The role of apoptosis in megakaryocytes and platelets

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Summary

The role of apoptotic pathways in the development and function of the megakaryocyte lineage has generated renewed interest in recent years. This has been driven by the advent of BH3 mimetic drugs that target BCL2 family proteins to induce apoptosis in tumour cells: agents such as ABT-263 (navitoclax, which targets BCL2, BCL2L1 and BCL2L2) and ABT-199 (a BCL2 specific agent) are showing great promise in early stage clinical trials. However, the major dose-limiting toxicity of navitoclax has proven to be thrombocytopenia, an on-target effect of inhibiting BCL2L1. It turns out that the anucleate platelet contains a classical intrinsic apoptosis pathway, which at steady state regulates its life span in the circulation. BCL2L1 is the critical pro-survival protein that restrains apoptosis and maintains platelet viability. These findings have paved the way to a deeper understanding of apoptotic pathways and processes in platelets, and their precursor cell, the megakaryocyte.

Keywords

Megakaryocytes

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Apoptosis

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BH3 mimetic

Apoptosis: the original programmed cell death

In 1972, Kerr and colleagues published their landmark study proposing that a form of cell death previously referred to as “shrinkage necrosis” is in fact a distinct mechanism of “cell deletion” (Kerr*, et al* 1972). Characterised by “ultrastructural features that are consistent with an active, inherently controlled phenomenon”, the authors called this type of cell death “apoptosis”, a Greek word meaning the "dropping off" or "falling off" of leaves from trees. In the 40 years since, enormous progress has been made in elucidating the molecular pathways that regulate apoptotic cell death, and the roles they play in physiology and pathology. This is nowhere better demonstrated than the haematopoietic system. It is now clear, that, from stem cell through to terminally differentiated effector, the development, survival, function and turnover of every lineage is facilitated and controlled by apoptotic pathways and processes (Josefsson and Kile 2009).

The intrinsic and extrinsic apoptosis pathways

Apoptosis—as it is currently defined in mammals—refers to two convergent pathways of programmed cell death: the intrinsic and extrinsic (Youle and Strasser 2008) (Fig 1). The critical regulators and effectors of the former are proteins of the BCL2 family, which are characterised by the presence of one or more BCL2 homology (BH) domains. They are divided into three groups. The first comprises the multi-domain killers BAK1 and BAX, which are the essential effectors of the intrinsic pathway. The second group are the pro-survival proteins (BCL2, BCL2L1 (also known as BCL-XL), BCL2L2, MCL1 and BCL2A1), whose function is to prevent the activation of BAK1 and BAX. While the precise mechanisms are still being clarified, it is believed they achieve this by physically restraining BAK1 and BAX directly, and by sequestering a third group of pro-apoptotic BCL2 family members known as “BH3-only” proteins (BIM (BCL2L11), BID, BAD, PUMA (BBC3), NOXA (PMAIP1), BMF, BIK and HRK).

In a healthy cell, BCL2 family pro-survival activity is sufficient to keep BAK1 and BAX in check. Apoptotic signals such as DNA damage trigger the BH3-only proteins, either by transcriptional upregulation or post-translational modification, thereby allowing them to overwhelm the function of the pro-survival proteins and activate BAK1 and BAX. This is thought to be the result of BH3-only proteins binding to and inhibiting pro-survivals, but also by directly binding and activating BAK1 and BAX (Ren*, et al* 2010, Willis*, et al* 2007). Once unleashed, the latter oligomerise and induce mitochondrial outer membrane permeabilisation (MOMP), facilitating the efflux of apoptogenic factors such as cytochrome *c* and SMAC (DIABLO) into the cytoplasm, and eventually leading to the dissipation of mitochondrial potential ($\Delta\psi_m$). Once MOMP has occurred, the apoptotic caspase cascade is engaged, beginning with the “initiator”, or “apical” caspase, Caspase-9. Together with

APAF1 and Cytochrome *c*, the inactive Caspase-9 zymogen is recruited to form the "apoptosome" complex, the function of which is to proteolytically activate Caspase-9. Active Caspase-9 then triggers the rest of the caspase cascade, culminating in the activation of the "effector" caspases, Caspase-3 and Caspase-7.

