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Lessons from gain- and loss-of-function models of pro-survival Bcl2 family proteins: implications for targeted therapy

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Abstract

Cell survival depends on the maintenance of mitochondrial integrity controlled by a well-balanced interplay between anti- and pro-apoptotic B cell lymphoma 2 (Bcl2) family members. Given their frequent deregulation in human pathologies, including autoimmunity and cancer, significant research efforts have increased our molecular understanding of how Bcl2 proteins control cell death. This has fostered the development of small non-peptidic compounds, so-called BH3-mimetics, that show excellent prospects of passing clinical trials and entering daily use for targeted therapy. Possible limitations in clinical application may, to a certain degree, be predicted from loss-of-function phenotypes gathered from studies using gene-modified mice that we attempt to summarize and discuss in this context.

Keywords

apoptosis; Bcl-2 family; cancer; mouse models; targeted therapy

Introduction

Within the B cell lymphoma 2 (Bcl2) family, BH3-only proteins, such as Bim, Puma or Bid, act as sentinels, activated in response to a broad range of developmental or environmental cues to trigger mitochondrial apoptosis. They do so by neutralizing anti-apoptotic Bcl2 proteins and by directly activating the highly redundant but rate-limiting cell death effectors Bax and Bak (Fig. 1). Once activated, these form homodimers that then assemble into higher order oligomers enabling activation of proteases of the caspase family (Casp-9, -3, -6, -7) and cell death [1,2].

Upon ectopic expression in tissue culture, however, pro-survival members of the Bcl2 family (Bcl2, BclX, Mcl1, A1/Bfl1, Bclw and BclB) are all able to mediate cell death

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resistance to a broad range of genotoxic stimuli. Yet, overexpression phenotypes often fail to faithfully reflect the physiological role(s) of a protein. Nonetheless, first *in vivo* studies in transgenic mice galvanized critical roles in cell death control but also pointed towards possible differences in physiological function [3]. However, only loss-of-function studies provided clear evidence that each individual Bcl2 family member can exert highly selective roles in apoptosis signalling, often even in a strictly cell-type-dependent manner (Table 1).

Unfortunately, peri-implantation stage embryonic lethality of mice lacking myeloid cell leukemia 1 (Mcl1) [4], the early embryonic death of BclX-deficient embryos [5,6] and the severely reduced lifespan of mice lacking Bcl2 [7-9] have slowed down our progress in understanding the role of the individual Bcl2 pro-survival proteins in development, tissue homeostasis and disease. Similarly, gene quadruplication of the *Bcl2a1* locus encoding for A1/Bfl1 in mice has prevented classical gene targeting studies leaving the physiological role of this protein largely undefined [10,11]. Deletion of Bclw, on the one hand, has revealed essential roles in spermatogenesis [12,13], while loss of *Boo/Diva*, the mouse homologue of *BCLB/BCL2L10* in humans, on the other hand, showed no obvious defects [14], leading to a drop in research efforts aiming to address the role of the latter two proteins in regulating mitochondrial apoptosis.

In this review, we aim to give an overview of our current knowledge of pro-survival Bcl2 family proteins in normal physiology, as evidenced by gain- or loss-of-function studies in mice, and discuss possible implications for Bcl2-targeting therapy [15].

B cell lymphoma 2 (Bcl2)

Bcl2 was the first discovered regulator of apoptosis when it was found as translocated and subsequently overexpressed in patients suffering from follicular B cell lymphoma, 30 years ago [16,17]. High-level expression of BCL2 was confirmed in numerous human tumours sparking extensive gain- and loss-of-function studies in different model systems. Early studies in mice have focused largely on the effects of Bcl2 overexpression in the immune system (*Eμ-BCL2*; *H2K-BCL2*; *hBCL2-Ig*; *Vav-BCL2* transgenic mice) highlighting important roles in lymphocyte development and the induction and maintenance of tolerance. These mice developed autoimmune phenotypes as well as a predisposition for spontaneous and oncogene-driven lymphoma development and drug resistance [18-23]. Similar predispositions have been reported subsequently in mice expressing BCL2 in epithelial cells such as the mammary gland that showed increased rates of breast cancer upon concomitant MYC or SV40 LT overexpression [24,25]. Paradoxically overexpression of BCL2 in the liver delayed diethylnitrosamine (DEN) driven hepatocellular carcinoma [26]. This phenomenon may actually be due to the lack of compensatory proliferation upon DEN treatment by BCL2 [27]. Drug resistance phenotypes caused by BCL2 overexpression were also recapitulated in human disease [28].

