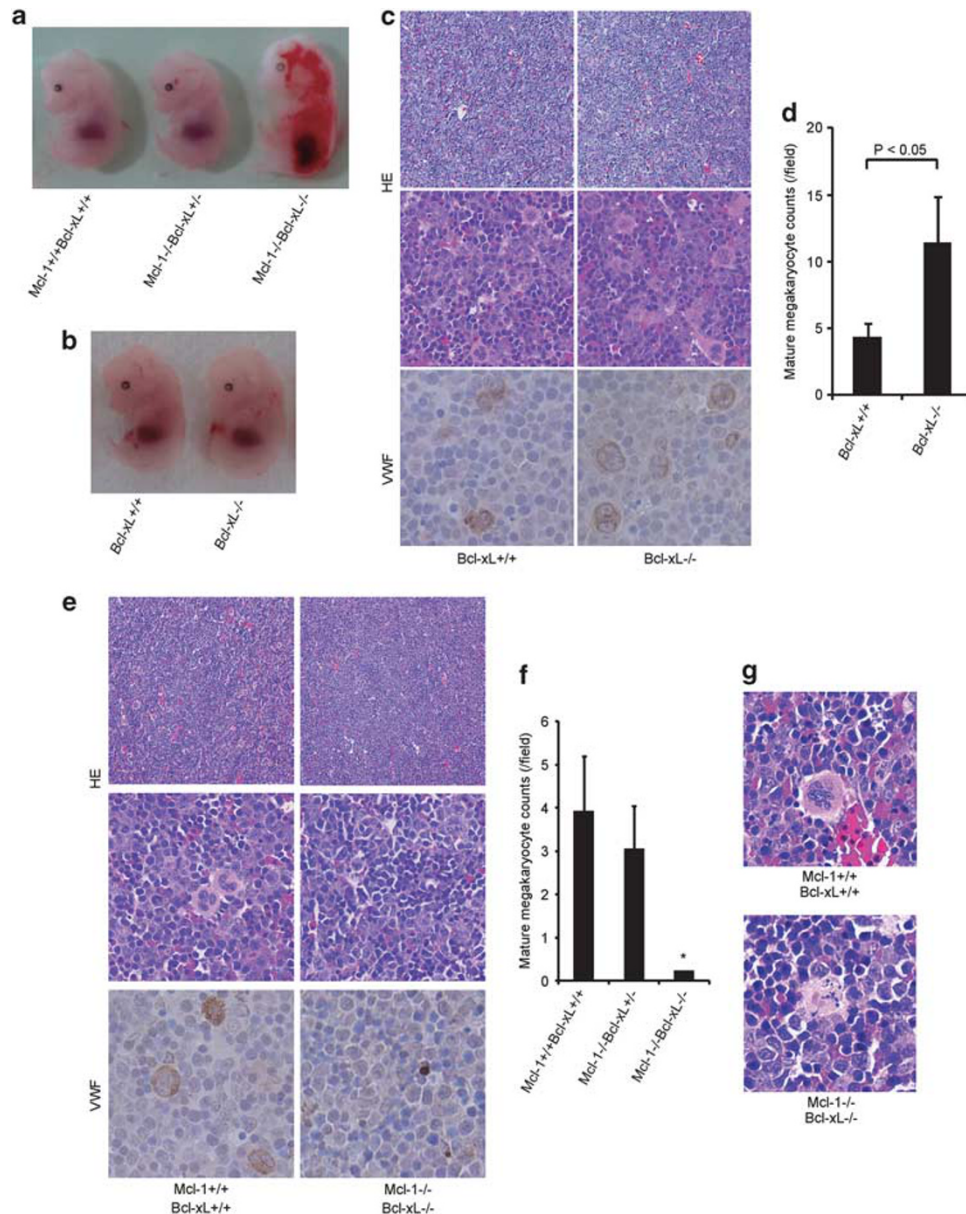


Figure 2 Embryo analysis of megakaryocytic lineage-specific knockout of Mcl-1 or Bcl-xL or both. Offspring embryo from mating of *mcl-1^{fllox/fllox}bcl-x^{fllox/+}pf4-Cre* mice and *mcl-1^{fllox/fllox}bcl-x^{fllox/fllox}* mice and mating of *bcl-x^{fllox/fllox}pf4-Cre* mice and *bcl-x^{fllox/fllox}* mice was analyzed at ED 13.5, respectively. Mcl-1 +/+ Bcl-xL +/+ stands for *mcl-1^{fllox/fllox}bcl-x^{fllox/fllox}* mice or *mcl-1^{fllox/fllox}bcl-x^{fllox/+}* mice. Mcl-1 -/- Bcl-xL +/+ and Mcl-1-/-Bcl-xL -/- stand for *mcl-1^{fllox/fllox}bcl-x^{fllox/+}pf4-Cre* mice and *mcl-1^{fllox/fllox}bcl-x^{fllox/fllox}pf4-Cre* mice, respectively. Bcl-xL +/+ and Bcl-xL -/- stand for *bcl-x^{fllox/fllox}* mice and *bcl-x^{fllox/fllox}pf4-Cre* mice, respectively. Representative images of macro findings of each strain (a and b). Representative images of HE and VWF staining of the fetal liver of each strain (original magnification: top $\times 100$, middle and bottom $\times 400$) (c and e). Mature megakaryocyte counts in the fetal liver of each strain; six mice per group (d) and five mice per group, * $P < 0.05$ versus the other two groups (f). Representative images of HE staining of the fetal liver of *mcl-1^{fllox/fllox}bcl-x^{fllox/fllox}* mice and *mcl-1^{fllox/fllox}bcl-x^{fllox/fllox}pf4-Cre* mice (original magnification: $\times 400$) (g)

their control littermates and did not recover even 48 h after the treatment (Figure 4e). Of importance are the findings that the Mcl-1 knockout mice displayed severe anemia, demonstrated by significant reduction of red blood cell counts compared with the control littermates at 48 h after the treatment (Figure 4f). Systemic screening revealed that all Mcl-1 knockout mice tested developed spontaneous mucosal hemorrhage of the stomach (Figure 4g). ABT-737-induced phenotypes in the Mcl-1 knockout mice, displaying megakaryocyte loss, platelet decrease and severe anemia, were

fully prevented if Bak and Bax were also deficient (Figures 4h–k). These results indicated that apoptotic loss of megakaryocytic lineage via the Bak/Bax-dependent mitochondrial pathway was responsible for the spontaneous hemorrhagic anemia observed in the ABT-737-treated Mcl-1 knockout mice.

