Megakaryocytes possess a functional intrinsic apoptosis pathway that must be restrained to survive and produce platelets

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Abbreviations used: BMC, BM cell; Fasl., Fas ligand; FLc, fetal liver cell; MPV, mean platelet volume; PDW, platelet distribution width; PRP, platelet-rich plasma; STS, staurosporine.

It is believed that megakaryocytes undergo a specialized form of apoptosis to shed platelets. Conversely, a range of pathophysiological insults, including chemotherapy, are thought to cause thrombocytopenia by inducing the apoptotic death of megakaryocytes and their progenitors. To resolve this paradox, we generated mice with hematopoietic- or megakaryocyte-specific deletions of the essential mediators of apoptosis, Bak and Bax. We found that platelet production was unperturbed. In stark contrast, deletion of the pro-survival protein Bcl-xL resulted in megakaryocyte apoptosis and a failure of platelet shedding. This could be rescued by deletion of Bak and Bax. We examined the effect on megakaryocytes of three agents that activate the intrinsic apoptosis pathway in other cell types: etoposide, staurosporine, and the BH3 mimetic ABT–737. All three triggered mitochondrial damage, caspase activation, and cell death. Deletion of Bak and Bax rendered megakaryocytes resistant to etoposide and ABT–737. In vivo, mice with a Bak−/− Bax−/− hematopoietic system were protected against thrombocytopenia induced by the chemotherapeutic agent carboplatin. Thus, megakaryocytes do not activate the intrinsic pathway to generate platelets; rather, the opposite is true: they must restrain it to survive and progress safely through proplatelet formation and platelet shedding.

Megakaryocytes are specialized mammalian cells responsible for the production of blood platelets. The latter are generated by an extraordinary feat of cellular remodeling (Italiano et al., 1999; Patel et al., 2005; Junt et al., 2007). Massive cytoskeletal rearrangements drive the formation of cytoplasmic extensions called proplatelets, structures along which large-scale trafficking of granules and organelles occurs. These components are packaged into nascent platelets, which are then released from the BM into the circulation. Effectively the entire cytoplasm of a megakaryocyte is converted in this manner, each giving rise to hundreds, or potentially thousands, of platelets. It is widely held that to produce platelets, megakaryocytes deliberately activate apoptosis (Kaluzhny and Ravid, 2004; Fadeel and Orrenius, 2005; Patel et al., 2005; Siegel, 2006; Galluzzi et al., 2008; Solary et al., 2008). A considerable
body of work supports this notion, most of it centered on
the intrinsic (or mitochondrial) apoptosis pathway. Mice car-
rying mutations that impair apoptosis—by overexpression of
prosurvival Bcl-2 (Ogilvy et al., 1999) or deletion of the
proapoptotic Bcl-2 family member Bin (Bouillet et al.,
1999)—exhibit reduced circulating platelet counts (thrombo-
cytopenia). Overexpression of another close relative of pro-
survival Bcl-2, Bcl-xL, impairs proplatelet formation by cultured
megakaryocytes (Kaluzhny et al., 2002). Similarly, pharma-
cological inhibition of caspases, the proteolytic enzymes re-
ponsible for dismantling dying cells, blocks the generation of
proplatelets (De Botton et al., 2002; Clarke et al., 2003).

However, the precise requirement for apoptosis in platelet
shedding has not been fully clarified. It is not understood
how a megakaryocyte can undergo apoptosis and yet produce
viable platelets that circulate in the bloodstream for days. Fur-
thermore, the idea that apoptosis is required for platelet pro-
duction is inconsistent with evidence that a range of insults,
including chemotherapeutic agents, autoantibodies, and viruses,
cause thrombocytopenia by inducing the apoptotic death of
megakaryocytes and their progenitors (Ballem et al., 1987;
Zucker-Franklin et al., 1989; Zauhli et al., 1996; Chang et al.,
2003; Houwerzijl et al., 2004; McMillan and Nugent, 2005;
Zeuner et al., 2007).

Given the postulated role of the intrinsic apoptosis path-
way in platelet production, we analyzed the consequences of
blocking or activating apoptosis in megakaryocytes, using
both genetic and pharmacological approaches. The intrinsic
pathway to apoptosis is regulated by the Bcl-2 protein family
(Youle and Strasser, 2008). The key mediators of this process
are Bak and Bax, which if unrestrained, trigger mitochondrial
outer membrane permeabilization (MOMP), an irreversible
step toward apoptotic cell death (Green and Kroemer, 2004).

Combined deficiency of Bak and Bax renders multiple cell
populations insensitive to apoptosis (Lindsten et al., 2000; Cheng et al.,
2001). Selective deletion of Bak and Bax within the megakaryocyte
lineage, we were able to fully evaluate the consequences of
blocking apoptosis on megakaryocyte biology both in vitro
and in vivo. Much to our surprise, loss of Bak and Bax had no
impact upon platelet production, even though platelet life
span, as anticipated (Mason et al., 2007), was prolonged.

Normally, Bak and Bax are kept in check, either directly
or indirectly, by the prosurvival Bcl-2 family members; Bcl-2
itself, or close relatives such as Bcl-xL (Willis et al., 2007;
Ren et al., 2010). We found that selectively inactivating
prosurvival Bcl-xL triggered megakaryocyte apoptosis and
failure of platelet production. Three agents that activate the
intrinsic apoptosis pathway in other cell types, etoposide, stauro-
sporine (STS), and the BH3 mimetic ABT-737, all triggered
mitochondrial damage, caspase activation and cell death in
megakaryocytes in vitro. Deletion of Bak and Bax blocked
the response to etoposide and ABT-737, but not STS. In vivo,
loss of Bak and Bax rescued the defects caused by deletion of
Bcl-xL, and ameliorated the thrombocytopenia caused by the
chemotherapeutic agent carboplatin. Thus, our studies

overturn the notion that classical mitochondrial apoptosis is
essential for platelet production. Instead, megakaryocytes must
restrain apoptosis to survive and progress safely through pro-
platelet formation and platelet shedding. This striking finding
has important implications for our understanding of human
diseases where the apoptotic death of megakaryocytes has
been implicated.

RESULTS
Platelet number and life span are increased in the absence
of Bak and Bax
To determine the role of the intrinsic apoptosis pathway in
platelet production, we generated mice with a constitutive
deletion of Bak and a megakaryocyte-specific deletion of Bax.
Bak−/− animals were crossed with mice harboring a floxed
allele of Bax (Baxflfl; Takeuchi et al., 2005) and a Pf4-Cre trans-
gene from which Cre recombinase is expressed under the control of the Platelet factor 4 promoter (Tiedt et al., 2007). The latter mediates efficient deletion of floxed alleles from the earliest stages of megakaryocytic differentiation (Hitchcock et al., 2008; Pleines et al., 2010; Tiedt et al., 2007). At weaning, Bak−/− Baxflfl;Pf4Cre−/− mice were present in the expected Mendelian ratios and were outwardly healthy and fertile. Western blot analysis of platelet and megakaryocyte lysates confirmed that Bak and Bax were efficiently deleted from the lineage (Fig. 1 a).