While the vast body of evidence describing the anti-apoptotic effects of Bcl2 in various cell types and tissues predicted a prominent role in embryonic development and tissue homeostasis, loss-of-function analyses revealed a surprisingly restricted role in normal physiology. *Bcl2* knockout mice showed largely normal embryogenesis but newborns displayed postnatal growth retardation, melanocyte loss and early death. Reduced lifespan

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was due to impaired renal cell differentiation and increased apoptosis leading to fatal polycystic kidney disease [7,8], but the severity of this phenotype [29] and phenotypes reported for the gastrointestinal tract [9] or in the postnatal nervous system [29] showed significant variability between studies, most probably due to differences in genetic background of the individual knockout strains [30].

In accordance with the noted massive splenic involution, no *Bcl2*$^{-/-}$ mice were able to maintain their lymphoid system. At the age of 4 weeks Bcl2 deficiency already caused massive loss of mature lymphocytes, accompanied by a relative increase in myeloid cells. Notably, the few surviving lymphocytes from *Bcl2*$^{-/-}$ mice did not exhibit any disadvantage when activated with mitogens [7-9].

Transplantation of bone marrow (BM) or fetal liver derived hematopoietic stem cell (HSC) enriched populations showed that Bcl2 loss favoured the generation of myeloid over lymphoid cells in a cell autonomous manner [31,32]. Transplanted *Bcl2*$^{-/-}$ HSCs failed to produce T cells and were unable to maintain B cell numbers. However, the presence of B cells in the periphery indicated that Bcl2 is an important pro-survival factor during early B cell development and homeostatic survival of mature B cells but no longer essential in activated B cells or Ig-producing plasma cells. Of note, adoptive transfer experiments suggest minor roles in activated and regulatory T cells [33].

Together these and subsequent studies pinpointed essential roles for Bcl2 in (most) mature lymphocyte subsets, melanocytes and kidney epithelium. Importantly, all these phenotypes were restored by loss of one or two alleles of Bim, the most critical physiological Bcl2 antagonist [34]. However, due to the drastically limited lifespan of *Bcl2*$^{-/-}$ mice, its physiological role in many important cell types is still poorly understood. Conditional *Bcl2*$^{fl/fl}$ mice have been made available [35] and will allow detailed follow-up studies investigating the role of Bcl2 in a cell-type and tissue-dependent context.

B cell lymphoma like 1 (Bcl2L1/BclX)

Bcl2's next of kin, BclX, was noted early on to display a largely reciprocal expression pattern with Bcl2 in developing lymphocytes (Fig. 2). In fact, Bcl2 appears to be exchanged for BclX on a regular basis when massive expansion of lymphocyte precursors or mature lymphocytes is required, e.g. at the pre-B to naïve B cell transition in bone marrow, the CD4/CD8 double-negative to CD4/CD8 double-positive transition in the thymus or upon antigen encounter in spleen or reactive lymph nodes [36-38]. The reasons for this remain poorly understood. It may be possible, though, that next to reported negative effects of Bcl2 on proliferation (e.g. via blocking Ca^{2+} release from the endoplasmic reticulum, increased p27 levels or repressed autophagy), changes in the expression pattern of pro-apoptotic genes may select for a switch to BclX in activated lymphocytes (Fig. 1).

In analogy to studies on Bcl2, a series of transgenic mouse lines was generated addressing the role of BclX in lymphocyte development, immunity and malignant disease (*SV40-Eμ-BclX*; *Eμ-tkP-BclX*; *Lck-BclX*). These models largely recapitulated findings in BCL2 transgenic mice, although some of the phenotypes observed were more variable [37-42]. Combined overexpression of BclX and BCL2 further enhanced cell death resistance of

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lymphocytes, e.g. that of B cells exposed to high concentrations of anti-IgD, normally driving their depletion *in vivo,* or upon cytokine deprivation *in vitro*, suggesting functional redundancy [37]. Functional differences between BclX and BCL2 were reported upon overexpression in the liver that sparked some controversy as to whether Bcl2 family proteins are able to block death receptor mediated apoptosis [43-45]. Similarly, while BCL2 prevented DEN-driven liver cancer, BclX overexpression did not [44]. Ultimately, these discrepancies, next to a difference in transgene expression levels, may in part be explained by differences in binding to the BH3-only protein Bid (activated by caspase-8) or Bak by BclX (but less stringently by Bcl2), as well as possible anti-proliferative effects of Bcl2.