TPO/Jak signaling positively regulates both Mcl-1 and Bcl-xL in human megakaryoblastic cells. To further examine the involvement of these anti-apoptotic proteins in

the survival of megakaryocytes, we performed *in-vitro* study using CMK cells, well-established human megakaryoblastic cells,¹⁹ which expressed both Mcl-1 and Bcl-xL (Figure 5a). Via an electroporation procedure, *mcl-1* siRNA could reduce the level of Mcl-1 expression without any change of Bcl-xL expression in CMK cells (Figure 5a). Mcl-1 knockdown showed neither effect on caspase-3/7 activity nor on cell survival (Figures 5b and c), when monitored by the specific cleavage of the Ac-DEVD-pNA substrate or WST assay, respectively. On the other hand, while ABT-737 treatment itself moderately activated caspase-3/7 and impaired cell survival, Mcl-1 knockdown significantly augmented these effects (Figures 5b and c) and caused massive apoptotic cell death in CMK cells (Figures 5d and e). These *in-vitro* results are consistent with *in-vivo* findings that both deletion of Mcl-1 and Bcl-xL lead to massive megakaryocyte apoptosis.

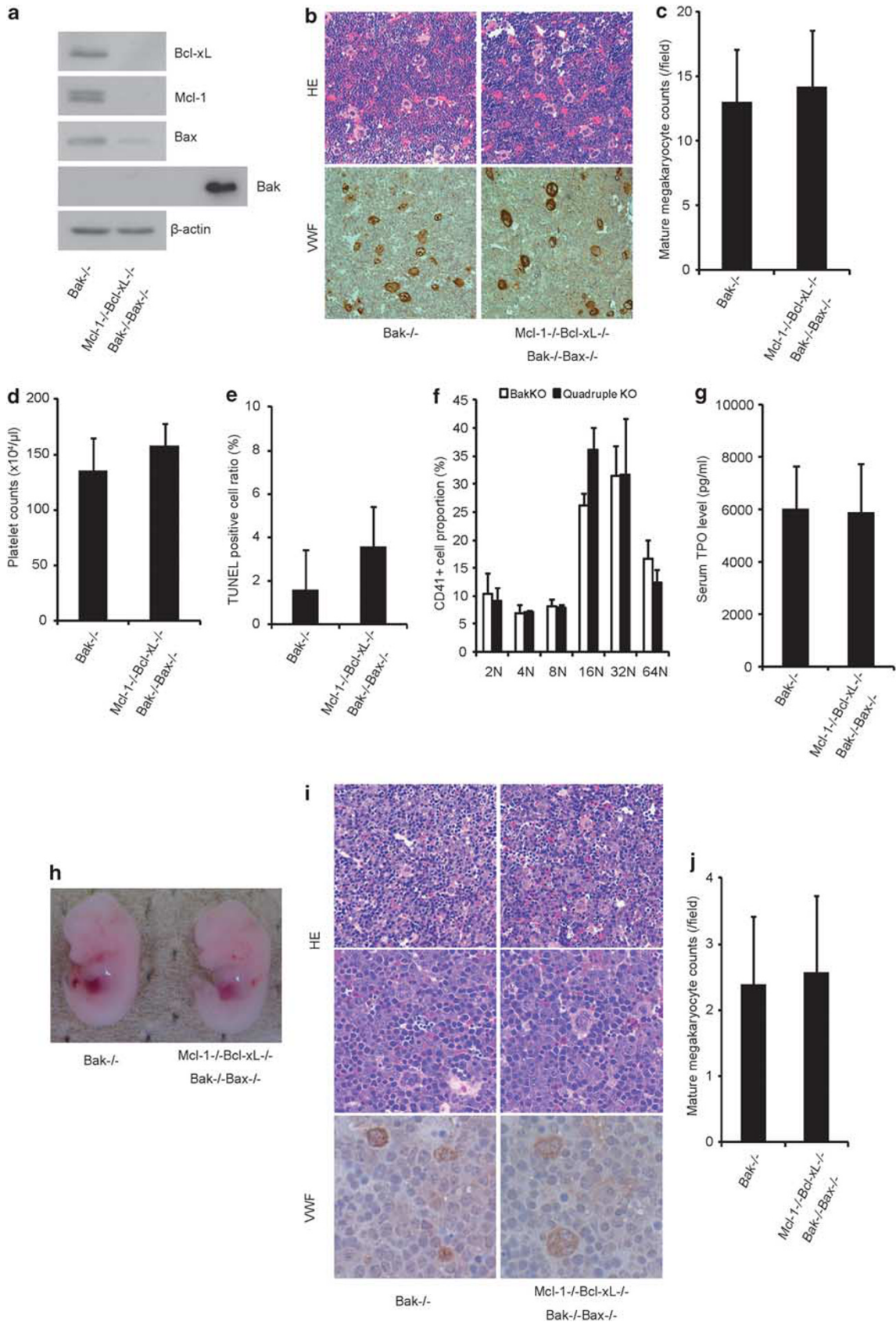
We next examined the regulatory mechanism of these proteins in CMK cells. Previous reports revealed that TPO signaling regulated Bcl-xL expression through the Jak/Stat5 pathway.²⁰ We found that mRNA and protein levels of Mcl-1 as well as Bcl-xL increased upon TPO administration in CMK cells, which were blocked by the Jak inhibitor (at 1.0 μM) (Figures 5f–h). These findings demonstrated that TPO/Jak signaling regulated Mcl-1 expression as well as Bcl-xL in megakaryoblastic cells. While several pathways including Stat5, ERK, p38 and Akt were previously reported as being downstream of TPO/Jak signaling,²¹ TPO induced phosphorylation of Stat5 and ERK in these cells, both of which were prevented by the Jak inhibitor (Figure 5h). Pharmacological inhibition of the Jak signaling was found to cause caspase activation, demonstrated by the appearance of a cleaved form of caspase-3, and impaired cell survival with downregulation of both Mcl-1 and Bcl-xL expression (Figures 5h and i), suggesting the involvement of Jak signaling in the survival of these cells.