Caspases are the executioners of the intrinsic apoptosis pathway, mediating many of the hallmarks of apoptosis, such as DNA fragmentation, phosphatidylserine (PS) exposure and membrane blebbing. However, MOMP is generally accepted to be the point of no return. Cells lacking BAK1 and BAX are refractory to a wide range of apoptotic stimuli, and do not exhibit Cytochrome *c* release or caspase activation (Ekert*, et al* 2006, Ekert*, et al* 2004, Lindsten*, et al* 2000, Lindsten and Thompson 2006, Wei*, et al* 2001). Most importantly, they are able to maintain clonogenicity. In contrast, cells in which caspases or APAF1 have been deleted, or cells treated with caspase inhibitors, show short-term resistance to the effects of apoptotic stimuli, but do not retain clonogenic potential. The haematopoietic system is a striking case in point: $BAK1^{-/-}$ $BAX^{-/-}$ mice exhibit a massive accumulation of mature blood cells, particularly lymphocytes (Lindsten*, et al* 2000), as do animals reconstituted with $BAK1^{-/-}$ $BAX^{-/-}$ bone marrow (Rathmell*, et al* 2002). However, mice with an APAF1 or Caspase-9-deficient haematopoietic system show no significant perturbations in mature blood cell number (Marsden*, et al* 2002, van Delft*, et al* 2010, White*, et al* 2012). Thus, it appears that primary role of the apoptotic caspase cascade is to accelerate the process of cell death. The physiological consequences—if any—of the delayed apoptotic deaths that must presumably occur in the absence of the apoptotic caspase cascade remain to be established.

The other apoptotic pathway, the extrinsic, is activated by ligands binding to cell surface death receptors that belong to the tumour necrosis factor (TNF) receptor family, such as FAS or TNF receptor-1 (TNFR1). Once activated, the adaptor protein FAS-associated death domain (FADD) and Caspase-8 are recruited to the receptor complex. Caspase-8 is the essential mediator of the extrinsic apoptosis pathway. Following proteolytic cleavage, the activated form of Caspase-8 directly triggers activation of Caspase-3/7. In so called Type I cells, such as lymphocytes, this is sufficient to induce death (Scaffidi*, et al* 1998). In Type 2 cells, such as hepatocytes, the additional recruitment of the intrinsic pathway is required to induce killing. This is achieved via Caspase-8-mediated activation of the BH3-only protein BID, which then triggers the activation of BAK1 and BAX (Jost*, et al* 2009, Yin*, et al* 1999).

Platelet survival

In 1910, William Duke made the seminal observation (amongst many others) that "platelets are short-lived bodies" (Duke 1910). In the manuscript that followed, in which presented a study of dogs subjected to experimentally-induced thrombocytopenia, Duke went on to suggest that "platelets under the usual conditions of life are rapidly disappearing either through utilization or disintegration and that the count is kept constant under a given set of conditions by as rapid a rate of formation" (Duke 1911). With the blossoming of radiation and chemotherapy treatments for cancer in the 1940s and 1950s, the demand for platelet transfusions provoked a concerted effort to accurately measure platelet life span, and to maximise platelet survival pre- and post-transfusion (Freireich*, et al* 1959, Stefanini and Dameshek 1953). It was determined that in resting, healthy humans, circulating platelet life span is 8-9 days (Leeksma and Cohen 1955). The reasons for this finite existence remained obscure. The most widely accepted explanation for several decades was the "multiple hit" model, which posits that platelets suffer external damage as they circulate, eventually becoming so damaged that they are recognised by the reticuloendothelial system and removed from the circulation (Mustard*, et al* 1966). While it is largely consistent with mathematical modelling of platelet survival curves, the nature of the hits and the mechanism by which they trigger clearance have not yet been established.

In the last few years, it has become apparent that the intrinsic apoptosis pathway plays an essential role in platelet survival, and is an important regulator of platelet life span *in vivo*. Whilst the presence of BCL2 family members and caspases in platelets had been documented (Vanags and Orrenius 1997, Wolf*, et al* 1999), it wasn't until the advent of the "BH3 mimetic" anti-cancer drugs that the vital contribution the pathway makes to platelet biology was revealed. As the name suggests, BH3 mimetics are small molecules designed induce apoptosis in cancer cells by recapitulating the activity of the BH3-only proteins. The latter possess differing affinities for BCL2 family pro-survival proteins. BIM and PUMA, for example, can inhibit all 5 pro-survivals, whereas BAD binds to tightly to BCL2, BCL2L1, and BCL2L2, but only weakly to BCL2A1, and not to MCL1 (Chen*, et al* 2005). The first bona fide BH3 mimetic, ABT-737, exhibits a target profile similar to BAD (Oltersdorf*, et al* 2005). An orally available analogue of ABT-737 called ABT-263 (now known as navitoclax (Tse*, et al* 2008)) entered clinical trials in 2007 for a range of human malignancies, and is showing promising anti-tumour activity (Gandhi*, et al* 2011, Roberts*, et al* 2012, Wilson*, et al* 2010). However, its major dose-limiting toxicity has proven to be thrombocytopenia. This is because platelets are absolutely dependent on BCL2L1 for survival (Mason*, et al* 2007). In contrast, ABT-199, a BCL2-specific BH3 mimetic, was recently shown to

have potent anti-tumour activity in patients with chronic lymphocytic leukemia, but no significant effect on platelet counts (Souers, *et al* 2013).