Ubiquitous BclX deficiency, however, is embryonic lethal and mice die at embryonic day 13 post fertilization. Analysis of foetuses revealed strong accumulation of apoptotic cells in the fetal liver and defective primitive and definite erythropoiesis as well as massive cell death in the central nervous system (CNS). Chimeric mice generated by transplantation of $BclX^{-/-}$ embryonic stem cells into C57BL/6 or $RAG2^{-/-}$ blastocysts showed reduced numbers of pre-B and immature B cells in the bone marrow and a shifted T cell ratio in the thymus [5,6]. This was accompanied by a significant loss of the T and B cells in the spleen while lymph nodes of $BclX^{-/-}$ chimeric mice remained unaffected. Moreover, bone marrow B cells and immature thymocytes manifested highly increased spontaneous apoptosis rates *ex vivo* while mature lymphocytes showed largely normal cell death responses. These experiments proved that BclX-deficient lymphocytes can mature, but in strongly reduced numbers. Once mature, they no longer depend on BclX but on Bcl2 and Mcl1. Similarly, despite the fact that BclX is prominently expressed in double-positive thymocytes, conditional deletion of *BclX* by *CD4-Cre* mediated recombination led only to a minor reduction in cell numbers. However, this can be explained by functional redundancy with Mcl1 at that stage as only combined deletion of both genes causes the near complete loss of CD4 + CD8 + double positive thymocytes [46]. Thus, lack of BclX shortens the lifespan and increases the apoptosis rate of immature lymphocytes. This notion is also supported by the finding that *Cre* expression, under control of the *Rag1* promoter in $BclX^{fl/fl}$ mice, does not affect pro-B cell numbers, but these cells cannot progress to the pre-B cell stage, consistent with the onset of BclX expression downstream of a functional pre-BCR [47]. Conditional ablation of *BclX* in germinal centre (GC) B cells using *Aicda-Cre* showed minimal impact on GC or memory B cell formation [48] nor did *Ncr1-Cre* mediated deletion affect natural killer (NK) cell numbers [49].

A crucial physiological role for BclX was revealed in $BclX^{fl/fl}$ *MMTV-Cre* mice [50] and was confirmed later in a targeted *N*-ethyl-*N*-nitrosourea (ENU) screen that highlighted its role as a timer for platelet lifespan [51]. In the latter, ENU mutagenesis introduced destabilizing point mutations into BclX (C15T or N182I) that led to accelerated degradation of BclX thereby triggering Bak-dependent apoptosis. BclX also determines megakaryocyte function, as assessed by platelet factor 4 (*Pf4*)-Cre deletion, and, similar to immature thymocytes, controls their survival in conjunction with Mcl1 [52]. It may be speculated that BclX may exert a similar timer function in erythrocytes, as its absence only affects advanced erythroid differentiation stages, causing the massive anaemia observed upon its deletion during embryogenesis [53] and in $BclX^{fl/fl}$ *MMTV-Cre* mice [50]. However, results from

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clinical trials with navitoclax (see below), which is able to target BclX, did not show anaemia as a major side effect. Thus, whether all platelet, enucleated reticulocyte or erythrocyte death is mediated by simple degradation of BclX or whether BH3-only proteins can modulate time to death upon hypoxia, injury or during inflammation still remains to be investigated.

Less is known about the physiological functions of BclX outside the hematopoietic compartment. Haplo-insufficiency reduces fertility in male mice associated with Bax-dependent testicular degeneration [54]. Conditional deletion of *BclX* was performed in the skin using *K5-Cre* and in the mammary gland epithelium by *MMTV-Cre*. This study revealed normal mammary gland development but exacerbated involution phenotypes upon forced weaning [55]. *K5-Cre* mediated keratinocyte-specific BclX deletion did not compromise skin barrier function although more apoptotic cells were noted in the epidermis and *BclX*$^{-/-}$ keratinocytes were found to be more susceptible to UVB radiation damage *in vitro* [56] but less susceptible to UVB or 7,12-dimethylbenz[α]anthracene (DMBA)-driven skin cancer *in vivo* [57]. Loss of BclX in hepatocytes triggered increased cell death and fibrosis [58]. Of note, this effect was rescued by co-deletion of Bax/Bak (hepatocytes actually express little/no Bak) and, interestingly, also by co-deletion of the BH3-only protein Bid, found present in its active form at low levels in the liver [59]. Finally, deletion of BclX in the respiratory epithelium caused the death of about 50% of newborns but a significant number developed to adulthood. In summary, this study proposed that BclX is dispensable for normal lung maturation but functions to protect respiratory epithelial cells against oxygen-induced toxicity [60].