Platelet survival is dependent on Bcl-xL due to proteasomal degradation of Mcl-1, while both are involved in the survival of reticulated platelets. Since platelets are generated from mature megakaryocytes by the shedding of their bodies²² and do not have any transcriptional machinery,²³ the molecular contents of both may be quite similar. Nevertheless, platelets possess Bcl-xL alone, unlike megakaryocytes in which two functional anti-apoptotic proteins, Bcl-xL and Mcl-1, are present.^{11,12,16} We thus studied the effect of post-transcriptional degradation of Mcl-1 in platelets *in vivo*. At 1 or 2 days after the administration to wild-type mice of MG-132, a proteasome inhibitor, western blot revealed that the Mcl-1 protein was observed in platelets isolated from the MG-132-treated mice in contrast to platelets from the vehicle-treated mice (Figure 6a). This finding suggested that the proteasomal degradation may rapidly diminish Mcl-1 protein in circulating platelets when there is a lack of *de-novo* protein synthesis, leading to the dependence of platelet survival on Bcl-xL. Next, we examined the characteristics of circulating platelets in the Bcl-xL knockout mice. They mostly consisted of reticulated platelets (Figure 6b), known as young platelets with higher hemostatic function^{24,25} and Mcl-1 proteins were clearly detected in lysates derived from their circulating platelets (Figures 6c

and d). In contrast, Mcl-1 proteins were hardly detected in the lysates derived from the circulating platelets in the wild-type mice (Figures 6c and d), which mostly consisted of non-reticulated platelets (Figure 6b). In addition, given that reticulated platelets contain some rough ER and messenger RNA and that they retain a weak ability of protein synthesis,²⁶ we speculated that Mcl-1 may still remain in the reticulated platelets. We thus investigated the functional involvement of Mcl-1 and Bcl-xL in the survival of reticulated platelets *in vivo*. Disruption of Mcl-1 neither affects their proportion in circulation (Figure 6e) nor apoptosis which was assessed by their Annexin V positivity (Figure 6f). Inhibition of Bcl-xL by ABT-737 administration slightly induced apoptosis in reticulated platelets of the wild-type mice (Figure 6f) but increased their proportion in circulation (Figure 6e) similar to the Bcl-xL knockout mice (Figure 6b), probably due to a higher susceptibility of non-reticulated platelets to ABT-737-induced apoptosis.^{27,28} Meanwhile, ABT-737 treatment induced moderate apoptosis of reticulated platelets in the Mcl-1 knockout mice (Figure 6f) and their circulating proportion was significantly lower than the wild-type reticulated platelets (Figure 6e). These results suggested that the presence of either Bcl-xL or Mcl-1 may be important for the survival of reticulated platelets like mature megakaryocytes.

Discussion

Among the previously recognized five members of the anti-apoptotic Bcl-2 family, systemic knockout mice of the *bcl-2*, *bcl-w* or *bfl-1/a1* gene could survive, and the number of megakaryocytes and platelets was normal in the Bcl-2 or Bcl-w knockout mice; it was not evaluated in the Bfl/A1 knockout mice.^{7,8,13,14} On the other hand, the mice with systemic deletion of the *mcl-1* or *bcl-x* gene died in the embryo stage due to implantation failure or erythrocyte and neuronal apoptosis, respectively,^{6,29} which did not provide information about their involvement in the survival of the megakaryocytic lineage. However, Pf4-Cre transgenic mice have recently been developed, and any target gene can be deleted exclusively from their megakaryocytes and platelets.³⁰ We thus generated megakaryocytic lineage-specific Mcl-1 knockout mice, which display no obvious effect in their development and survival *in vivo* (Figures 1a–i), in agreement with our *in-vitro* results (Figures 5b–e). With regard to Bcl-xL, previous *in-vitro* studies reported not only its expression profile during megakaryocyte differentiation but also its anti-apoptotic involvement.^{10,31} This agrees with our *in-vitro* study showing moderate apoptosis of megakaryoblastic cells upon ABT-737 treatment (Figures 5b–e). On the other hand, in *in-vivo* settings, Josefsson *et al.*¹⁶ very recently analyzed this in detail and reported that Bcl-xL played an important role in preventing mature megakaryocytes from apoptosis at the proplatelet formation stage but was not required for their growth and development. In agreement with these findings, in our *in-vivo* study, although apoptosis of mature megakaryocytes was observed in the Bcl-xL knockout mice, they showed an increased number of mature megakaryocytes, containing many large polyploid cells (Figures 1k–n). This is probably due to compensatory megakaryopoiesis induced by thrombocytopenia. Such a compensative response may be also



observed in the acute inhibition of Bcl-xL by ABT-737 administration into wild-type mice (Figure 4a). These findings show that Bcl-xL was involved in preventing mature megakaryocytes from apoptosis but was dispensable for their growth and development *in vivo*. We have recently reported that Mcl-1 and Bcl-xL cooperatively maintain hepatocyte integrity,³² which led us to generate mice with megakaryocytic lineage-specific deletion of both genes. The double knockout mice developed loss of mature megakaryocytes in the fetal liver. In addition, in the absence of Mcl-1, ABT-737 diminished them in the adult BM. These results indicated that, among the five members of the anti-apoptotic Bcl-2 family, either existence of Mcl-1 or Bcl-xL is required for the development and survival of megakaryocytes in both developing and adult mice. During revision of this manuscript, Debrincat *et al.*³³ published a paper on line reporting the importance of these anti-apoptotic proteins in megakaryocyte survival, which agree with our current findings. Our present study now provides solid evidence that Mcl-1 and Bcl-xL have an important pro-survival role in preventing Bak/Bax-dependent megakaryocyte apoptosis in both developing and adult mice.

Regarding the regulatory mechanism of anti-apoptotic Bcl-2 family proteins, Stat is known to directly upregulate transcription levels of Mcl-1 in macrophages, neutrophils and T cells³⁴ and Bcl-xL in mast cells and erythroid lineage cells.^{35,36} In megakaryocytes, previous reports revealed that Bcl-xL expression is upregulated by TPO through the Stat5 and PI3k pathways.²⁰ In the present *in-vitro* study, we demonstrated that mRNA and protein expression of Mcl-1 was also upregulated via TPO/Jak signaling in megakaryoblastic cells as well as Bcl-xL. Among its known downstream pathways including Stat5, Akt, Erk and p38,²¹ we found that TPO administration phosphorylated Erk as well as Stat5; the phosphorylation of both was blocked by Jak inhibition. Thus,

these pathways might be involved in the downstream portion of TPO/Jak signaling regulating these anti-apoptotic protein expression. Disruption of Jak signaling caused caspase activation and impaired cell survival in these cells concomitant with a decrease in Mcl-1 and Bcl-xL expression, suggesting that the Jak pathway may be involved in megakaryocyte survival via the induction of anti-apoptotic proteins, Mcl-1 as well as Bcl-xL. Recent human studies have revealed that about half of the patients with essential thrombocythemia (ET) carry a dominant gain-of-function mutation of JAK2.³⁷ Jak2^{V617F} transgenic mice displayed ET-like phenotypes³⁸ and constitutive Stat5 phosphorylation was observed in Jak2^{V617F} knock-in mice with megakaryocyte hyperplasia and thrombocythemia.³⁹ In addition, our *in-vitro* study showed that activation of Jak signaling suppressed the endogenous caspase activation with increases in Mcl-1 and Bcl-xL. These findings suggest that increases in these anti-apoptotic proteins via activation of Jak signaling might be involved in the excessive megakaryocyte survival of ET patients.