Apoptosis and the regulation of platelet life span

The limit of circulating platelet life span in mice at steady state is approximately 5 days (Ault and Knowles 1995). Mutations in BCL2L1 cause dose-dependent cell-intrinsic reductions in platelet survival: in mice heterozygous or homozygous for a hypomorphic allele of BCL2L1, platelet life span is decreased to 3.5 and 1.0 days, respectively (Mason, *et al* 2007). In animals with a megakaryocyte-specific deletion of BCL2L1, platelet life span is reduced to only 5 hours (Josefsson, *et al* 2011). Abbreviated platelet survival results in thrombocytopenia, the severity of which correlates with the decrement in life span. The role of BCL2L1 in platelets is to restrain the activity of BAK1 and BAX. Accordingly, mice lacking BAK1 and BAX exhibit a doubling of circulating platelet life span at steady state (Josefsson, *et al* 2011, Mason, *et al* 2007). Furthermore, as proof of the critical link between the members of this pro- and anti-apoptotic triumvirate, deletion of BAK1 and BAX fully rescues the near-total thrombocytopenia observed in BCL2L1 conditional knockout mice (Kodama, *et al* 2011). Thus, platelet life span is dictated by an intrinsic apoptosis mechanism. Those platelets that are not consumed in a haemostatic process are destined to die at the hands of BAK1 and BAX.

This mechanism explains why pharmacological inhibition of BCL2L1 by either ABT-737 or navitoclax triggers thrombocytopenia in humans, dogs and mice (Gandhi, *et al* 2011, Mason, *et al* 2007, Roberts, *et al* 2012, Wilson, *et al* 2010, Zhang, *et al* 2007). These drugs directly induce platelet death. *In vitro*, the process is characterised by many of the hallmarks of apoptosis in nucleated cells: MOMP, caspase activation, loss of mitochondrial potential and PS exposure (Dasgupta, *et al* 2010, Mason, *et al* 2007, Schoenwaelder, *et al* 2011, Schoenwaelder, *et al* 2009, Vogler, *et al* 2011, Zhang, *et al* 2007). It is also accompanied by calcium store depletion, although the extent and physiological relevance of the latter remains controversial (Harper and Poole 2012, Schoenwaelder and Jackson 2012, Vogler, *et al* 2012). By definition, ABT-737-induced platelet death is mediated by the intrinsic apoptosis pathway: deletion of BAK1 and BAX renders platelets entirely refractory to its effects, both *in vitro* and *in vivo* (Mason, *et al* 2007, Schoenwaelder, *et al* 2009).

Interestingly, whereas deletion of BAK1 in mice confers an extension of platelet life span equivalent to that caused by the combined loss of BAK1 and BAX, deletion of BAK1 or BAX alone offers platelets little protection against ABT-737 (Josefsson, *et al* 2011, Kodama, *et al* 2011,

Mason, *et al* 2007). Both BAK1 and BAX must be ablated to prevent platelet apoptosis caused by the BH3 mimetic, suggesting that, while BAK1 is the primary killer of senescent platelets under steady state conditions *in vivo*, ABT-737 (and presumably navitoclax) treatment activates both BAK1 and BAX. This dichotomy highlights one of the major questions in the field: how is platelet apoptosis initiated? Pharmacological inhibition of BCL2L1 is clearly an effective means of liberating BAK1 and BAX, but what is the physiological mechanism that underpins the entry of individual platelets into apoptosis as they reach the end of their circulating life span? The "molecular clock" model holds that BCL2L1 degrades more quickly than BAK1, and, given their limited capacity for protein synthesis, as platelets circulate they gradually lose pro-survival activity, eventually reaching a point where BAK1 can no longer be restrained (Mason, *et al* 2007). This is an attractive hypothesis, and there is evidence BCL2L1 levels decrease during platelet storage (Bertino, *et al* 2003, Kodama, *et al* 2011). However, western blot analyses of platelets purified from wild-type, *BAK1*$^{-/-}$ and *BAK1*$^{-/-}$ *BAX*$^{-/-}$ mice detected no changes in the amount of BCL2L1 protein, despite the populations having significantly different age profiles (Josefsson, *et al* 2013).

If, therefore, the regulation of platelet life span is not simply a matter of differences in protein stability, then a more complex mechanism must be invoked. Is a pro-apoptotic signal actively generated and delivered to BCL2L1/BAK1/BAX? The most obvious candidates for doing so would be the BH3-only family. Data from BID, BIM and PUMA triple knockout mice indicates that, at least in some cell types, including neurons and T lymphocytes, direct activation of BAK1 and BAX at the mitochondria requires BH3-only proteins (Ren, et al 2010). Of the 8 family members, only BAD has been linked to platelet survival thus far, with BAD knockout mice exhibiting a modest but significant cell-intrinsic extension of platelet life span (Kelly, *et al* 2010). AKT-mediated phospho-inactivation of BAD has been reported to increase the viability of human platelets stored at 37^{o}C (Catani, *et al* 2010), and a recent study suggested that activation of the ADP receptor $P2Y_{12}$ can ameliorate the effects of ABT-737 and navitoclax via PI3K/AKT-mediated inactivation of BAD (Zhang, *et al* 2013). Given the rather modest changes associated with decreased BAD function, and the scope for redundancy amongst the BH3-only proteins, it seems likely that multiple family members might contribute to the regulation of platelet survival.