Timed depletion of BclX in the nervous system leads to interesting phenotypes, e.g. the loss of retinal ganglion cells (RGC) in the developing embryo when using *Six3-Cre* mediated mosaic deletion, leading to reduced thickness of the retina. However, BclX dependence of maturing RGCs was lost in adult mice where deletion was mediated by tamoxifen using a *Cre-ER*^{T2} allele [61]. Similarly, deletion of BclX in dopaminergic neurons using rat tyrosine hydroxylase promoter (*TH-Cre* mice) showed that it is required for the survival of catecholaminergic cells in the developing substantia nigra [62]. Hence, in post-mitotic neurons with established synaptic connections in the adult animal the role of BclX in survival may no longer be as prominent as during embryogenesis and Mcl1 may take over a more critical role (see below). Of note, concomitant loss of Bim acting upstream of MOMP can ameliorate several defects caused by loss of BclX, including the fetal liver apoptosis and testicular atrophy noted in *BclX*$^{+/-}$ mice, but not, however, neuronal loss during development [63].

Myeloid cell leukemia 1 (Mcl1)

Based on published findings one is tempted to speculate that, for reasons still poorly understood, Mcl1 is the most crucial pro-survival protein in the Bcl2 family as its deletion in the zygote stops embryogenesis already at the blastocyst stage [4]. Furthermore, most cells upon conditional deletion in the adult mouse succumb to pre-mature cell death. Notably, rescue from death usually requires co-deletion of Bax and Bak, while co-deletion of one or more BH3-only proteins, such as Bim and/or Puma, usually fails to do so. Whether this is

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due to proposed effects on mitochondrial structure and respiration remains to be clarified [64].

In contrast to Bcl2 or BclX, Mcl1 expression in lymphocytes is broad with little variation under steady state or during development with a propensity to higher levels in stem/progenitor cells (Fig. 2). While pro-B cells express little Mcl1, pre-B, immature, follicular and marginal zone B cells express higher but similar levels [65]. Similarly, most thymocyte and mature T cell subsets show comparable Mcl1 expression [46] and the protein is also prominently expressed outside the hematopoietic compartment. A crucial feature of Mcl1 is its short half-life allowing swift integration of different signalling inputs by stabilizing post-translational modification or its rapid proteasomal degradation. Reduction in Mcl1 expression is frequently a prerequisite for cell death induction in response to various forms of stress, including cytokine deprivation, UV radiation [66] or extended mitotic arrest [67], assigning a molecular 'timer' function to Mcl1 [68].

Similar to observations made in BCL2 or BclX transgenic mice, MCL1 overexpression led to splenomegaly with extended extra-medullary haematopoiesis correlating with enhanced leukocyte survival *ex vivo* [69,70]. *Vav-Mcl1* transgenic mice, similarly to *hMCL1* transgenic mice, were also predisposed to different types of late onset B cell lymphomas that in the first model were mainly derived from pre-B or stem/progenitor cells [69] while the latter model presented frequently with follicular lymphoma and diffuse large B cell lymphomas later in life [71]. H2K-MCL1 transgenic mice showed accelerated *Eμ-Myc*-driven lymphomagenesis comparable to those overexpressing BCL2 but, due to Mcl-1 shorter half-life, MCL1-overexpressing tumours were more prone to cell death upon drug treatment impinging on protein stability, such as etoposide or vincristine, than those from BCL2 transgenic mice [69,72].

Mcl1 loss-of-function was studied extensively in different models (Table 1). Poly-IC-triggered timed deletion in *Mcl1*$^{fl/-}$ *Mx-Cre* mice resulted in rapid mortality 12 to 21 days after Mcl1 ablation. These mice were highly anaemic and exhibited symptoms of bone marrow failure, due to impaired stem cell survival upon Mcl1 depletion. qPCR analysis of Mcl1 mRNA levels in cells from wild-type mice confirmed high expression levels in HSCs induced by stem cell factor (SCF) treatment, moderate levels in common lymphoid progenitors or common myeloid progenitors and low expression in megakaryocyte-erythrocyte progenitors and granulocyte-monocyte progenitors. Notably, HSC and committed progenitor populations were all diminished upon Mcl1 deletion, while erythropoiesis was not affected [73]. Low-level Mcl1 expression may explain in part the lack of phenotype in monocytes/macrophages upon *LysM-Cre* mediated deletion [74,75] or in megakaryocytes upon *Pf4-Cre* mediated deletion [52].