Reticulated platelets are young platelets with some residual mRNA.²⁶ While previous reports emphasized their utility as biomarkers of thrombopoiesis,²⁶ *in-vitro* study revealed that they display higher hemostatic activity shown by increased aggregation capacity and greater amounts of surface CD62P expression upon stimulation.^{24,25} Moreover, their pathophysiological involvement has shown that their increase may contribute to the prothrombotic phenotype of patients with thrombocythemia and the maintenance of hemostasis despite thrombocytopenia in patients with immune thrombocytopenic purpura.^{25,40} Recent advances in cancer therapy make it possible to selectively target some of the anti-apoptotic Bcl-2 proteins. ABT-263, an orally bioavailable Bcl-2 homology domain 3 (BH3) mimetic, is a promising anticancer agent against lung cancer and leukemia.²⁷ The side effect of this drug is thrombocytopenia due to the inhibition of Bcl-xL in platelets similar to ABT-737. However, these drugs have been considered to affect only circulating platelets, unlike the chemotherapy-induced thrombocytopenia acting through myelosuppression, and increase the proportion of reticulated platelets in circulation.^{27,28} Our present results indicated that Mcl-1 has an indispensable role in the survival of megakaryocytes and reticulated platelets in the absence of Bcl-xL, which prevents severe hemorrhagic complications from these BH3 mimetics.

In conclusion, the present *in-vivo* study clearly demonstrates that Mcl-1 and Bcl-xL have crucial anti-apoptotic roles at multistages in the megakaryocytic lineage, making possible

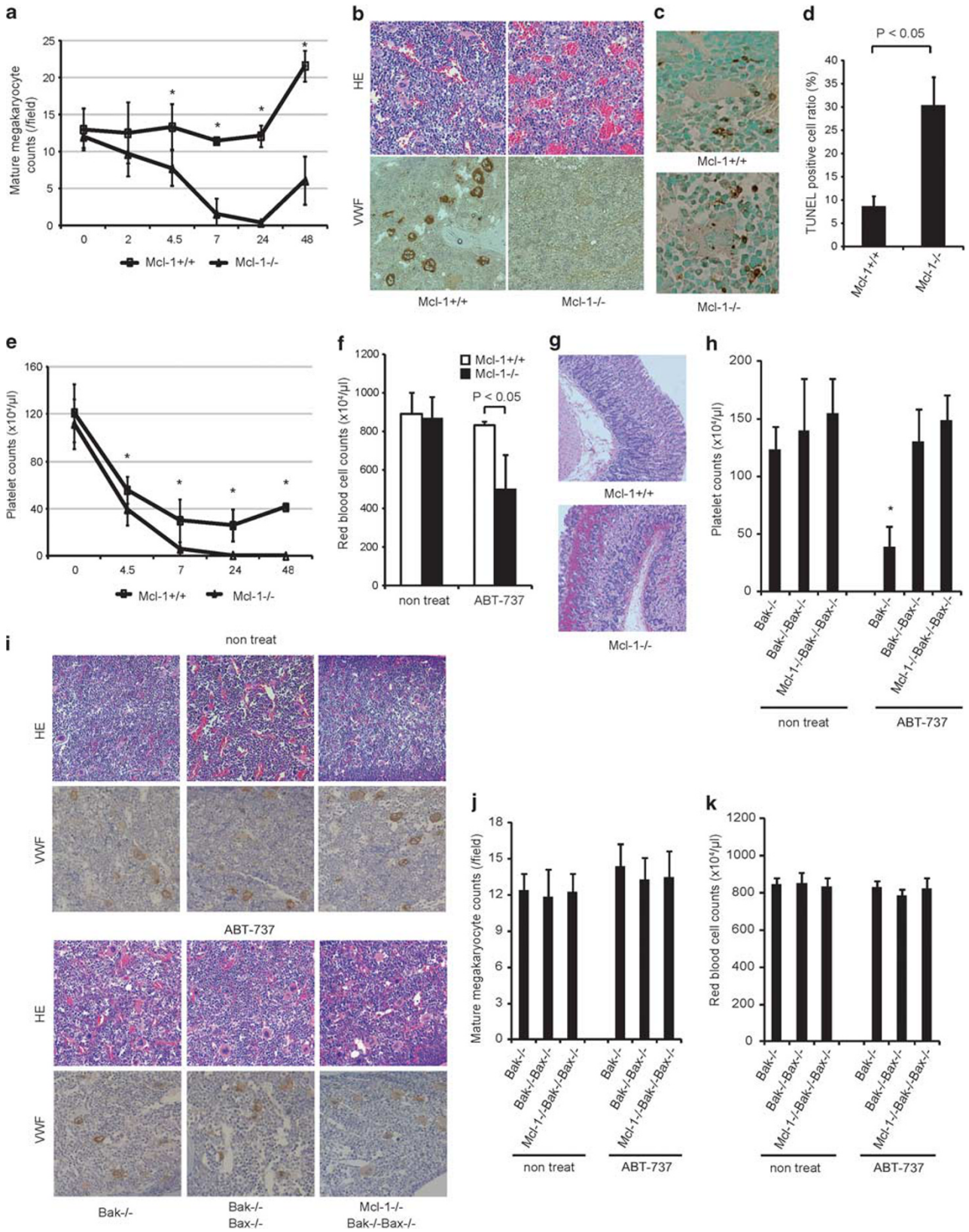
Table 2 Genotyping of offspring obtained by crossing $mcl-1^{flox/flox}bcl-x^{flox/flox}$ bak - / - bak^{flox/flox}pf4-cre mice and $mcl-1^{flox/flox}bcl-x^{flox/flox}$ bak - / - bak^{flox/flox} mice

	ED 13.5 3 Weeks	
$mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$	15	20
$mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$	12	16
Total	27	36

Abbreviation: ED, embryonic day

Note that each genotype is expected to account for one-half of the offspring from this mating