Apoptosis and activation

Once classified according to morphological features, apoptosis is now defined on the basis of biochemical pathways. This is not simply an exercise in reductionism: new forms of regulated cell death continue to be discovered. Two that have generated intense interest in recent years are pyroptosis and necroptosis. The former is associated with inflammatory and infectious stresses, and

is mediated by Caspase-1 and/or Caspase-11 (Lamkanfi and Dixit 2012). The latter is induced by cytokines such as TNF, and is mediated by the protein serine/threonine kinase receptor interacting protein 1 and 3 (RIPK1/RIPK3) complex (Vanlangenakker*, et al* 2012). For these and other forms of cell death, there still is much to be learnt with regard to specific physiological and pathogenic triggers, and the cell types, developmental processes and disease states in which each is relevant. Furthermore, while the extent to which pathways are co-activated and/or regulate each other is still largely unexplored, there are clearly some profoundly important interactions between key components, e.g. the requirement for Caspase-8 in suppressing RIPK3-dependent necrosis during embryonic development (Kaiser*, et al* 2011, Oberst*, et al* 2011, Zhang*, et al* 2011). It is therefore important to define as carefully as possible the biochemical mechanisms underpinning a given process, and avoid the temptation to use the term "apoptosis" as an all-purpose descriptor whenever (for example) active caspases are detected, or loss of mitochondrial potential is observed.

In the platelet context, activation of the intrinsic apoptosis pathway must be distinguished from cellular behaviours that, while traditionally associated with apoptotic cells, are either independent of BAK1 and BAX, or reflect their incidental activation. A prime example is PS exposure. Highly activated platelets expose PS in order to facilitate the formation of the tenase complex, which drives the production of thrombin (Bevers*, et al* 1982, Zwaal*, et al* 1977). Platelets stimulated to undergo apoptosis also expose PS (Zhang*, et al* 2007). However, while agonists promote PS exposure in BAK1 and BAX-deficient cells, BCL2L1 inhibitory BH3 mimetics do not (Schoenwaelder*, et al* 2009). Agonist-induced PS is calcium dependent, apoptotic PS is unaffected by inhibitors of platelet activation or extracellular calcium chelators. Caspase inhibitors block apoptotic PS, but have no effect on PS externalisation in activated platelets. These data clearly demonstrate that platelets have evolved two distinct pathways by which they can translocate PS to the cell surface, something that was first hypothesised more than a decade ago (Wolf*, et al* 1999). Thus, PS exposure in and of itself does not constitute definitive evidence that a platelet is undergoing apoptosis.

The presence of active caspases is also not sufficient to warrant the term "apoptosis". Like most nucleated cells, platelets possess a canonical intrinsic apoptosis caspase cascade, with Caspase-9 acting as the initiator, and Caspase-3/7 lying downstream (White*, et al* 2012). The cascade can be triggered by BAK1/BAX activation, and at least *in vitro*, mediates the shape change and PS exposure caused by exposure to ABT-737. However, it appears dispensable for platelet life span and function. Mice with a haematopoietic system lacking Caspase-9 exhibit no changes in the kinetics of platelet survival. *Casp9*$^{-/-}$ platelets are protected from ABT-737, but the effect is short term, and platelets succumb eventually, both *in vitro* and *in vivo*. They also respond normally to

agonist, and *Casp9*$^{-/-}$ knockout mice manifest no defects in haemostasis. The function of the apoptotic caspase cascade in platelets, therefore, is as mysterious as it is in nucleated cells—it accelerates the process of death *in vitro*, but appears entirely dispensable *in vivo*. Furthermore, the cascade can be activated independently of BAK1 and BAX. Agents such as ionomycin can very effectively induce caspase activation (along with loss of mitochondrial potential and PS exposure), but do so with equal efficiency in wild-type and *BAK1*$^{-/-}$ *BAX*$^{-/-}$ platelets (Schoenwaelder*, et al* 2009). Thus, while a wide variety of agents, agonists, pathological states and even shear have been suggested to cause or be associated with apoptotic caspase activation (Leytin 2012), it cannot be assumed that BAK1 and BAX-mediated apoptosis is responsible or required. For that to be established, BAK1 and BAX must be deleted, since there are, as yet, no chemical inhibitors that target these essential mediators. This is not a trivial exercise, but it is the definitive assay for activation of the intrinsic apoptosis pathway in platelets. In the absence of this evidence, it is my contention that the presence of active caspases and/or mitochondrial damage and/or PS exposure should not be referred to as "apoptosis", but simply reported as observed.