Consistent with the initial study (Mason et al., 2007), we
found that peripheral blood platelet counts in Bak−/− mice were significantly increased relative to wild-type counter-
parts (Fig. 1 b). Surprisingly, with the additional loss of Bax,
platelet numbers remained elevated. Given the role these
two proteins play in the regulation of platelet life span, we
examined the kinetics of platelet clearance in vivo. Deletion
of Bax had a minor effect, with a modest but statistically sig-
nificant extension of platelet survival apparent (Fig. 1 c). In
contrast, and as previously documented (Mason et al., 2007),
platelet life span in Bak−/− mice was almost doubled. This
was also true of Bak−/− Baxfl/+;Pf4Cre−/+ animals; in fact, platelet survival curves in Bak−/− Baxfl/+;Pf4Cre−/+ and
Bak−/− Baxflfl;Pf4Cre−/− were indistinguishable. Transfusion of biotinylated platelets into wild-type recipients
established that life span in all cases was independent of host genotype, i.e., cell intrinsic (Fig. S1 d). These data con-
firmed that at steady state, Bak is the critical factor limiting
platelet production in vivo.

Megakaryopoiesis is normal in the absence of Bak and Bax
Given the platelet data, we examined the megakaryocyte
compartment in detail. Megakaryocyte numbers in the
BM, as determined by histological (Fig. 1 d) and flow cyto-
nometric analyses (unpublished data) were comparable across all
genotypes. Megakaryocyte ploidy (Fig. 1 e), and serum
thrombopoietin (TPO) levels (Fig. 1 f) were unchanged in
Bak−/− BakPf4−/− mice. Semisolid agar cultures of BM cells (BMCS) and splenocytes demonstrated that committed myeloid (Bak−/− Bak+/+, 87.5 ± 15.2; Bak−/− BakPf4−/−, 70.3 ± 7.3) and megakaryocyte progenitor numbers (Bak−/+ Bak+/+, 21.3 ± 7.1; Bak−/− BakPf4−/−, 16.3 ± 0.5) were normal. Next, we derived mature megakaryocytes from the BM of adult Bak−/− BakPf4−/− mice and assessed their ability to form proplatelets. To circumvent any potential issues relating to the timing of Pf4-Cre-mediated excision of Bak, megakaryocytes were also derived from fetal liver cells (FLC) harvested from constitutive Bak−/− BakPf4−/− mice (Fig. 2 a). No decrease in the rate of proplatelet formation was observed in either case (Fig. 2 a and not depicted). Interestingly, proplatelet-bearing Bak−/− and Bax-deficient megakaryocytes exhibited increased survival, persisting with proplatelets intact for several days longer than wild-type counterparts (Fig. 2, a and b).

Bak- and Bax-mediated apoptosis is not required for platelet production

Collectively, our data indicated that there is no requirement for Bak- and Bax-mediated apoptosis in the production of platelets by megakaryocytes. To rule out the possibility that this is true only at steady state, we tested the ability of Bak−/− BakPf4−/− mice to recover from acute thrombocytopenia induced by antiplatelet serum (APS), 24 h after a single dose of APS, platelet counts had fallen to almost undetectable levels (Fig. 2 c). Subsequently, each genotypic class mounted a robust recovery of similar kinetics, with platelet counts exceeding baseline by 6 d after APS (Fig. 2 c). Interestingly, visual inspection of BM sections taken from mice during the recovery phase revealed an abundance of naked megakaryocyte nuclei (Fig. 2, d and e). This phenomenon was significantly increased in Bak−/− BakPf4−/− mice relative to wild-type or Bak−/− Baxfl/fl counterparts. Although less pronounced, the difference was also observed at steady state.

Given the surprising nature of our results, we elected to confirm them by generating chimeric animals in which the hematopoietic system was reconstituted with constitutive knockout Bak−/− Bak−/− FLC (Fig. S1). These animals develop a complex disease characterized by an accumulation of lymphocytes in the peripheral blood, BM, and spleen (Rathmell et al., 2002; Fig. S1 e). In our hands, Bak−/− Bak−/− reconstituted mice developed splenomegaly, with spleens weighing 2.5 times more than wild type at 8 wk after transplant (Fig. S1 f). Therefore, we splenectomized a group of wild-type C57BL/6 CD45.1 recipients, and 4 wk later transplanted them with FLC. 8 wk after reconstitution, platelet counts and platelet life span in mice that had received Bak−/− Bak−/− FLC were the same as those seen in Bak−/− and Bax−/−/− FLC animals (Fig. 2 f and Fig. S1, b and c). The response to APS-induced thrombocytopenia was normal (Fig. 2 i). These data demonstrate that at both steady state and under conditions of thrombopoietic stress, the intrinsic mitochondrial apoptosis pathway is dispensable for platelet production.

Bcl-xL is required for platelet survival

Despite the fact that they express Bak and Bax, and a range of other apoptotic regulators (Krajewska et al., 1994; Sanz et al., 2001; Fig. S2 i), our results raised the question of whether megakaryocytes possess a functional intrinsic apoptosis pathway at all. If megakaryocytes are susceptible to activation of the intrinsic pathway to apoptosis, then one would expect that one or more members of the Bcl-2 family of prosurvival proteins must keep Bak and Bax in check to maintain cellular integrity. Given the known role of one such prosurvival, Bcl-xL, in regulating platelet life span (Mason et al., 2007), and given its documented expression in megakaryocytes (Terui et al., 1998; Sanz et al., 2001; Kozuma et al., 2007), we generated a megakaryocyte-specific deletion of Bcl-xL. Mice harboring a
littermates. The life span of the vast majority of labeled platelets were labeled in wild-type mice, but only 20% in body derivative. 15 min after treatment, with a fluorescently labeled platelet-specific anti-GPIb with a fluorescently labeled platelet-specific anti-GPIb b). Only 20–30% of the cells present in PRP expressed the creased phosphatidylserine (PS) exposure (Fig. 3 b and Fig. S2 b). To investigate circulating platelet life span, we intravenously injected mice with a fluorescently labeled platelet-specific anti-GPIbβ antibody derivative. 15 min after treatment, ~95% of platelets were labeled in wild-type mice, but only 20% in Bcl-xPf4/−/Pf4−/− littermates. The life span of the vast majority of labeled platelets was dramatically reduced to ~5 h, versus 5 d for wild-type and Bcl-xPfl/fl mice (Fig. 3 c). A very small number of platelets survived in the circulation up to 24 h after labeling (Fig. S2, c and d), potentially the product of megakaryocytes in which Bcl-xL had not been completely excised by the Cre enzyme. Collectively, the data confirm that Bcl-xL is absolutely required for platelet survival in vivo.