Figure 3 Bak/Bax-dependent apoptotic loss of mature megakaryocytes and embryonic lethality caused by Mcl-1/Bcl-xL deletion. (a–g) Offspring from mating of $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$ mice and $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$ mice were analyzed. Bak - / - stands for $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$ mice. Mcl-1 - / - Bcl-xL - / - Bak - / - Bax - / - stands for $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$ mice. Western blot for Mcl-1, Bcl-xL, Bak, Bax and β-actin protein of cultured megakaryocytes derived from BM, lysates of cultured wild-type megakaryocytes were used as the positive control of Bak protein (a). Representative images of HE (upper) and VWF (bottom) staining of the BM (original magnification: × 200) (b). Mature megakaryocyte counts in the BM; > 5 mice per group (c). Circulating platelet counts; > 8 mice per group (d). TUNEL-positive cell ratio of morphologically recognizable megakaryocytes in the BM; five mice per group (e). Ploidy distribution of primary megakaryocyte of the BM; data are presented as the proportion among CD41-positive cells of the BM, two mice per group, Bak KO and Quadruple KO stand for $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$ mice and $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$ mice, respectively (f). Serum thrombopoietin levels; five mice per group (g). (h–j) Offspring from mating of $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$ mice and $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$ mice were analyzed at ED 13.5. Bak - / - and Mcl-1 - / - Bcl-xL - / - Bak - / - Bax - / - stand for $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}$ mice and $mcl-1^{flox/flox}bcl-x^{flox/flox}bak - / - bak^{flox/flox}pf4-cre$ mice, respectively. Representative images of Macro findings (h). Representative images of HE and VWF staining of the fetal liver (original magnification: top × 100, middle and bottom × 400) (i). Mature megakaryocyte counts in the fetal liver; N = 11/group (j)



prevention of lethal or severe spontaneous hemorrhaging in developing and adult mice. Our study findings shed light on the important involvement of these anti-apoptotic Bcl-2 family members in the physiology of the megakaryocytic lineage and also its pathology.

Materials and Methods

Cells and reagents. CMK cells, a human megakaryoblastic cell line,¹⁹ were maintained with RPMI medium supplemented with 10% fetal bovine serum at 37°C under 5% CO₂. Recombinant human TPO and rabbit anti-mouse platelet serum were purchased from R&D Systems Inc. (Minneapolis, MN, USA) or Inter Cell Technologies (Jupiter, FL, USA), respectively. Jak inhibitor I was purchased from Merck (San Diego, CA, USA) and dissolved with DMSO. ABT-737 was provided by Abbott Laboratories (Abbott Park, IL, USA) and dissolved with DMSO.

Mice. The following mice have been described previously: mice carrying a *bcl-x* gene with 2 *loxP* sequencers at the promoter region and a second intron (*bcl-x^{fllox/fllox}*),¹⁵ a *mcl-1* gene encoding amino acids 1 through 179 flanked by 2 *loxP* sequencers (*mcl-1^{fllox/fllox}*),⁴ and heterozygous *pf4-Cre* transgenic mice expressing Cre recombinase gene under regulation of the promoter of the platelet factor 4 gene.³⁰ Megakaryocytic lineage-specific Bcl-xL knockout mice (*bcl-x^{fllox/fllox} pf4-Cre*) have been also described previously.¹⁵ We purchased C57BL/6J mice from Charles River (Osaka, Japan) and conditional Bak/Bax double knockout mice (*bak^{-/-} bax^{fllox/fllox}*) from the Jackson Laboratory (Bar Harbor, ME, USA). We generated megakaryocytic lineage-specific Mcl-1 knockout mice (*mcl-1^{fllox/fllox} pf4-Cre*), Mcl-1/Bcl-xL double knockout mice (*mcl-1^{fllox/fllox} bcl-x^{fllox/fllox} pf4-Cre*), Mcl-1/Bak/Bax triple knockout mice (*mcl-1^{fllox/fllox} bak^{-/-} bax^{fllox/fllox} pf4-Cre*) and Mcl-1/Bcl-xL/Bak/Bax quadruple knockout mice (*mcl-1^{fllox/fllox} bcl-x^{fllox/fllox} bak^{-/-} bax^{fllox/fllox} pf4-Cre*) by mating the strains. They were maintained in a specific pathogen-free facility. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of Osaka University Medical School. All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Hematological analyses. Blood was collected from the inferior vena cava of mice. Complete blood cell counts were determined using Automated Cell Counter (Sysmex, Kobe, Japan).

Histological analyses. For immunohistochemistry analyses, femurs and embryonic livers were excised and fixed overnight in 4% paraformaldehyde. The femurs were then decalcified in 20% EDTA for 2 h. BM sections or embryonic liver sections were stained with HE or VWF antibody (Dako, Grostrup, Denmark). To detect apoptotic cells, the BM sections were also subjected to TUNEL staining, according to a previously reported procedure.¹⁵ For immunocytochemistry, BM cells suspended in PBS were spun down (5 × 10⁵ cells/slide) on microscope slides using a Cytospin4 (Thermo Shandon Inc., Pittsburgh, PA, USA) for 6 min at 600 × g. The cells were air-dried and fixed in 4% paraformaldehyde for 20 min at 4°C. Slides were then permeabilized with 0.2% Triton X-100 in TBS for 10 min at room temperature and stained with Mcl-1 antibody (Abcam, Cambridge, MA, USA). After washing with TBS, the slides were incubated with Alexa Fluor 555-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA,

USA), followed by incubation with FITC-conjugated CD41 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Flow cytometric analysis of reticulated platelets. The proportion of reticulated platelets was examined by a previously described procedure.¹² Briefly, 1 μl of whole blood collected from the inferior vena cava of C57BL/6J mice was mixed with 50 μl Thiazole Orange (TO) (0.1 μg/ml) (Invitrogen, Carlsbad, CA, USA), 0.25 μl APC-conjugated CD41 antibody (0.5 μg/ml) (BD Pharmingen, San Diego, CA, USA) and 9 μl PBS, and incubated in the dark at room temperature for 15 min. Next, the samples were fixed by addition of 1 ml of PBS containing 1% paraformaldehyde and analyzed with a Becton Dickinson FACS Canto II flow cytometer (BD Pharmingen).

Flow cytometric analysis of Annexin V positivity of reticulated platelets. Whole blood, 1 μl, collected from the inferior vena cava of C57BL/6J mice was mixed with 50 μl TO (0.1 μg/ml) (Invitrogen), 0.25 μl APC-conjugated CD41 antibody (0.5 μg/ml) (BD Pharmingen), 5 μl Pacific Blue-conjugated Annexin V antibody (BD Pharmingen) and 9 μl PBS, and incubated in the dark at room temperature for 15 min. Samples were diluted with 400 μl of Annexin V binding buffer (BD Pharmingen) and analyzed with a Becton Dickinson FACS Canto II flow cytometer (BD Pharmingen) within 1 h after staining.