Having identified the existence of two convergent pathways to PS exposure, the question now is to what extent there is crosstalk between them. Is there a role beyond platelet life span for BAK1/BAX-mediated platelet apoptosis *in vivo*? Does dysregulation of the pathway contribute to disease? One conceivable possibility is that highly activated platelets involved in haemostatic or thrombotic processes might trigger BAK1 and BAX to augment PS exposure and subsequent procoagulant function. Arguing against this is *in vitro* evidence that ABT-737 and navitoclax exposure blocks platelet activation, and that, conversely, agonists cause platelets to become more resistant to ABT-737 and navitoclax (Zhang*, et al* 2013). It will require careful study *in vivo* to tease out the requirement for apoptosis in haemostasis and thrombosis. The same is true of the various disease states where phenomena such as PS exposure and caspase activation have been linked to pathological platelet apoptosis; these include immune thrombocytopenia (Winkler*, et al* 2011) type 2 diabetes (Cohen*, et al* 2002), and infectious conditions such as Dengue virus (Alonzo, et al 2012) and *Helicobacter pylori* (Yeh, et al 2010). Most compelling is recent work demonstrating that *Escherichia coli* and *Staphylococcus aureus* isolates from sepsis patients induce features of apoptosis in platelets in vitro, including the calpain-mediated degradation of BCL2L1 (Kraemer, et al 2012). The fact that pathogenic bacteria can target the key platelet pro-survival protein certainly lends weight to the notion that the intrinsic apoptosis pathway might be aberrantly activated in some human diseases.

Megakaryocytes and apoptosis: productive death?

The role of apoptosis in megakaryocytes has been something of a conundrum. Thirty years ago, Radley and Haller described "degenerate senescent megakaryocytes" in the bone marrow of mice, the ultrastructural features of which resembled "death by apoptosis" (Radley and Haller 1983). They were referring to megakaryocytes post-platelet release, cells with "naked" nuclei and a thin ring of cytoplasm. The evidence led them to suggest that once shedding has occurred, megakaryocytes undergo apoptosis and phagocytosis by macrophages. Interest in denuded megakaryocyte nuclei initially centred on their increased frequency in conditions such as human immunodeficiency virus (HIV) infection and myelodysplastic syndromes (Hatfill*, et al* 1992, Thiele*, et al* 1983, Zauli*, et al* 1996, Zucker-Franklin*, et al* 1989). However, the identification of TPO and development of primary megakaryocyte culture systems led to the observation that peak platelet production by mature megakaryocytes "corresponded to the onset of apoptosis" (Zauli*, et al* 1997). When mice lacking the pro-death BH3-only protein Bim (Bouillet*, et al* 1999), or overexpressing pro-survival BCL2 were found to be thrombocytopenic, the idea that megakaryocytes might undergo apoptosis deliberately in order to facilitate platelet shedding was born (Ogilvy*, et al* 1999).

Seminal work by Debili and colleagues cemented this hypothesis (De Botton*, et al* 2002). They reported the presence of active apoptotic caspases in the cytoplasm of cultured primary human $CD34^{+}$-derived megakaryocytes, and showed that proplatelet formation was impaired in the presence of the fluoromethylketone caspase inhibitors Z-VAD.fmk, Z-LEDH.fmk or Z-DEVD.fmk. Caspase activity was discrete and punctuate, leading the authors to suggest that proplatelet formation requires localised apoptosis to facilitate the cytoskeletal rearrangements required for platelet shedding. Given that Cytochrome *c* was released from mitochondria, and the fact that proplatelet formation was blocked in cells transduced with a BCL2 overexpressing retrovirus, caspase activation was suggested to reflect the activity of the intrinsic apoptosis pathway. This conclusion was supported by evidence from transgenic megakaryocytes expressing BCL2 under the control of the *Platelet Factor 4* (PF4) promoter, which produced fewer proplatelets in culture (Kaluzhny*, et al* 2002). It was further bolstered by data from a case of familial thrombocytopenia, in which affected members were found to carry a variant form of Cytochrome *c* with normal redox function, but an enhanced capacity to activate the apoptosome (Morison, et al 2008). Patient-derived megakaryocytes produced platelet-like particles at an accelerated rate, fitting with a model whereby aberrant activation of the intrinsic apoptosis pathway in megakaryocytes leads to premature release of platelets into the marrow space rather than the sinusoids.

Collectively, these data point to a productive role for the intrinsic apoptosis pathway in platelet production. However, they are difficult to reconcile with evidence that pathophysiological insults can trigger the apoptotic death of megakaryocytes and their progenitors, with the result being thrombocytopenia, rather than enhanced thrombopoiesis. Perhaps the most important examples are myelosuppressive or myeloablative chemotherapy and/or radiation (Kaushansky 1996). Megakaryocyte apoptosis has also been implicated in of range of infection-related thrombocytopenias, including those associated with arenaviruses (Schattner*, et al* 2013), HIV (Zauli*, et al* 1996), dengue (Noisakran*, et al* 2012) and anthrax (Chen*, et al* 2013). It is thought to contribute to thrombocytopenia in myelodysplastic syndrome (MDS) (Houwerzijl*, et al* 2005), and in immune-mediated thrombocytopenia (ITP) where IVIg treatment correlated with a reduction in the number of "para-apoptotic" and "apoptotic" megakaryocytes in patient bone marrow (Houwerzijl*, et al* 2004).