Similarly, *Lck*- or *CD19-Cre* mediated deletion of the *Mcl1* locus arrested T and B cell development at the DN2/3 to DN4 transition in the thymus or the pre-pro-B cell stage in the bone marrow, respectively, and caused a subsequent reduction of all mature lymphocyte subsets [46,76]. Interestingly, the surviving cells had overcome the Mcl1 deletion. This observation is consistent with the Stat5a-driven onset of Mcl1 expression in early B cell development, after successful rearrangement of the Ig heavy chain [47]. Moreover, a

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reduction in B cell numbers was already noted in Mcl1 hemizygous mice, indicating a gene dosage effect, a phenomenon not noted in T cells [76]. However, these models did not allow assessment of the requirement for Mcl1 in mature T or B cell survival. Adoptive transfer of *Mcl1*$^{f/-}$ *Mx-Cre* lymphocytes in *Rag2*$^{-/-}$ mice and subsequent treatment with poly-IC resulted in severe lymphopenia within 2 weeks, indicating beyond doubt that Mcl1 is also critical for the survival of mature lymphocytes [73,76]. Similarly, lymphocytic choriomeningitis virus (LCMV) driven interferon production triggering Mx-Cre deletion of Mcl1 led to a lack of virus-specific $CD4^+$ and $CD8^+$ T cells that could only partially be rescued by concomitant overexpression of BclX, usually strongly induced upon T cell activation [77]. In contrast, BCL2 transgene expression did not rescue Mcl1-deficient T cells from developmental cell death while co-deletion of Bak, its key effector target, partially restored T cell numbers [78].

As Mcl1 expression, along with BclX, was noted to be higher in GC B cells than non-GC B cells, the consequences of loss of BclX and Mcl1 in GC formation, class switching and B cell memory formation were compared. Mcl1 deletion was thereby targeted to B cells initiating somatic hypermutation or class-switch recombination using *Aicda*-driven *Cre* recombinase [48]. Alternatively, B cells from *CreER*^{T2}*Mcl1*$^{fl/fl}$ mice were adoptively transferred into isogenic recipients that were then immunized with NP-KLH, and tamoxifen treatment was used to assess the impact of Mcl1 deletion on B cell survival. Strikingly, no antigen-specific isotype-switched B cells or GC-derived memory B cells were detected and serum IgG1 (but not IgM) levels were low in *Aicda-Cre/Mcl1*$^{f/f}$ mice after immunization. *CreER*^{T2} mediated deletion of adoptively transferred B cells confirmed Mcl1 dose dependence in pre-formed GC and memory B cells [48]. In a follow-up the same group showed that timed Mcl1 deletion limits formation of plasma blasts *in vitro* as well as of pre-formed plasma cells in the bone marrow and in the spleen [79]. BclX, on the other hand, only appeared critical for the survival of long-lived plasma cells in the bone marrow but not GC or memory B cell formation [48,79]. The role of Bcl2 in all these processes and possible redundancies with Mcl1 remain to be investigated.

Deletion of Mcl1 in innate immune cells including granulocytes by *LysM-Cre* or in mast cells by *Cpa3-Cre* deletion confirmed essential survival roles while monocytes and macrophages became only Mcl1-dependent upon microbial challenge [74,80]. A recent report also documents crucial roles in NK cell survival rendering *Mcl1*$^{fl/fl}$ *Ncr1-Cre* mice highly susceptible to tumour metastasis but resistant in models of multi-bacterial sepsis [49].

The facts that Bcl2 family proteins can co-regulate the cell death of hepatocytes under inflammatory conditions and growth factor treatment induces Mcl1 levels in primary hepatocytes, prompted studies investigating its role in liver homeostasis. *Mcl1*$^{fl/fl}$ *Alb-Cre* mice presented with reduced liver size due to spontaneous hepatocyte apoptosis subsequently triggering increased rates of compensatory proliferation that fostered liver cancer in aged mice [81]. In addition, mice lacking Mcl1 in the liver were more susceptible to Concanavalin A (ConA) - driven hepatitis [82], demonstrating a relevant contribution of the intrinsic cell death pathway to this type of liver damage [83]. In line with a possible redundancy with BclX that triggers similar phenotypes when deleted, double deficiency in both genes using *Alb-Cre* causes liver failure and perinatal death [84].