Flow cytometric analysis of megakaryocyte ploidy. BM was harvested from the femurs and tibias and flushed into 10 ml of PBS containing 0.38% sodium citrate and 2.5% BSA. The cell suspension was centrifuged at 400 × g for 4 min at RT, resuspended with 1 ml of PBS containing 0.38% sodium citrate and 2.5% BSA, and incubated with an antibody to CD16 and CD32 (BD Pharmingen) for 15 min to block non-specific bindings of the subsequently introduced antibody. The cells were then stained with FITC-conjugated CD41 antibody (BD Pharmingen) for 30 min at 4°C and washed twice. After centrifugation at 400 × g for 4 min at 4°C, the cells were resuspended with 300 μl PBS, fixed with 700 μl 100% cold methanol for 30 min at 4°C and washed twice. After centrifugation at 400 × g for 4 min at 4°C, the cells were incubated with 9 μg/ml propidium iodide (BD Pharmingen) and 200 μg/ml RNase A (Qiagen, Valencia, CA, USA) for 30 min at 4°C and washed once. After centrifugation at 400 × g for 4 min at 4°C, the cells were resuspended with PBS and then analyzed with a Becton Dickinson FACS Canto II flow cytometer (BD Pharmingen).

In-vivo ABT-737 experiment. ABT-737 was dissolved with a mixture of 30% propylene glycol, 5% Tween 80 and 65% D5W (5% dextrose in water), pH 4–5. ABT-737 (100 mg/kg) was intraperitoneally administered to the Mcl-1 knockout mice (*mcl-1^{fllox/fllox} pf4-cre*) or Mcl-1/Bak/Bax triple knockout mice (*mcl-1^{fllox/fllox} bak^{-/-} bax^{fllox/fllox} pf4-cre*) or each group of control littermates (*mcl-1^{fllox/fllox}* or *mcl-1^{fllox/fllox} bak^{-/-} bax^{fllox/fllox}*, respectively).

In-vivo APS experiment. Mice were given intraperitoneal injection of 200 μl rabbit anti-mouse platelet serum (Inter Cell Technologies) at 1:40 dilution and killed according to the indicated time courses.

Enzyme-linked immunosorbent assay (ELISA). Mouse serum TPO levels were measured by using the Quantikine Mouse TPO immunoassay kit (R&D Systems) according to manufacturer's protocol.

siRNA-mediated knockdown. CMK cells were transfected with siRNA against *MCL1* (Stealth select RNAi siRNA, Oligo ID: HSS181043) (Invitrogen) by

Figure 4 Bak/Bax-dependent apoptotic loss of mature megakaryocytes and hemorrhagic anemia in the ABT-737-treated Mcl-1 knockout mice. (a–g) ABT-737 (100 mg/kg) was intraperitoneally administered to *mcl-1^{fllox/fllox} pf4-Cre* and *mcl-1^{fllox/fllox}* mice. Mcl-1 +/+ and Mcl-1 -/- stand for *mcl-1^{fllox/fllox}* and *mcl-1^{fllox/fllox} pf4-Cre* mice, respectively. Mature megakaryocyte counts in the BM at indicated time courses; > 3 mice per group; *P < 0.05 (a). Representative images of HE (upper) and VWF (bottom) staining of the BM 7 h after ABT-737 administration (original magnification: upper × 200, lower × 200) (b). Representative images of TUNEL staining of the BM 4.5 h after ABT-737 treatment (original magnification: × 400) (c). TUNEL-positive cell ratio of morphologically recognizable megakaryocytes in the BM 4.5 h after ABT-737 treatment; five mice per group (d). Circulating platelet counts for the indicated time courses; > 3 mice per group; *P < 0.05 (e). Red blood cell counts 48 h after ABT-737 administration; six mice per group (f). Representative images of HE staining of the stomach 48 h after ABT-737 administration (original magnification: × 200) (g). (h–k) Offspring from mating of *mcl-1^{fllox/+} bak^{-/-} bax^{fllox/fllox} pf4-cre* mice and *mcl-1^{fllox/+} bak^{-/-} bax^{fllox/fllox}* mice were given intraperitoneal injection of ABT-737 (100 mg/kg) and killed 24 h later. Bak -/- stands for *mcl-1^{fllox/fllox} bak^{-/-} bax^{fllox/fllox}* mice or *mcl-1^{fllox/+} bak^{-/-} bax^{fllox/fllox}* mice. Bak -/- Bax -/- stands for *mcl-1^{fllox/+} bak^{-/-} bax^{fllox/fllox} pf4-cre* mice. Mcl-1 -/- Bak -/- Bax -/- stands for *mcl-1^{fllox/fllox} bak^{-/-} bax^{fllox/fllox} pf4-cre* mice. Circulating platelet counts; > 3 mice per group, *P < 0.05 versus all (h). Representative images of HE and VWF staining of the BM (original magnification: × 200) (i). Mature megakaryocyte counts in the BM; > 3 mice per group (j). Red blood cell counts; > 3 mice per group (k)