In recent years, the role of the intrinsic apoptosis pathway in megakaryocytes has been explored in detail, primarily through studies conducted in genetically modified mice. Spurred on by the data linking the pathway to the regulation of platelet life span, several groups have examined the requirement for BCL2 family proteins and apoptotic caspases in megakaryocyte life and death. The results to date clearly indicate that megakaryocytes, like their anucleate progeny, possess a fully functional intrinsic apoptosis pathway. In addition to BCL2L1, megakaryocyte growth and development requires MCL1 (Debrincat*, et al* 2012, Josefsson*, et al* 2011, Kodama*, et al* 2012). Megakaryocyte-specific deletion of BCL2L1 results in severe thrombocytopenia, a reflection not only of dramatically reduced platelet life span, but aberrant platelet shedding by dying megakaryocytes (Josefsson*, et al* 2011). It appears that at least in mice, megakaryocytes become dependent on BCL2L1 just at the point at which they are ready to produce proplatelets. While BCL2L1-deficient megakaryocytes develop normally, they are unable to form proplatelets *in vitro*, and manifest striking ultrastructural defects *in vivo*, with large vacuolated chunks of cytoplasm being sloughed off into the sinusoids. Loss of both MCL1 and BCL2L1 results in a failure of megakaryopoiesis, systemic haemorrhage and embryonic lethality, indicating that the combined functions of both pro-survivals are required for megakaryocyte growth and development (Debrincat*, et al* 2012, Kodama*, et al* 2012). If their role is to prevent the activation of the intrinsic apoptosis pathway, then it would be expected that deletion of BAK1 and BAX would completely rescue the defects caused by loss of MCL1 and BCL2L1, and this is indeed the case: quadruple MCL1/BCL2L1/BAK1/BAX knockout mice are healthy and exhibit normal megakaryopoiesis (Kodama*, et al* 2012).

A corollary of these studies is that—somewhat disappointingly, given the appealing nature of the model—there is little to suggest megakaryocytes require the intrinsic apoptosis pathway to produce platelets. Deletion of BAK1 and BAX does not impair thrombopoiesis, in fact platelet counts are significantly elevated due to the extended platelet life span that loss of BAK1 confers. Rather than be activated to facilitate cytoskeletal rearrangements, the intrinsic pathway must be restrained in order to survive and proceed safely through the process of platelet shedding. Treatment of mature wild-type mouse megakaryocytes in culture with ABT-737 induces mitochondrial damage and effector caspase activation (Josefsson*, et al* 2011). The result is failure of proplatelet formation and death. These effects are mediated by the intrinsic apoptosis pathway, since *BAK1*$^{-/-}$ *BAX*$^{-/-}$ megakaryocytes are resistant to ABT-737. Other apoptotic stimuli such as the topoisomerase II inhibitor etoposide and the broad-spectrum kinase inhibitor staurosporine (STS) also trigger mitochondrial damage and caspase activation. Interestingly, however, while deletion of BAK1 and BAX can prevent etoposide-induced mitochondrial damage and caspase activation, it does not rescue proplatelet formation, suggesting that DNA damage signals suppress platelet shedding independently of the apoptotic pathway being engaged. While some differences are observed *in vivo*, the basic premise is the same. ABT-737 does not appear to kill wild-type megakaryocytes *in situ*, but in mice with a megakaryocyte-specific deletion of MCL1, it induces the fulminant apoptotic death of megakaryocytes within 3 hours (Debrincat*, et al* 2012, Kodama*, et al* 2012). These data suggest there might be subtle differences in MCL1 expression or protein-protein interactions in cultured megakaryocytes vs their counterparts *in vivo*, potentially the result of the latter's exposure to a range of pro-megakaryocytic cytokines and growth factors. Regardless, activation of apoptosis is accompanied by a failure of platelet shedding and severe thrombocytopenia. As expected, deletion of BAK1 and BAX renders MCL1-deficient megakaryocytes entirely refractory to the effects of ABT-737 *in vivo* (Kodama*, et al* 2012).