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Highly detrimental also are the consequences of Mcl1 deletion using muscle creatine kinase promoter driven Cre (*Ckmm-Cre*) in the heart and skeletal muscle, leading to early postnatal death with signs of severe cardiomyopathy and fibrosis. Timed ablation of Mcl1 using *Myh6-Mer-Cre-Mer* (*Myh-CreER*) mice that express a tamoxifen-inducible version of Cre under control of the cardiac-specific a-myosin heavy chain promoter triggered dilated cardiomyopathy, a phenomenon that can be counteracted by Bcl2 transgene expression [85]. Additionally, *Mcl1$^{fl/fl}$ Myh-CreER* mice suffered from loss of heart muscle contractility leading to heart failure within 3 weeks of tamoxifen administration. Co-deletion of Bax and Bak could rescue these effects but the noted distortion of mitochondrial ultrastructure and respiratory capacity was not fully restored [85].

Finally, neuroscientists also explored the role of Mcl1 in the survival of neuronal precursor cells (NPCs) that have the capacity to regenerate damaged regions in the brain. The size of the NPC pool is in part controlled by apoptosis and reducing NPC apoptosis may enhance regenerative capacity after injury. *Nestin-Cre* mediated deletion of Mcl1 impaired neurogenesis in the embryo leading to early lethality [86] and subsequent analyses also documented a critical role for Mcl1 in adult NPC survival [87]. Ablation of Mcl1 in cortical neurons of the cortex using a *CamKIIα-Cre* BAC transgene, on the other hand, triggered an autophagic stress response and caused early postnatal death of these animals (< 8 weeks) [88]. Whether Mcl1 is indeed a negative regulator of autophagy or whether absence of Mcl1 leads to increased cell death susceptibility for neurons that try to overcome this stress (e.g. impaired respiratory capacity) by activating autophagy remains to be dissected experimentally.

Bcl2a1 (A1/Bfl1)

While humans and rat encode A1 in a single gene locus, mice harbour three functional genes encoding for A1 protein (A1a, A1b and A1d) and one pseudogene (A1c) [10]. All isoforms are highly conserved at the DNA and protein level, suggesting a large degree of functional redundancy, and show > 70% homology to human *A1/BFL-1* [89]. Another limitation is that commercial antibodies recognizing endogenous A1/Bfl1 are not of great quality and hence most expression data are based on mRNA analysis. Unquestionably, expression levels increase upon successful rearrangement of the T cell receptor β (TCR-β) chain and pre-TCR expression in developing thymocytes [90] and upon TCR mediated activation in T cells [91,92]. During B cell maturation A1 mRNA levels gradually increase and activated B cells show highest levels [93] but A1 is downregulated again in plasma cells by the transcription factor Blimp-1. Myeloid cells express A1 either constitutively, e.g. granulocytes, or in response to inflammatory cytokines (e.g. TNF, G-CSF) or TLR ligation (LPS) in macrophages, or upon FcεRI mediated activation in an NF-AT-dependent manner in mast cells [89]. Outside of the haematopoietic system A1 is usually poorly expressed but is found in some solid tumours, best documented in melanoma [94,95]. Similar to Mcl1, A1 has a very short half-life and is subjected to rapid proteasomal turnover suggesting critical roles in adaptation and selection processes upon antigen challenge, inflammation or drug treatment [96]. The nature of E3-ligases involved in A1 turnover, however, is currently unknown.

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In *Eμ-A1a* transgenic mice, which do overexpress the mouse A1a isoform, defective pro- to pre-B cell transition was reported, leading to the accumulation of pro-B cells ($B220^+CD43^+IgM^-$) and reduced numbers of mature B cells in the periphery. However, this phenotype was quite variable between different founder lines [97]. Bone marrow B lineage cells showed reduced spontaneous apoptosis *ex vivo* and thymocytes were less sensitive to spontaneous death, γ-irradiation or dexamethasone, again in support of redundancy with Mcl1, Bcl2 and BclX. Similarly, *Lck*-driven expression of A1a in T cells caused cell death resistance of thymocytes, resting and activated T cells in response to different triggers of mitochondrial cell death [98]. Neither study reported on pronounced lymphadenopathy that might enable spontaneous tumour formation indicating that the expression levels achieved were insufficient to facilitate transformation.