**Bcl-xL is required for normal platelet shedding**

The abnormal morphology and cell surface phenotype of Bcl-xPfl/fl/−/− platelets suggested that platelet production had been perturbed by the loss of the Bcl-xL in megakaryocytes. Mathematical modeling of production rates further supported the notion that the reduction in platelet life span could not explain the severity of thrombocytopenia (Fig. 3 d). Relative to Bcl-xPfl/fl littermates, megakaryocyte numbers were significantly increased in both the BM and spleen of Bcl-xPfl/fl/−/− mice (Fig. 3 e, Fig. S2 e, and not depicted). Megakaryocyte ploidy was skewed toward 32N (Fig. 3 f). Megakaryocyte progenitor numbers were somewhat increased in Bcl-xPfl/fl/−/− mice (Fig. 3 g), and serum TPO levels were significantly reduced (Fig. 3 h). Collectively, these data were reminiscent of...
those from mice lacking the transcription factor NF-E2, which although able to generate mature megakaryocytes, suffer a profound defect in platelet shedding and severe thrombocytopenia (Shivdasani et al., 1995; Lecline et al., 1998; Levin et al., 1999).

To elucidate the consequences of Bcl-x deletion on megakaryocyte development, we cultured Bcl-x<sup>fl/fl</sup> and Bcl-x<sup>Pf4</sup>/Pf4<sup>fl/fl</sup> FLC in serum-free media with TPO. To control for any variability in the deletion of the floxed allele of Bcl-x, FLC from mice homozygous for a constitutive knockout allele, denoted Bcl-x<sup>−/−</sup> (Motoyama et al., 1995), were cultured in parallel. Bcl-x<sup>−/+</sup>, Bcl-x<sup>+/+</sup>, Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup>, and Bcl-x<sup>−/−</sup>/Pf4<sup>−/−</sup> FLC cultures produced similar numbers of megakaryocytes (Fig. 3 i). This agreed with our in vivo data, confirming that Bcl-x<sub>L</sub> is not required for the development and growth of megakaryocytes. We next assessed the ability of mature megakaryocytes to generate proplatelets. In striking contrast to gradient-purified Bcl-x<sup>−/+</sup> and Bcl-x<sup>+/+</sup> megakaryocytes, Bcl-x<sup>−/−</sup> and Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> cells exhibited an almost total failure of proplatelet formation (Fig. 4, a and b). This was accompanied by a rapid loss of viability.

To investigate these phenomena more closely, time-lapse video microscopy of mature megakaryocytes in culture was performed (Fig. 4 c and Videos 1 and 2). Bcl-x<sup>−/−</sup> megakaryocytes sent out long extensions and formed elaborate proplatelets. In contrast, Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> megakaryocytes generated extended extensions, but they were significantly fewer, much shorter, and less detailed. Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> megakaryocytes died soon after they had formed these short extensions. To visualize phosphatidylserine exposure, Alexa Fluor 488–conjugated Annexin V was included in the media (Fig. 4 d and Videos 3 and 4). Minimal PS was observed on Bcl-x<sup>−/+</sup> megakaryocytes as they proceeded through proplatelet formation. However, several hours after the process appeared to be complete, proplatelet-bearing megakaryocytes were seen to collapse in on themselves and stain intensely positive for Annexin. Blood samples were collected 0.25, 1, 3, and 5 h after labeling. Data represent mean ± SEM. n = 8 mice for Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup>; n = 4 for wild type; n = 3 for Bcl-x<sup>−/−</sup>; n = 2 for Bcl-x<sup>−/−</sup>. Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup>, P < 0.0001 at 5 h.

(d) Calculated platelet production rates in wild-type and Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> mice as described in Materials and methods. P < 0.0001, two-tailed unpaired Student’s t test. (e) Morphologically recognizable MGKs in H&E-stained sections of BM from Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> mice. Each symbol represents mean number per field of view (200x) from five fields per mouse. Data represent overall mean ± SEM. (f) Ploidy distribution profile of CD41<sup>−/−</sup> BMCs. Data represent mean ± SEM. n = 6 mice per genotype. (g) Hematopoietic progenitor cell numbers in adult mice. 25,000 BMCs were cultured with stem cell factor, IL-3, and erythropoietin in semisolid agar for 7 d. Non-MGK colonies represent the total of blast, granulocyte, mixed granulocyte/macrophage, macrophage, and eosinophil colonies. Data represent mean ± SEM. n = 7–11 mice per genotype. **, P = 0.0021.

(h) Serum TPO levels. Each symbol represents an individual mouse. Data represent mean ± SEM. ***, P < 0.0001. (l) MGK differentiation from mouse FLC E12.5 Bcl-x<sup>−/−</sup> and Bcl-x<sup>−/−</sup>/Pf4<sup>−/−</sup> and E13.5 Bcl-x<sup>−/−</sup> and Bcl-x<sup>Pf4</sup>/Pf4<sup>−/−</sup> fetal livers were cultured in TPO for 3–5 d and the development of CD41<sup>−/−</sup> cells measured by flow cytometry. Data represent mean ± SD. n = 3–5 biological replicates (except day 3, n = 2). **, P < 0.005; ***, P < 0.0001.
Bcl-x<sup>+/−</sup> and Bcl-x<sup<y4Δ/Δ</sup> BM sections. As expected, many megakaryocytes displaying normal ultrastructure were observed in the knockout (Fig. 5 a, bottom left). However, strikingly abnormal cells, projecting large vacuolated fragments of cytoplasm into the sinusoidal spaces were also evident (Fig. 5 a, bottom middle; and Fig. S3). Discrete fragments that had been shed were seen in the circulation; they appeared to be highly vacuolated and morphologically abnormal (Fig. 5 a, bottom right; and Fig. S3). These observations were consistent with late-stage megakaryocyte death, but did not absolutely prove an intrinsic physiological dependence upon Bcl-x<sub>L</sub>, given the associated dramatic reduction in platelet life span and platelet number exhibited by Bcl-x<sup>y4Δ/Δ</sup> mice.

To establish whether megakaryocytes lacking Bcl-x<sub>L</sub> could produce platelets normally in the absence of severe thrombocytopenic stress, we generated BM chimeras by transplanting 50:50 ratios of Bcl-x<sup>+/+</sup> or Bcl-x<sup>y4Δ/Δ</sup> BMCs with wild-type GFP-expressing Bcl-x<sup>fl/fl</sup> and Bcl-x<sup>y4Δ/Δ</sup> BM sections. As expected, many megakaryocytes displaying normal ultrastructure were observed in the knockout (Fig. 5 a, bottom left). However, strikingly abnormal cells, projecting large vacuolated fragments of cytoplasm into the sinusoidal spaces were also evident (Fig. 5 a, bottom middle; and Fig. S3). Discrete fragments that had been shed were seen in the circulation; they appeared to be highly vacuolated and morphologically abnormal (Fig. 5 a, bottom right; and Fig. S3). These observations were consistent with late-stage megakaryocyte death, but did not absolutely prove an intrinsic physiological dependence upon Bcl-x<sub>L</sub>, given the associated dramatic reduction in platelet life span and platelet number exhibited by Bcl-x<sup>y4Δ/Δ</sup> mice.