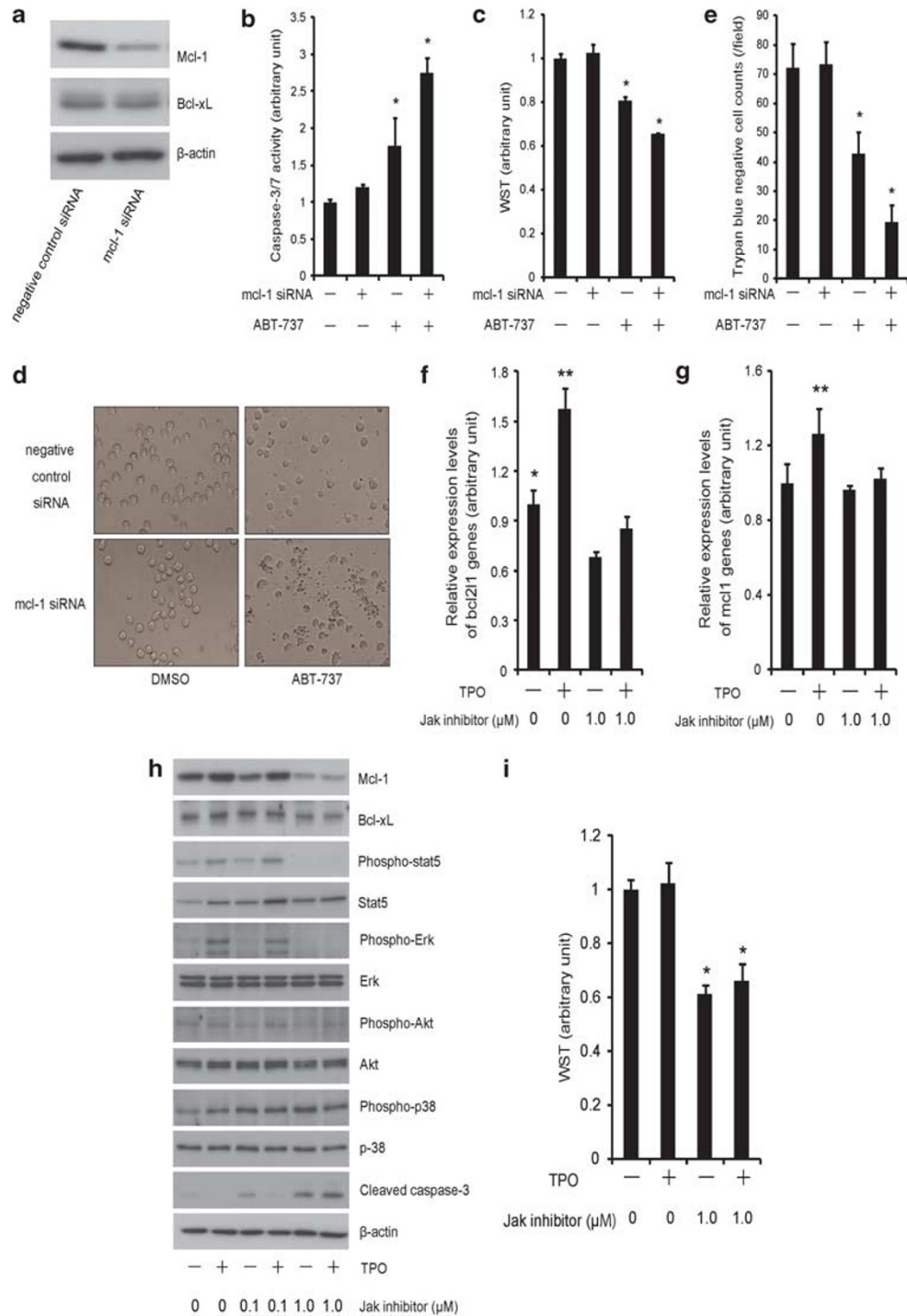


Figure 5 TPO/Jak signaling positively regulates both Mcl-1 and Bcl-xL in human megakaryoblastic cells. (a) CMK cells were transfected with *mcl-1* siRNA or negative control siRNA for 2 days by electroporation procedure. Western blot analysis for Mcl-1 and Bcl-xL protein. (b and c) CMK cells were transfected with *mcl-1* siRNA or negative control siRNA for 2 days and then cultured with 0.5 μ M ABT-737 or vehicle for 4 h. Caspase-3/7 activity of the cultured supernatant ($N=4$ /group, $*P<0.05$ versus all) (b). WST assay ($N=5$ /group, $*P<0.05$ versus all) (c). (d and e) CMK cells were transfected with *mcl-1* siRNA or negative control siRNA for 3 days and then cultured with 0.3 μ M ABT-737 or vehicle for 4 h. Representative images of Macro findings (original magnification: $\times 100$) (d). Trypan blue negative cell counts ($N=5$ /group, $*P<0.05$ versus all) (e). (f and g) CMK cells were treated with human TPO (50 ng/ml) and/or Jak inhibitor (1.0 μ M) for 6 h. mRNA levels of *bcl-x* (f) and *mcl-1* (g) genes were assessed by real-time RT-PCR ($N=4$ /group, $*P<0.05$ versus TPO or Jak inhibitor group, $**P<0.05$ versus all). (h) CMK cells were treated with human thrombopoietin (TPO) (50 ng/ml) and/or Jak inhibitor at the indicated dosage for 8 h. Western blot analysis for Mcl-1, Bcl-xL, cleaved caspase-3, Stat5, phospho-Stat5, Akt, phospho-Akt, Erk, phospho-Erk, p38 and phospho-p38 protein. (i) CMK cells were treated with TPO (50 ng/ml) and/or Jak inhibitor (1.0 μ M) for 12 h. WST assay ($N=5$ /group, $*P<0.05$ versus without Jak inhibitor groups)