Targeted deletion of *A1a* in mice was shown to affect neutrophil survival *in vitro* but failed to reveal other defects in lymphocyte development or homeostasis that might be explained by the rather poor expression of A1a in T and B cells and functional redundancy with A1b and A1d [11]. Mast cells derived from these mice lacked the ability to respond with increased survival upon *ex vivo* stimulation of FcεRI [99] and peritoneal macrophages showed a similar deficit upon exposure to microbial challenge [100].

A recent study using *in vivo* RNAi to knockdown all A1 isoforms present in mice revealed signs of delayed thymic development and impaired B cell homeostasis [101]. Two different model systems were generated and both were predicted to lead to a constitutive knockdown of A1 in all hematopoietic cells but the phenotypes observed were only partially overlapping. While in both models a reduction of the colony formation potential of granulocyte progenitors in the bone marrow was noted the effects on mature granulocyte survival varied significantly. More convincingly, A1 RNAi diminished the numbers of mature follicular B cells in the spleen and impaired their activation by mitogens [101]. Moreover, B cells displayed enhanced apoptosis rates upon BCR ligation suggesting that, in contrast to Bcl2 or BclX, Mcl1 and A1 ensure the survival of mature B cells upon mitogen-induced stimulation. Similarly, mast cell homeostasis and survival upon activation were impaired in mice with a constitutive A1 knockdown leading to protection from systemic and cutaneous anaphylaxis [102]. This suggests that mast cell homeostasis is also co-regulated by Mcl1 and A1 [80]. However, due to limitations of the employed RNAi systems the generation of sophisticated conditional alleles of *A1* in mice or a Cas/CRISPR knockout rat model are needed to increase our understanding of A1 in cell death control.

Implications for BCL2 targeting therapy

A critical question often asked in biomedical research is, of course, can we learn anything from the analysis of all these mouse mutants that would help us to predict the efficacy or possible side effects of BCL2-targeted therapy? As BH3-only proteins usually antagonize one or more anti-apoptotic BCL2 pro-survival proteins, can we anticipate that mimicking their function in patients causes some of the knockout or hypomorphic phenotypes observed in mice?

A number of so-called BH3-mimetics are currently in pre-clinical and clinical development, mainly for the treatment of malignant disorders [103]. The best and most specific agents of

this group share the mechanism of structurally displacing BH3-domains from the hydrophobic groove of anti-apoptotic Bcl2 family members, breaking up or preventing protein–protein interactions and thereby promoting Bax/Bak-dependent apoptosis [104-106]. Ideally, BH3-mimetics display specificity for one anti-apoptotic protein over the others. While in theory this might then affect all cells expressing the relevant anti-apoptotic protein, in practice it turns out that these treatments derive some measure of specificity for tumour cells providing a therapeutic window [103]. Reminiscent to this, not all healthy cells that express a certain anti-apoptotic Bcl2 family protein suffer consequences from its deletion. In fact sensitive tumour cells may have a balance between pro- and anti-apoptotic proteins that is much closer to the threshold for cell death than is the case in normal tissues, rendering them sensitive to drug treatment. This ‘primed to death’ phenotype may be mimicked to a degree in those cells that do suffer from gene ablation in mice, because they are in a vulnerable state near an apoptotic threshold, e.g. under the influence of developmental cues. Both seem to be the case and at least for some tumours early clinical experiences hint at feasibility and initial measures of success of the overall approach [103].

However, the development of these agents is in its early stages and has already encountered some major obstacles that have led to discontinuation of developmental programmes for some substances. A major obstacle in clinical development was, paradoxically, an extremely fast efficacy, which in a clinical context produces significant problems with so-called tumour lysis syndromes, but this problem was successfully met by application schedules using slow initial dose escalation [103,106]. In addition, the first generation BH3-mimetic navitoclax (which was derived from the lead compound ABT-737) was acting as a so-called ‘Bad-like mimetic’, thus achieving a significant inhibition of BCLX, BCL2 and Bcl2L2 [105,107]. This turned out to lead to problematic thrombocytopenia by directly killing platelets [108] and this side effect led to the discontinuation of the navitoclax programme in hematological malignancies in favour of the development of the Bcl2-specific ABT-199 [103]. However, ABT-263 is still investigated in smaller trials in solid tumours, usually in combination therapy [109,110]. A number of BH3-mimetics with other specificities are currently in development and pre-clinical analyses as well as clinical trials will discover their critical side effects [103]. The question is whether the knowledge acquired in loss-of-function models, as reviewed in this paper, may help to foresee problems in clinical development of these agents and whether this may aid the choice of directions these programmes should take.