Table 1. Blood counts in mice lacking Bcl-x<sub>L</sub> in the megakaryocyte lineage

<table>
<thead>
<tr>
<th></th>
<th>Bcl-x&lt;sup&gt;fl/fl&lt;/sup&gt;</th>
<th>Bcl-x&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Bcl-x&lt;sup&gt;y4Δ/Δ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>1,139 ± 150</td>
<td>1,163 ± 170</td>
<td>697 ± 100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelets (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>939 ± 92</td>
<td>731 ± 91</td>
<td>73 ± 8</td>
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<td>MPV (femtoliters)</td>
<td>7.7 ± 0.6</td>
<td>7.1 ± 0.9</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>53.2 ± 4.2</td>
<td>51.2 ± 5.6</td>
<td>52.3 ± 7.1</td>
</tr>
<tr>
<td>Erythrocytes (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>10.7 ± 0.4</td>
<td>10.7 ± 0.5</td>
<td>10.7 ± 0.5</td>
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<tr>
<td>Hematocrit (%)</td>
<td>50.8 ± 1.7</td>
<td>51.1 ± 2.2</td>
<td>51.4 ± 2.3</td>
</tr>
<tr>
<td>Leukocytes (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>10.5 ± 1.7</td>
<td>9.4 ± 2.0</td>
<td>10.2 ± 2.4</td>
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<tr>
<td>Neutrophils (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
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<tr>
<td>Lymphocytes (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>9.1 ± 1.5</td>
<td>8.0 ± 1.8</td>
<td>8.8 ± 2.3</td>
</tr>
<tr>
<td>Monocytes (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>Eosinophils (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>Erythrocytes (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
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Peripheral blood cell counts from mice at 7 wk of age. MPV, mean platelet volume; PDW, platelet distribution width. Data represent mean ± SD.

<sup>a</sup>Platelet counts determined by flow cytometry with Sphero blank calibration particles (3.5–4.0 µm), gating on CD41<sup>+</sup> and TER-119<sup>+</sup> cells. n = 3 mice per genotype. Unpaired Student’s t test with two-tailed p-values.

<sup>b</sup>P < 0.001, with respect to control mice Bcl-x<sup>fl/fl</sup> or Bcl-x<sup>+/+</sup>, 1 way ANOVA with Bonferroni’s multiple comparison correction.

Figure 4. Megakaryocytes require Bcl-x<sub>L</sub> for survival during platelet formation.

Proplatelet formation (a) and viability (b) of cultured fetal liver–derived MGKs harboring a constitutive or conditional deletion of Bcl-x<sub>L</sub>. Data represent mean ± SEM. Bcl-x<sup>+/+</sup>, n = 7; Bcl-x<sup>−/−</sup>, n = 8; Bcl-x<sup>y4Δ/Δ</sup>, n = 18; and Bcl-x<sup>y4Δ/Δ</sup>, n = 13 technical replicates. n = 3 biological replicates for Bcl-x<sup>−/−</sup>; n = 5 for Bcl-x<sup>y4Δ/Δ</sup> and corresponding controls. (c) Representative still images from time-lapse video microscopy of cultured fetal liver-derived Bcl-x<sup>+/+</sup> (Video 1) and Bcl-x<sup>y4Δ/Δ</sup> (Video 2) MGKs. Frames were captured every minute for 24 h. Videos run at 25 frames/s. Time (h). Bar, 100 µm.

(d) Representative still images from time-lapse video microscopy of cultured fetal liver-derived Bcl-x<sup>+/+</sup> (Video 3) and Bcl-x<sup>y4Δ/Δ</sup> (Video 4) MGKs binding Alexa Fluor 488–conjugated Annexin V (green). Frames were captured every 15 min for 24 h. Videos run at 6 frames/s. Time (h). Bar, 100 µm.
BMCs into lethally irradiated adult recipients. 7 wk after transplant, recipients of Bcl-x<sup>+/+</sup>/GFP BMCs had platelet counts of 959 ± 145, and recipients of Bcl-x<sup>fl/fl</sup>/GFP had platelet counts of 354 ± 69, an ~16-fold increase relative to Bcl-x<sup>fl/fl</sup>/GFP<sup>−/−</sup> mice (Fig. 5 b, left). As expected, given their short life span, <10% of platelets in recipients of Bcl-x<sup>fl/fl</sup>/GFP BMCs were GFP<sup>−</sup> (Fig. 5 b, middle). In both models, wild-type platelet morphology, whether GFP<sup>+/+</sup> or GFP<sup>−</sup>, was normal (Fig. 5 b, right). In contrast, Bcl-x<sup>fl/fl</sup>/GFP<sup>−</sup> platelets displayed the same disturbed morphological profile as those from unmanipulated Bcl-x<sup>fl/fl</sup>/GFP<sup>−/−</sup> mice (Fig. 5 b). Thus, even in the absence of the severe thrombocytopenic stress, megakaryocytes lacking Bcl-x<sub>L</sub> undergo an abnormal platelet shedding process. Collectively, these data demonstrate that megakaryocytes are dependent on Bcl-x<sub>L</sub> to maintain survival during platelet shedding.

Inhibition of Bcl-x<sub>L</sub> triggers megakaryocyte apoptosis

To more fully understand the death process in Bcl-x<sup>fl/fl</sup>/GFP<sup>−/−</sup> megakaryocytes, we measured apoptotic effector caspase activity in mature, unmanipulated FLC-derived cells. Bcl-x<sup>−/−</sup> and Bcl-x<sup>fl/fl</sup>/GFP<sup>−/−</sup> megakaryocytes exhibited a fivefold increase in the activity of caspase-3/7 (Fig. 6 a) and ~9 (not depicted) relative to Bcl-x<sup>+/+</sup> and Bcl-x<sup>fl/fl</sup>/GFP<sup>−/−</sup> counterparts. ATP levels, a marker of mitochondrial function, were dramatically decreased after 24 h of culture (Fig. 6 b). These results are consistent with loss of Bcl-x<sub>L</sub> inducing the intrinsic apoptosis cascade. We then asked whether pharmacological inhibition of Bcl-x<sub>L</sub> could achieve the same effect. Wild-type FLC- and BM-derived mature megakaryocytes were incubated with the small molecule ABT-737, a BH3 mimetic drug that antagonizes Bcl-x<sub>L</sub> and the related prosurvival proteins Bcl-2 and Bcl-w (Oltersdorf et al., 2005). After 5 h, a sixfold increase in caspase activity and a concomitant reduction in ATP levels were observed (Fig. 6, c and d). ABT-737 inhibited proplatelet formation in a dose-dependent manner (Fig. 6 e). Collectively, these data indicate that genetic deficiency or pharmacological inhibition of Bcl-x<sub>L</sub> triggers the intrinsic apoptosis pathway, leading to caspase activation and failure of proplatelet formation. We conclude that caspase activation has a negative impact on the ability of megakaryocytes to produce platelets. This runs counter to previous studies suggesting that megakaryocytes require apoptotic caspases to facilitate platelet shedding (De Botton et al., 2002; Clarke et al., 2003).