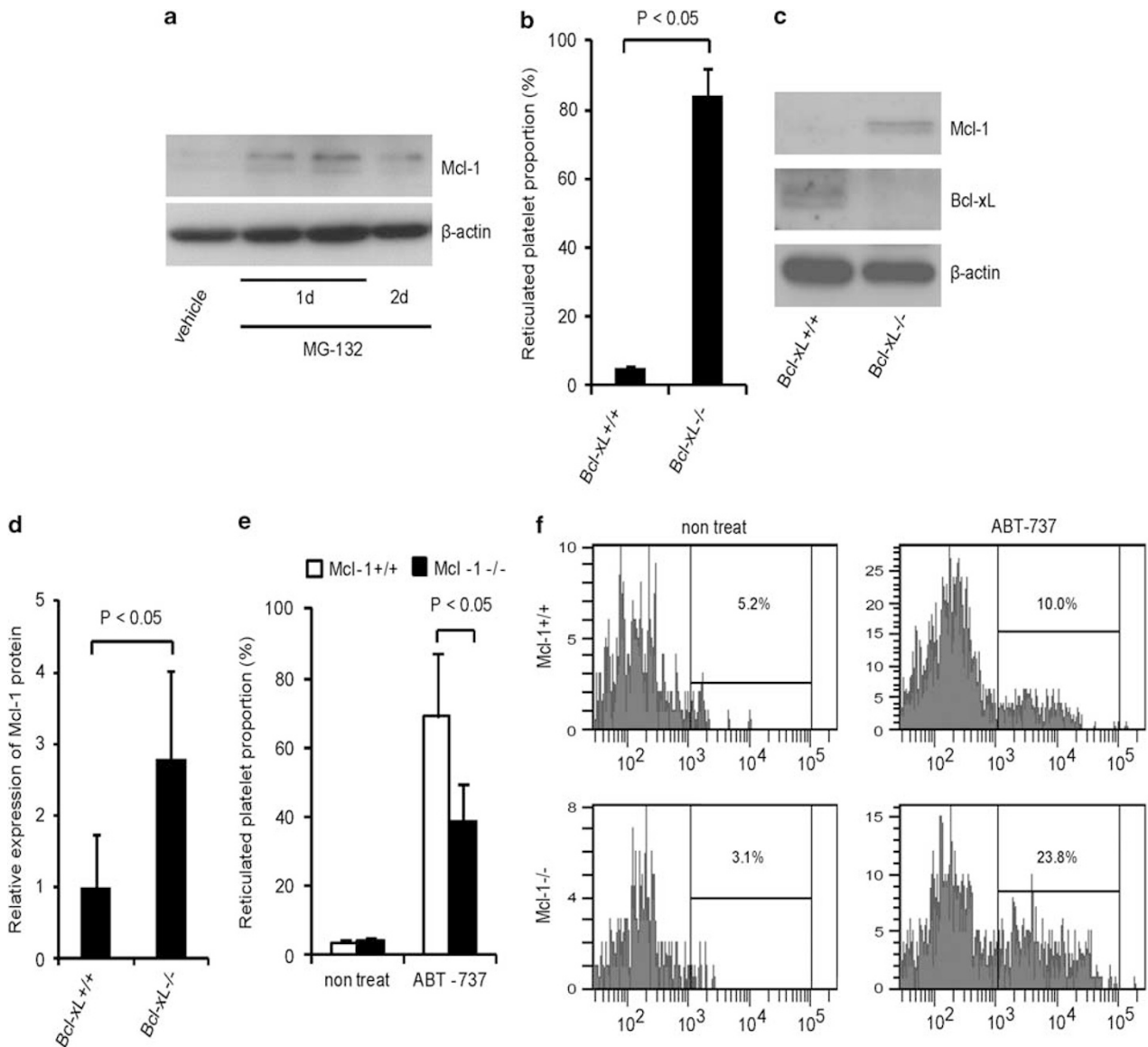


Figure 6 Platelet survival is dependent on Bcl-xL due to proteasomal degradation of Mcl-1, while both are involved in the survival of reticulated platelets. (a) MG-132 (20 mg/kg) or vehicle was intraperitoneally administered to C57BL/6J mice. Western blot for Mcl-1 and β -actin protein in isolated platelets 1 or 2 days after MG-132 or vehicle administration. (b) Reticulated platelet proportion of *bcl-x^{fllox/fllox}pf4-Cre* and *bcl-x^{fllox/fllox}* mice was determined by staining with thiazole orange; five mice per group, *Bcl-xL +/+* and *Bcl-xL -/-* stand for *bcl-x^{fllox/fllox}* and *bcl-x^{fllox/fllox}pf4-Cre* mice, respectively. (c and d) Western blot for Mcl-1, Bcl-xL and β -actin protein in platelets isolated from *bcl-x^{fllox/fllox}pf4-Cre* and *bcl-x^{fllox/fllox}* mice. *Bcl-xL +/+* and *Bcl-xL -/-* stand for *bcl-x^{fllox/fllox}* and *bcl-x^{fllox/fllox}pf4-Cre* mice, respectively. Representative blots of three independent experiments are shown (c). Relative expression of Mcl-1 protein was calculated as the optical densities of the Mcl-1 blots normalized with the β -actin blots (d). (e and f) *mcl-1^{fllox/fllox}pf4-Cre* and *mcl-1^{fllox/fllox}* mice were given intraperitoneal injection of ABT-737 (100 mg/kg) and analyzed 6 h later. *Mcl-1 +/+* and *Mcl-1 -/-* stand for *mcl-1^{fllox/fllox}* and *mcl-1^{fllox/fllox}pf4-Cre* mice, respectively. The reticulated platelet proportion was determined by staining with thiazole orange, three mice per group; statistical analysis was performed using Mann-Whitney's *U*-test (e). Apoptosis of reticulated platelets (CD41⁺thiazole orange⁺) was determined by staining with Annexin V, three mice per group; representative histograms with positive cell ratio are shown (f)

electroporation procedure. Stealth RNAi negative control Med GC (Invitrogen) was used as the control.

In-vivo MG-132 experiment. MG-132 was purchased from Sigma-Aldrich (St. Louis, MO, USA). MG-132 (20 mg/kg) was intraperitoneally administered to C57BL/6J mice.

WST assay. The WST assay was performed with a cell proliferation kit (CellTiter 96 AQ_{UEOUS}, Promega, Tokyo, Japan) according to manufacturer's protocol. Upon addition of MTS solution, the reaction plate was incubated at 37°C

for 1 h then the absorbance was read at 490 nm using a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Caspase-3/7 activity. Supernatant caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega) according to manufacturer's protocol.

Western blot analysis. Western blot was performed as previously described.⁴¹ For immunodetection, the following antibodies were used: rabbit polyclonal antibody to stat5 (Santa Cruz Biotechnology), rabbit polyclonal antibody

to phospho-stat5 (Tyr694) (Cell Signaling Technology), rabbit polyclonal antibody to Akt (Cell Signaling Technology), rabbit polyclonal antibody to phospho-Akt (Thr308) (Cell Signaling Technology), rabbit polyclonal antibody to Erk (Cell Signaling Technology), rabbit polyclonal antibody to phospho-Erk (Thr202/Tyr204) (Cell Signaling Technology), rabbit polyclonal antibody to p38 (Cell Signaling Technology), rabbit polyclonal antibody to phospho-p38 (Tyr182) (Cell Signaling Technology), rabbit polyclonal antibody to cleaved caspase-3 (Cell Signaling Technology), rabbit polyclonal antibody to Bcl-xL (Santa Cruz Biotechnology), rabbit polyclonal antibody to Mcl-1 (Rockland, Gilbertsville, PA, USA), rabbit polyclonal antibody to Mcl-1 (Santa Cruz Biotechnology), mouse monoclonal antibody to β -actin (Sigma-Aldrich), rabbit polyclonal antibody to Bax (Cell Signaling Technology), and rabbit polyclonal antibody to Bak (Millipore, Billerica, MA, USA). Protein expression levels were quantified using ImageJ 1.40 software (NIH, Bethesda, MD, USA).

Real-time reverse transcription PCR for mRNA. Total RNA extracted from CMK cells using the RNeasy mini kit (Qiagen) was reverse-transcribed and subjected to real-time reverse transcription PCR (real-time RT-PCR) as previously described. mRNA expression of the specific genes was quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) as follows: homo sapiens BCL2L1 (Assay ID: Hs99999146_m1), homo sapiens MCL1 (Assay ID: Hs03043899_m1) and homo sapiens ACTB (Assay ID: Hs999903_m1). Transcript levels are presented as fold induction.

Statistical analysis. Data are expressed as mean \pm S.D. unless otherwise indicated. Statistical analyses were performed by unpaired Student *t*-test, Mann-Whitney's *U*-test or one-way ANOVA. When ANOVA analyses were applied, differences in the mean values among the groups were examined by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

TK and TT designed the study and wrote the paper. HH, TK, MS, YH, WL and TM performed the mouse analyses. KK, ST and YT performed the *in-vitro* experiments and provided experimental advice. SS and TT performed the *in-vitro* experiments. AH, TK, NH and NH interpreted the data and provided experimental advice.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)