Collectively, the *in vitro* and *in vivo* data clearly indicate that activation of BAK1 and BAX leads to megakaryocyte death and failed platelet production. The major question that follows is what pathophysiological insults—beyond BH3 mimetics—might trigger the intrinsic pathway in megakaryocytes. Preliminary evidence suggests that traditional chemotherapeutic agents are good candidates. In mouse bone marrow chimeras, deletion of BAK1 and BAX could significantly ameliorate the drop in platelet counts induced by a single dose of carboplatin, a drug commonly associated with thrombocytopenia in humans (Josefsson*, et al* 2011). The protection afforded was likely the combined product of improved progenitor, committed precursor and developing megakaryocyte survival—not just megakaryocytes themselves—suggesting that the intrinsic

pathway may indeed play an important role in mediating the death of the megakaryocyte lineage in response to stress. Conversely, upregulation of pro-survival signalling might contribute to increased megakaryocyte proliferation and survival in essential thrombocythemia (ET) and megakaryoblastic leukemia. TPO has been shown to regulate BCL2L1 expression in cultured megakaryocytes and human megakaryoblastic leukemia cells lines such as UT-7 and CMK, and this is thought to be mediated in large part via JAK/STAT signalling (Kirito*, et al* 2002, Kozuma*, et al* 2007). Data from CMK cells demonstrated that MCL1 expression is also influenced by TPO/JAK signalling, and that treatment with a JAK inhibitor could reduce MCL1 protein levels (Kodama*, et al* 2012). It will be interesting to see whether the combination of BH3 mimetic drugs targeting BCL2L1, and small molecule JAK inhibitors such as such as the FDA-approved drug ruxolitinib (Pardanani 2012), has therapeutic value in megakaryocytic disorders, particularly those carrying activating mutations in JAK such as the V617F allele (James*, et al* 2005). Recent studies of JAK mutant human and mouse lymphoid leukemias indicate that this approach has significant potential (Waibel*, et al* 2013).

The extrinsic pathway and non-apoptotic roles for caspases

There is still much work to be done regarding the role of apoptotic pathways in megakaryocyte and platelet function and development. While it is clear that the intrinsic pathway is vital to the life and death of the lineage, the contribution of the extrinsic pathway has yet to be determined. It too has been implicated in platelet production. In the cultured MEG-01 cell line or primary murine megakaryocytes, treatment with FAS ligand (FASLG) or agonistic FAS antibodies increased the yield of platelet-like particles (Clarke*, et al* 2003). Direct delivery of a recombinant active form of Caspase-8 to cultured megakaryocytes doubled the number of cells that formed proplatelets. Similar results were obtained in explant cultures of human trabecular bone cores, which produced more platelet-like particles when incubated with anti-FAS antibodies, and fewer when treated with z-VAD.fmk. Although incubation with TNF did not promote particle generation, indicating that the effect might be specific to signalling through FAS, studies of the TNF-related apoptosis-inducing ligand (TRAIL), another death receptor ligand, have suggested that it can also promote megakaryocyte maturation (Melloni*, et al* 2005). This has been extended by reports that reductions in megakaryocyte apoptosis caused by decreased TRAIL expression might contribute to impaired thrombopoiesis in immune thrombocytopenia (ITP) patients (Yang*, et al* 2010). Genetic deletion studies will be required to define the role of the extrinsic apoptosis pathway in megakaryocyte biology. This is also true of platelets, which have been reported to express various components of the extrinsic pathway, including Caspase-8, but not the canonical death receptor FAS (Li*, et al* 2000, Mutlu*, et al* 2012, Plenchette*, et al* 2001).

Finally, a question remains as to the function of caspases in the megakaryocyte lineage. Like platelets, megakaryocytes have been shown recently to possess a canonical apoptotic caspase cascade, with Caspase-9 functioning as the initiator (White*, et al* 2012). As evidence of that role, deletion of Caspase-9 can protect cultured megakaryocytes from ABT-737-induced death, but only in the short term. Conversely, despite previous work suggesting that caspase inhibition blocks proplatelet formation (Clarke*, et al* 2003, De Botton*, et al* 2002), platelet production in mice with a Caspase-9-deficient hematopoietic system is normal, at least at steady state. The discrepancy may in part be explained by the differential effects of the various caspase inhibitors employed: whereas the fluoromethylketone caspase inhibitor z-VAD.fmk does block proplatelet formation, the difluorophenoxy-methylketone-based pan-caspase inhibitor Q-VD-OPh does not (Josefsson*, et al* 2011). z-VAD.fmk is known to target other enzymes such as cathepsins (Chauvier*, et al* 2007), and to promote necrosis (Temkin*, et al* 2006, Wu*, et al* 2011), so perhaps the classical apoptotic caspase cascade can be discounted as a means by which megakaryocytes promote platelet shedding. Nevertheless, the precise physiological role of the apoptotic caspases in megakaryocytes and platelets is yet to be established. Are they truly dispensable? It remains plausible that one or more caspases do in fact contribute to platelet production. Although this would likely be in a "non-apoptotic" capacity, there is no doubt that further work will be required to elucidate the possibility.