We therefore examined the role of caspases in proplatelet formation more closely. Wild-type megakaryocytes were incubated with two distinct broad-spectrum caspase inhibitors, z-VAD.fmk (Dolle et al., 1994) and Q-VD-OPh (Caserta et al., 2003). In agreement with previous studies, cells cultured with high doses of z-VAD.fmk exhibited a significant impairment of proplatelet formation and loss of viability (Fig. 6, f and g). In contrast, neither effect was observed with Q-VD-OPh (Fig. 6, f and g). In fact, proplatelets formed by megakaryocytes treated with Q-VD-OPh exhibited a similar increase in stability and survival to those formed by Bax<sup>−/−</sup> megakaryocytes (Fig. 2, a and b). Because Q-VD-OPh did not impair proplatelet formation or viability, we tested whether it could ameliorate the effects of ABT-737 on wild-type megakaryocytes. Interestingly, although preincubation with Q-VD-OPh could hinder the loss of mitochondrial function triggered by ABT-737 on wild-type megakaryocytes, intriguingly, although preincubation with Q-VD-OPh could hinder the loss of mitochondrial function triggered by ABT-737 (Fig. 7 a), it did not rescue proplatelet formation (unpublished data). Collectively, these data indicate that activation of the intrinsic apoptosis pathway induces mitochondrial damage and caspase activation, resulting in failure of platelet shedding and cell death.

Deletion of Bak and Bax protects megakaryocytes from apoptotic signals

Bak and Bax are the gatekeepers of the intrinsic apoptosis pathway in a wide range of cells, including platelets. We therefore asked whether...
no effect. In contrast, Bak/Bax double deficiency completely rescued the defects in platelet production caused by loss of Bcl-xL (Fig. 7 d). This encouraged us to examine the role of Bak and Bax in chemotherapy-induced thrombocytopenia, specifically carboplatin, an agent with a well-described propensity to cause thrombocytopenia in patients (Budd et al., 1999). Given that carboplatin, along with many other standard chemotherapeutics, is thought to kill both megakaryocytes and their progenitors (Zeuner et al., 2007), we generated wild-type and 
$\text{Bak}^{-/-}$ $\text{Bax}^{-/-}$ FLC-reconstituted animals. At 8 wk after transplantation, these were treated with a single dose of carboplatin. Loss of Bak and Bax significantly ameliorated both the thrombocytopenia (Fig. 7 e) and anemia (Fig. 7 f) triggered by the drug.

DISCUSSION

Our studies demonstrate that megakaryocytes possess an intrinsic apoptosis pathway, the key components of which are prosurvival Bcl-xL, and prodeath Bak and Bax. Contrary to the widely accepted model, this pathway is not activated by megakaryocytes to facilitate platelet shedding. Deletion of Bak and Bax, the gatekeepers of the intrinsic apoptosis pathway, had no adverse effect on megakaryocyte number, ploidy, they mediate megakaryocyte death in response to proapoptotic stimuli. Mature fetal liver- and BM-derived megakaryocytes were exposed to three compounds that are known to induce the intrinsic apoptosis pathway in other cell types: ABT-737, the topoiso- merase II inhibitor etoposide, and the broad-spectrum kinase inhibitor STS. In wild-type cells, all three compounds induced a dramatic reduction in ATP levels (Fig. 7 a and Fig. S4) and a concomitant increase in apoptotic caspase activity (Fig. 7 b). This was accompanied by failure of proplatelet formation (Fig. 7 c). Strikingly, in the case of ABT-737, genetic deletion of Bak and Bax could completely rescue megakaryocytes from these effects. Viability, caspase activity, and proplatelet formation were all normal in $\text{Bak}^{-/-}$ $\text{Bax}^{-/-}$ megakaryocytes treated with the drug (Fig. 7, a–c; and Fig. S4). Interestingly, $\text{Bak}^{-/-}$ $\text{Bax}^{-/-}$ megakaryocytes were protected from etoposide also, but still exhibited an impairment of proplatelet formation (Fig. 7, a–c). This was in contrast to the effects of STS, which were in no way ameliorated by loss of the prodeath proteins (Fig. 7, a–c).

To examine whether loss of Bak and Bax could protect the megakaryocyte lineage in vivo, we first deleted Bak and Bax in Bcl-xL$^{+/+}$Pf4 mice. In agreement with Kodama et al. (2011), we found that single deficiency of either protein had
or proplatelet formation. Platelet production at steady state, or in response to experimentally induced thrombocytopenia, was normal. This was true whether Bak and Bax were conditionally or constitutively deleted from the megakaryocyte lineage. The only abnormality we observed was an increase in the number of naked megakaryocyte nuclei when both Bak and Bax were deleted. Although apparent at steady state, the phenomenon was much more pronounced during recovery from thrombocytopenia. This suggests that Bak and Bax mediate the clearance of the megakaryocyte nuclei after platelet shedding. Whether or not this is an apoptotic process is unclear. It is certainly possible; we observed a thin ring of cytoplasm around naked nuclei, perhaps this is normally the site of postshedding apoptosis designed to ensure swift engulfment. In support of this notion, our live cell imaging data demonstrated that, in vitro, wild-type megakaryocytes expose significant levels of PS once proplatelet formation is complete.

In stark contrast to their absence, the activation of Bak and Bax had a profound and detrimental impact on megakaryocytes. Wild-type cells treated in vitro with the proapoptotic agent ABT-737, which inhibits Bcl-xL, Bcl-2, and Bcl-w (Oltersdorf et al., 2005), suffered mitochondrial damage, caspase activation, and death. A similar phenomenon was observed in mature, unmanipulated megakaryocytes lacking Bcl-xL. In both cases, proplatelet formation failed. Genetic deletion of Bak and Bax could block death and rescue proplatelet formation in the presence of ABT-737, and completely restored platelet production in mice lacking Bcl-xL. Thus, megakaryocytes require Bcl-xL to restrain the activity of Bak and Bax during platelet production.

In both cases, proplatelet formation failed. Genetic deletion of Bak and Bax could block death and rescue proplatelet formation in the presence of ABT-737, and completely restored platelet production in mice lacking Bcl-xL. Thus, megakaryocytes require Bcl-xL to restrain the activity of Bak and Bax during platelet production.

Our data indicate that megakaryocytes become dependent on Bcl-xL just as they enter into proplatelet formation. It is not required for their growth and development, as indicated by the abundance of polyploid megakaryocytes in the BM of Bcl-xL−/−/Bax−/− mice, and in the yield of large mature cells from cultures of Bcl-xL−/− deficient BM and fetal liver. Unlike their wild-type counterparts, however, cultured Bcl-xL−/−/Bax−/− megakaryocytes are unable to elaborate proplatelets, instead undergoing Bak/Bax-dependent death which is accompanied by significant exposure of PS. Although the situation in vivo is...
more difficult to visualize, there is no doubt that platelet production is disturbed, independently of the severe thrombocytopenia that results from loss of Bcl-xL in platelets. This is demonstrated by the ultrastructural analysis, and also the BM chimera experiments which confirmed that even in the presence of wild-type hematopoiesis, Bcl-xL−/−/− megakaryocytes produce morphologically aberrant platelets.