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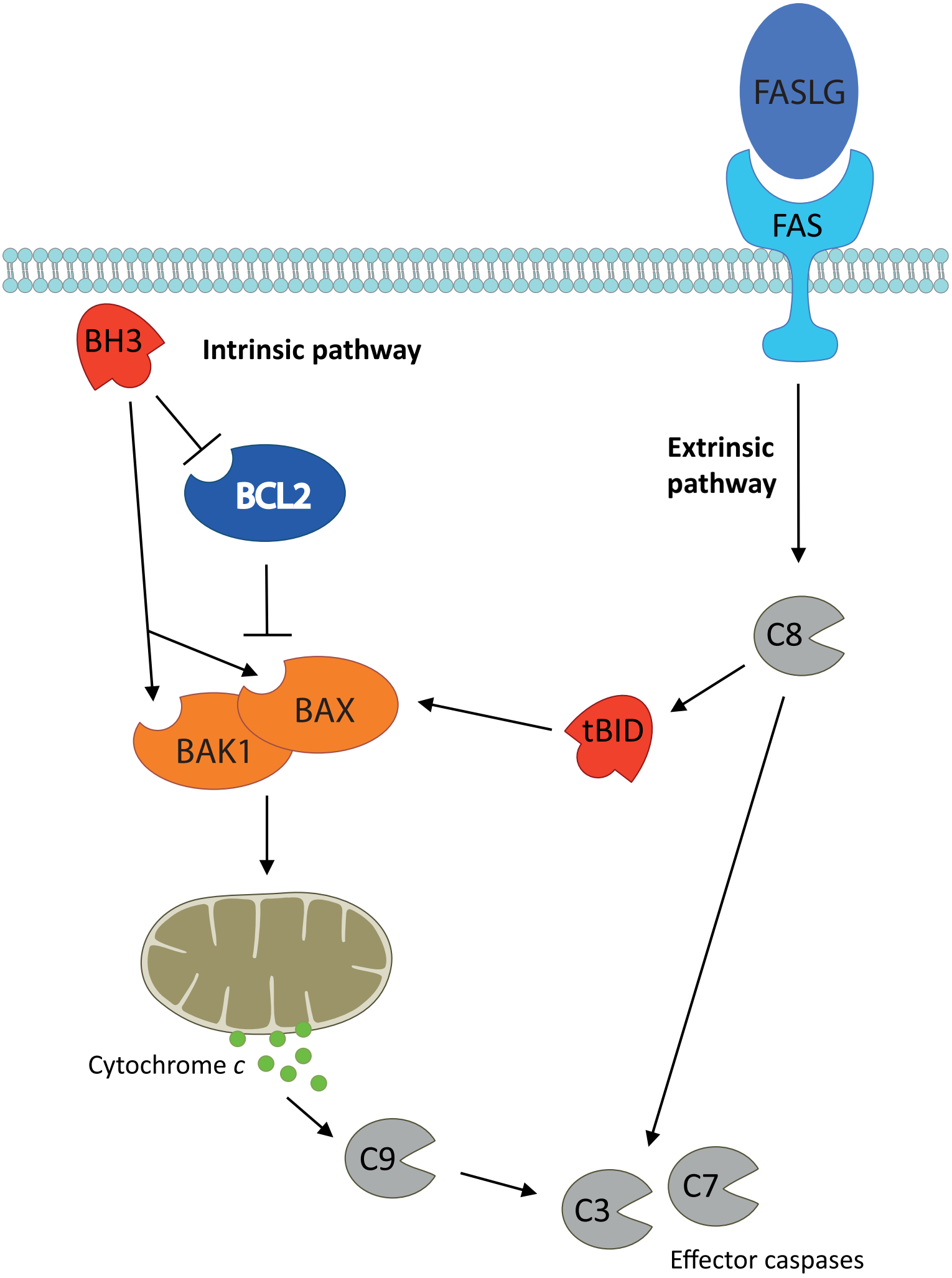
Figure Legend

Fig 1. Two convergent pathways regulate apoptosis in mammals. The intrinsic apoptosis pathway is controlled by the BCL2 family of proteins. In a healthy cell, pro-survival members (BCL2, BCL2L1, BCL2L2, MCL1 and BCL2A1) restrain the activity of pro-death BAK1 and BAX. Stress signals activate the BH3-only proteins (in red, labeled "BH3"), which liberate BAK1 and BAX to cause mitochondrial outer membrane permeabilisation. Diffusion of Cytochrome *c* from the mitochondria triggers formation of the apoptosome and subsequent activation of the apoptotic caspase cascade. Theas extrinsic apoptosis pathway is induced upon ligand binding to death receptors (e.g. FAS ligand (FASLG) to FAS receptor). This initiates recruitment of adaptor proteins to the death receptor intracellular domain (death domain), which results in cleavage and activation of Caspase-8. Active Caspase-8 can directly activate the effector caspases, Caspase-3 and Caspase-7. In addition, it also can trigger the intrinsic pathway by cleaving BID to produce tBID, which then activates BAK1 and BAX.


FASLG
FAS
BH3
Intrinsic pathway
BCL2
Extrinsic pathway
C8
BAX
BAK1
tBID
Cytochrome c
C9
C3
C7
Effector caspases