Clearly, induction of Bak- and Bax-mediated apoptosis results in a failure of platelet shedding, but it appears there are additional mechanisms by which proapoptotic stimuli can block proplatelet formation. Although Bak- and Bax-deficient megakaryocytes were resistant to etoposide-induced mitochondrial damage and caspase activation, they still exhibited a failure of proplatelet formation. This suggests that DNA damage may suppress platelet shedding independently of apoptosis occurring. Etoposide treatment results in DNA double-strand and single-strand breaks (Wozniak and Ross, 1983), which trigger activation of kinase signaling cascades, primarily the ATM–Chk2 and ATR–Chk1 pathways (Cimprich and Cortez, 2008). The role of these pathways in megakaryocytes, and the mechanism by which they might influence proplatelet formation is yet to be established. Interestingly, even in the absence of Bak and Bax, STS, a classical inducer of the intrinsic apoptosis pathway, was able to induce mitochondrial damage and caspase activation in megakaryocytes. This contrasts with Bak−/− Bax−/− mouse embryonic fibroblasts, which are resistant (Wei et al., 2001). These data indicate that megakaryocytes possess additional cell death signaling pathways, and, as Q-VD-OPh could not block STS-induced mitochondrial damage, they are likely to be caspase independent.

Our findings contradict a substantial body of literature which holds that megakaryocytes deliberately use the apoptotic machinery to facilitate platelet production. We believe that many of the apparent discrepancies can be explained. First, several of the key studies reporting that impaired apoptotic signaling diminishes platelet production involved overexpression of Bcl-2 family survival proteins, either in mice or in cell culture (Ogilvy et al., 1999; De Botton et al., 2002; Kaluzhny et al., 2002). Overexpression of any protein can impact on cellular processes in complex and unforeseen ways, and Bcl-2 has been linked to the cell cycle (Vairo et al., 1996), calcium homeostasis (Bassik et al., 2004), autophagy (Pattingre et al., 2005), and inflammasome function (Bruey et al., 2007). Indeed, it has recently been demonstrated that Bcl-2 transgenic mice, which exhibit thrombocytopenia (Ogilvy et al., 1999), recover normal platelet counts upon splenectomy (Kozuma et al., 2009). Second, data from in vitro culture systems, cell lines, and chemical inhibitor studies should be interpreted with caution. Our results agree with previous studies that high concentrations of the caspase inhibitor zVAD.fmk can inhibit proplatelet formation by megakaryocytes (De Botton et al., 2002; Clarke et al., 2003). In contrast, however, we found that the difluorophenoxymethylketone-based pan-caspase inhibitor Q-VD-OPh does not. Wild-type proplatelets in cultures treated with Q-VD-OPh survived significantly longer than untreated counterparts. Given that proplatelets lacking Bak and Bax exhibited a similar increase in stability and survival, we believe the most likely explanation for the discrepancy between the two inhibitors is that high doses of z-VAD.fmk trigger caspase-independent toxicity in megakaryocytes. It is known that z-VAD.fmk has potent activity against cathepsins B, H, and L (Chauvier et al., 2007), and in some cells can lead to necrosis (Temkin et al., 2006; Wu et al., 2011). In fact, in 2009 the Nomenclature Committee on Cell Death recommended that the term z-VAD.fmk-inhibitable should be used in preference to “caspase-dependent” (Kroemer et al., 2009).

In addition to the intrinsic apoptosis pathway, the extrinsic pathway has also been implicated in platelet shedding. Treatment of MEG-01 cells with Fas ligand (FasL) or an anti-Fas agonistic antibody was reported to increase proplatelet formation and production of platelet-like particles (Clarke et al., 2003). Similar results were obtained with primary mouse megakaryocytes or human bone core explants subjected to Fas agonism. This might suggest that although the intrinsic pathway is dispensable for platelet shedding, either the extrinsic pathway alone is required, or a combination of both is essential. Our results with Q-VD-OPh argue against this notion, as Fas signaling is mediated by caspase-8 (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996) and Q-VD-OPh is an effective inhibitor of this critical initiator (Chauvier et al., 2007). Genetic deletion studies will be required to define the role of the extrinsic apoptosis pathway in megakaryocyte biology.

Our results with carboplatin demonstrate that deletion of Bak and Bax can significantly protect the megakaryocyte lineage against a pathophysiological insult in vivo. Chemotherapy-induced thrombocytopenia (CIT) remains a significant unmet clinical need (Vadhan-Raj, 2009), and it will be interesting to see whether Bak/Bax-mediated killing contributes to CIT caused by other agents. In addition to cytotoxic drugs, it is conceivable the pathway might also be activated by auto-antibodies, viral infections such as HIV, or radiation. In support of the latter notion, ablation of Bak and Bax in hematopoietic and endothelial cells was recently shown to protect the BM and increase the survival of mice exposed to 12.5 Gy of whole-body irradiation (Kirsch et al., 2010). Whether loss of Bak and Bax can specifically protect the megakaryocyte lineage against radiation remains to be established.

Because carboplatin causes the death not just of megakaryocytes, but of megakaryoblasts and megakaryocyte progenitors (Zeuner et al., 2007), the protection conferred by loss of Bak and Bax suggests that the intrinsic pathway must be restrained throughout the developing megakaryocyte lineage. Prosurvival proteins expressed in megakaryocytes include Bcl-xL, Bcl-2, and Mcl-1 (Sanz et al., 2001; Zeuner et al., 2007; this study). One explanation for the fact that loss of Bcl-xL only appears to affect megakaryocytes undergoing platelet shedding might be that it is the sole prosurvival protein expressed at this critical juncture. The heterogeneous nature of megakaryocyte cultures makes this a difficult notion to test, and the fact that platelets express Bcl-2 suggests otherwise. Perhaps a
more likely scenario is that polyploidization, proplatelet formation, and platelet shedding generate acute proapoptotic stresses within the megakaryocyte, which multiple Bcl-2 family prosurvival proteins are required to resist. Loss of Bcl-xL is enough to disturb the pro- and antiapoptotic balance. It will be interesting to see whether deletion of Mcl-1 and Bcl-2 in megakaryocytes results in a similar phenotype. Given that agents specifically targeting Bcl-2 family prosurvival proteins are currently being developed for use in a range of human malignancies (Leber et al., 2010; Petros et al., 2010; Wilson et al., 2010; Roberts et al., In Press), it will be important to understand the contribution they make to the development and survival of the megakaryocyte lineage.

**MATERIALS AND METHODS**

**Animals.** Bak (Lindsten et al., 2000), Bax (Knudson et al., 1995) and Bcl-x (Motoyama et al., 1999) knockout, Bcl-x (Rucker et al., 2000) and Bax (Takeuchi et al., 2005) floxed, P4-Cre (Tiedt et al., 2007), and UBC-GFP (Schaefer et al., 2001) transgenic mice have been previously described. All mutations had been backcrossed to wild-type C57BL/6 at least 10 generations before this study. ChimERIC animals in which the hematopoietic system was reconstituted with Bak−/−/Bax−/− FLCs were generated as previously described (Schoenwaelder et al., 2009). All animal experiments complied with the regulatory standards of, and were approved by, the Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee.

**Materials.** Biotin N-hydroxy-succinimide ester (NHS-biotin), DMSO, thiazole orange, propidium iodide, and STS were purchased from Sigma-Aldrich. ABT-737 (5 µM), etoposide (50 µM), Q-VD-OPh (50 µM), vehicle controls DMSO, and ethanol if not otherwise stated. Megakaryocytes displaying proplatelets were counted by microscopy and percentage was calculated by dividing the number of proplatelet forming megakaryocytes with the total number of cells each day. Percentage of live megakaryocytes in Fig. 4 b was quantified by dividing the total cell number each day (proplatelet forming megakaryocytes + non proplatelet forming megakaryocytes) by the number of megakaryocytes at day 3.

**Megakaryocyte receptor expression.** E13.5 (or E12.5 when stated) FLCs were cultured in SFM and TPO for 3–5 d. The cells were stained with CD41 or negative control for 30 min on ice before washing and analysis on a FACSCalibur flow cytometer (BD). Megakaryocytes were also isolated for PCR analysis. In brief, FLCs were cultured in TPO for 1, 2, or 3 d and CD41 and propidium iodide− cells were sorted on MoFlo flow cytometer. Sorted megakaryocytes were lysed and used for PCR.

**Megakaryocyte ploidy.** BM was harvested from 8–10-wk-old mice, or 8 wk after BM reconstitution, and megakaryocyte ploidy was studied with propidium iodide as previously described (Kruse et al., 2009).

**Serum TPO.** Serum TPO levels were measured using the Quantikine Mouse TPO Immunoassay kit (R&D Systems) according to the manufacturer’s instructions.

**Caspase activity and viability assays.** Megakaryocytes were harvested from BSA gradients, counted, and reseeded in SFM with TPO into 96-well plates, and then returned to the 37°C incubator after the addition of ABT-737 (5 µM), etoposide (50 µM), STS (10 µM), Q-VD-OPh (50 µM), or vehicle controls DMSO and ethanol if not otherwise stated. Caspase-Glo 3/7 reagents (Promega) were added to the cells after 5 h. The luminescence of each sample was determined in a plate-reading LumiSTAR Galaxy lumino-meter (BMG Labtech) as directed by manufacturer. Alternatively, CellTiter Glo reagents (Promega) were added after 24 h to determine cell viability by measuring ATP levels.

**Video microscopy.** Megakaryocytes were generated as detailed in the proplatelet formation assay and transfected to optically transparent ibiTreat tissue culture treated sterile µ-slide 8-well (Ibidi) or 96-well optical bottom plates (Nunc). Experiments were performed on a Live Cell Observer with a fully motorized Axiovert 200 inverted microscope (Carl Zeiss, Inc.) driven by the Axiovision 4.7 software (Carl Zeiss, Inc.). Cells were maintained on the stage within a humidified chamber at 37°C and 5% CO2. Time-lapse images were acquired for 24 h with an AxioCam MRm camera (Carl Zeiss, Inc.) using a 20×/0.8 objective. Some experiments were performed with a 0.63× C-mount in place of the 1× C-mount to increase the camera field of view. In some experiments, CaCl2 (0.5 mM) and Alexa Fluor−488 conjugated Annexin V (1/100) were added immediately before commencing time-lapse images.
Platelet preparation. Blood was obtained by cardiac puncture into 0.1 volume of Aster Jandl (Aster and Jandl, 1964) citrate-based anticoagulant (88 mM sodium citrate, 69 mM citric acid, and 20 mg/ml glucose, pH 4.6). Mouse PRP was obtained by centrifugation of the murine blood at 125 g for 8 min, followed by centrifugation of the supernatant buffy coat at 125 g for 8 min. Mouse platelets were washed by two sequential centrifugations at 860 g for 5 min in 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose, pH 6.0 (buffer A). The platelet pellet was resuspended in 10 mM Hepes, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 10 mM glucose, and 0.5 mM NaHCO₃, pH 7.4 (buffer B). Alternatively, PRP was stained with CD41 or negative controls for 20 min at room temperature before dilution in buffer B and analysis on a FACS Calibur flow cytometer. FlowJo software (Tree Star) was used to determine platelet forward and side scatter profiles (CD41⁺ cells).

Platelet turnover studies. Platelet life span was assessed with the biotinylaton assay as previously described (Heilmann et al., 1993; Mason et al., 2007). Alternatively, platelet-specific X488 was injected intravenously, and platelet life span was measured as previously described in PRP by flow cytometry (Dowling et al., 2010). In addition, platelet life span was determined by X488 in whole blood by flow cytometry gating on CD41⁺ cells. Platelet transfection of biotinylated platelets from reconstituted mice (8 wk after) into wild-type animals was performed as previously described (Mason et al., 2007).

Platelet receptor expression. PRP was stained with platelet-specific antibodies or negative controls for 20 min. Samples were diluted with buffer B and directly acquired by flow cytometry. Annexin V binding was measured in PRP by incubation with fluorescently conjugated Annexin V (20 min) in Annexin V buffer (Invitrogen), followed by dilution with Annexin V buffer and direct acquisition by flow cytometry. Reticulated platelets were enumerated as previously described (Mason et al., 2007; Matic et al., 1998).

SDS-PAGE and Western blot analysis. Platelets and megakaryocytes were lysed in NP-40 lysis buffer (Josefsson et al., 2005) or RIPA buffer, respectively. Proteins were separated on 4–12% Bis-Tris gels (NuPAGE; Invitrogen) under reducing conditions, transferred onto Immobilon-P membranes (Micron Separation), and immunoblotted with various antibodies, followed by secondary HRP-conjugated antibodies and ECL.

Electron microscopy. Transmission electron microscopy was performed after fixation and decalcification of murine femurs, as previously described (Schaefer et al., 2001) BMCs (2 × 10⁶ cells total per recipient) into recipient male mice on a CD45.1 background or Bcl-x Pf4 mice. BM chimeras. GFP BM chimeras. 8 wk later, donor contribution was measured by CD45.1 and CD45.2 peripheral blood leukocytes, and measured using CD45.1 and CD45.2 peripheral blood leukocytes, and measured using Annexin V-Alexa Fluor 488 in green during proplatelet formation by Bcl-x⁻/⁻ control or Bcl-xl²⁺/²⁺ megakaryocytes, respectively. Videos 3 and 4 show IPS exposure visualized by Annexin V-Alexa Fluor 488 in green during proplatelet formation by Bcl-xl²⁺/²⁺ control or Bcl-xl²⁻/⁻ megakaryocytes, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110750/DC1.

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Author contributions: E.C. Josefsson, C. James, A.W. Roberts, D.C.S. Huang and B.T. Kile designed research, analyzed data, and wrote the paper. E.C. Josefsson, C. James, K.J. Henley, M.A. Debrincat, S. Elias, K.L. Rogers, M.R. Lane, K.D. Mason, M.R. Dowling, L.A. O'Reilly, E.A. Kruse, M.J. White, D. Metcalf, and P. Norden performed research and analyzed data. The Walter and Eliza Hall Institute of Medical Research has an ongoing research collaboration agreement with Genentech in the field of Bcl-2 family proteins. A.W. Roberts is an investigator on three clinical trials funded by Abbott and Genentech, and receives funding for laboratory research that is part of those trials. The work described in this manuscript was not funded by either Abbott or Genentech.

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REFERENCES


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Figure S1. Hematopoietic analysis of Bak/Bax BM chimeric mice. (a) Western blot of platelet protein lysates from FLC-reconstituted mice 8 wk after transplantation. Tubulin was included as loading control. (b) Platelet counts in lethally irradiated mice reconstituted with FLCs lacking Bak and Bax, 8 wk after transplantation. Data represent mean ± SD. n = 24–60 mice per group. (c) Platelet survival curves in lethally irradiated mice reconstituted with FLCs lacking Bak and Bax, 8 wk after transplantation. Platelets were labeled via intravenous injection of NHS-biotin. Data represent mean ± SD. n = 7 mice per group. (d) Platelet survival curves. FLC-reconstituted mice were injected with NHS-biotin 8 wk after transplant, and then labeled platelets were purified and transfused into wild-type recipients. Platelet clearance was measured by flow cytometric analysis at the indicated time points. Data represent mean ± SEM. n = 6–7 mice per group. ***, P < 0.0001. (e) Peripheral blood lymphocyte numbers in FLC-reconstituted mice 8 wk after transplant. Data represent mean ± SEM. n = 24–60 mice per genotype. ***, P < 0.001. (f) Spleen weight in FLC-reconstituted mice 8 wk after transplant. Data represent mean ± SEM. n = 5 male mice per genotype. ***, P < 0.0001. (g) Morphological recognizable MGKs in H&E-stained sections of BM. Each symbol represents the mean number per field of view (200x) from 6 fields per individual mouse. Data represent overall mean ± SD. (h) Megakaryocyte ploidy distribution profiles in FLC-reconstituted mice 8 wk after transplant. Bak+/−Bax+/− mice were not FLC-reconstituted. Data represent mean ± SD. n = 3–8 mice per genotype. ***, P = 0.0009. (i) Serum thrombopoietin levels in FLC-reconstituted mice 8 wk after transplant. Each symbol represents an individual mouse. Data represent mean ± SEM. *, P < 0.05. (j) Hematopoietic progenitor cell numbers in FLC-reconstituted mice 8 wk after transplant. 25,000 BMCs were cultured with stem cell factor, IL-3, and erythropoietin in semisolid agar for 7 d. MGK, megakaryocyte. Non-MGK colonies represent the total of blast, granulocyte, mixed granulocyte/macrophage, macrophage, and eosinophil colonies. Data represent mean ± SD. n = 3 Bak+/−Bax+/− and 3 Bak−/−Bax−/−; n = 2 Bak−/−Bax+/−. *, P = 0.0138; **, P = 0.0055; ***, P = 0.0005.
Figure S2. Conditional deletion of Bcl-x in the megakaryocyte lineage. (a) Peripheral blood smears from adult mice stained with May Grunwald Giemsa. Platelets are indicated by arrowheads. Representative images from five independent experiments. Bars, 20 μm. (b) Cell surface profile of platelets in PRP. Data represent 3–4 mice per genotype. (c) Platelet survival in vivo assessed by flow cytometric analysis of PRP. Platelets were labeled via intravenous injection of a DyLight 488-conjugated anti-GPⅡb/Ⅲa (CD42b) antibody. Blood samples were collected 0.25, 1, 3, 5 h, and after that once daily. Data represent mean ± SEM, n = 4 Bcl-x*+, 8 Bcl-x*+/+, 3 Bcl-x*Δ/Δ, 2 Bcl-x*Δ, Bcl-x*ΔΔ, P < 0.0001 at 5, 24 and 48 h when comparing Bcl-x*ΔΔ to Bcl-x*Δ, P = 0.004 at 48 h when comparing Bcl-x*Δ to Bcl-x*ΔΔ. (d) Platelet survival in vivo assessed by flow cytometric analysis of whole blood. Platelets were labeled in vivo as above and blood collected 0.5, 1, 3, 5, 8 h, and after that once daily. Data represent mean ± SEM. n = 4 animals per genotype. (e) H&E-stained sections of BM from adult mice. MGKs are indicated by arrowheads. Scale bar: 50 μm. (f) Western blot of protein lysates from fetal liver-derived MGKs. FLCs were cultured in serum free media in TPO for 5 d, then large MGKs were purified from a BSA gradient. Actin was used as control for protein loading. (g) Detection of the recombinant Bcl-x locus in MGKs. FLCs were cultured in serum free media in TPO for 1, 2, or 3 d. At each stage, CD41+ve cells were sorted by flow cytometry, DNA extracted and subjected to PCR. (h) Reticulated platelet fraction in adult conditional knockout mice. Thiazole orange positive platelets were measured by flow cytometry. Each symbol represents an individual mouse. Data represent mean ± SEM. *** P < 0.0001. (i) Expression of intrinsic pro and anti-apoptotic proteins in MGKs. Shown are Western blots of protein lysates from fetal liver-derived MGKs derived as in (g). Negative controls were Bak*+/+, Bak*Δ/Δ, Bcl-x*+/+ or Mcl-1*+/+ mouse embryonic fibroblasts (MEFs). Actin was used as a protein loading control.
Figure S3. Transmission electron microscope images of BM megakaryocytes lacking Bcl-xL.
(a) Bcl-xLfl/fl BM megakaryocyte. Bar, 10 μm. (i and ii) High magnification of two areas showing the megakaryocyte ultrastructure demarcation membrane system (DMS). Bars, 1 μm. (b) Bcl-xLfl/fl BM sinus containing platelets. Bar, 5 μm. (i and ii) High magnification of two areas showing platelet ultrastructure, Bars, 1 μm. Red blood cell (R). (c) Bcl-xLfl/fl fl/fl BM sinus containing megakaryocytic fragments and an actively shedding megakaryocyte. Bar, 10 μm. (i and ii) High magnification of two areas showing the megakaryocytic ultrastructure. Bars, 1 μm. (d) Bcl-xLfl/fl fl/fl BM sinus containing two megakaryocytic fragments. Bar, 10 μm. (i and ii) High magnification of two areas from the same fragment showing the megakaryocytic ultrastructure. Bars, 1 μm. Nucleus (N).
Figure S4. Deletion of Bak and Bax protects BM derived megakaryocytes from death signals. Viability of Bak- and Bax-deficient MGKs in response to (a) ABT-737, (b) etoposide and (c) STS. Mature BM-derived MGKs were purified from a BSA gradient and cultured in serum-free media with TPO and either ABT-737 (5 μM), Etoposide (50 μM), STS (5 μM), vehicle controls DMSO (0.05% ABT-737, 0.5%, STS), or ethanol (EtOH) 0.27%. Viability was measured 24 h after reseeding using the CellTiter-Glo assay system. DMSO controls were set as 100%. Data represent mean ± SEM. (a) n = 3–6 independent experiments except for Bak−/−/Bax−/−, n = 1. (b) n = 2–4 independent experiments except for Bak−/−/Bax−/−, n = 1. (c) n = 3–4 independent experiments except for Bak−/−/BaxΔPFAΔ, n = 1.

Video 1. Proplatelet formation by Bcl-xΔPFA control megakaryocytes.

Video 2. Proplatelet formation by Bcl-xΔPFAΔ megakaryocytes.
Video 3. Proplatelet formation by Bcl-x<sup>+/+</sup> control megakaryocytes. PS exposure visualized by Annexin V-Alexa Fluor 488 in green.

Video 4. Proplatelet formation by Bcl-x<sup>−/−</sup> megakaryocytes. PS exposure visualized by Annexin V-Alexa Fluor 488 in green.