The phenotypes of animals may present relevant pointers as to what side effects may develop when attacking a BCL2 family protein with a BH3-mimetic in patients. However, the description of phenotypes of mice usually carries some biases, warranting a note of caution. For example, embryonic or early postnatal phenotypes may not allow observation of important phenotypic changes that would be otherwise clear consequences of the alteration in an adult animal (or human in the end). Furthermore, the gene function during embryogenesis may not be very informative regarding the consequences of targeting in the adult organism (beyond the obvious extreme caution one has to take not to employ any of these drugs during pregnancy). Also, the dominance of some phenotypes may mask and distract from clinically more relevant alterations not noted or overlooked when analysing these animal models.

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For example, early data on BclX targeting were hampered by the embryonic lethality from a neurological phenotype. It was thus slightly surprising to find that ABT-263 had a profound effect on platelets. This thrombocytopenia was clearly puzzling at the time but might have been predicted by careful evaluation of the early data presented from *BclX*$^{fl/fl}$ *MMTV-Cre* mice reporting a decline in the number of circulating platelets [50]. The relevance of this finding was clarified only later in an ENU screen revealing destabilizing point mutations in BclX as a cause for the thrombocytopenia observed in these mouse mutants [51]. However, patients were already on clinical trial at that time [108], some of whom also presented with severe neutropenia that remains unexplained. By contrast the severe developmental consequences of targeting BclX and Bcl2 on the developing neuronal system and on developing kidneys did not clearly predict relevant CNS or renal toxicities in clinical trials with ABT-263 or ABT-199, although the CNS penetrance of the drugs may play an important role. Such predictions would have required the generation of tissue specific gene targeted mice. In practice, one prediction from the tissue specific targeting experiments outlined above would be testicular degeneration or osteopaenia upon prolonged BCLX inhibition [54,111]. However, the former should not be a concern to the usually advanced-age cancer patient. Skin hypersensitivity and sunburns may be another side effect, impinging on quality of life during therapy. On the other hand, neuronal complications may arise, similar to those in mice that lack BclX in retinal ganglion cells, upon axon injury [61]. While we do not know of reports to this end from clinical trials, these are examples for possible side-effects, predicted based on data from mouse model analysis.

The story, however, is even more complex. Observations from doubly targeted mice, such as the *Bcl2*$^{-/-}$*Bim*$^{-/-}$ deficient mice, show that ultimately it is the balance between specific pro-apoptotic BH3-only proteins and anti-apoptotic Bcl2 family members, rather than the lack of one of the latter group, that determines the outcome. One major lesson to be learned here is that not all tissues have the same thresholds. Indeed, loss of one allele of *Bim* rescued all phenotypes in the kidney and haematopoietic system almost completely, whereas the melanocyte loss in the hair follicles was only prevented when Bim was removed completely [34]. This suggests important gene dosage effects that may not become apparent (or predictable) by *in vivo* targeting of the BCL2 rheostat on one side alone. The gene dosage effects, however, may also play to our advantage. Based on observations in Mcl1-deficient models it seems likely that targeting Mcl1 specifically may carry an enormous toxic potential in a large number of tissues (see Table 1). However, given a specific vulnerability of certain tumour models, loss of a small part of Mcl1 protection in ‘incomplete drug targeting’ may not be enough to create a relevant toxic effect in healthy tissue while it may be very effective in tumours, as has been proposed for murine models of Burkitt’s lymphoma [112], B-acute lymhoblastic laeukemia (B-ALL) [113] or acute myeloid leukaemia (AML) [114].

It is also very important to determine the specificity of BH3-mimetics for binding to anti-apoptotic Bcl2 family members, since this carries important information on possible mechanisms of drug resistance. Clearly, overexpression of Mcl1 or A1/Bfl1 will be able to serve as a mechanism of resistance regarding a substance like ABT-199 that is unable to bind to these pro-survival molecules [115,116], but this is also true with regard to toxicities,

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so that normal tissues with constitutively high Mcl1 expression may not experience drug-related toxicities. The genetic models surveyed in this review thus contain information on where a key resistance player shows important functional dominance. One may conclude that such tissues may have relevant means to escape toxicities when treated with a certain type of BH3-mimetic. Of note, basic scientists can also learn from side effects in (pre)clinical studies about the BCL2 dependence of certain cell types. This is nicely exemplified by the prevention of tamoxifen-driven endometrial hyperplasia by ABT-737-driven apoptosis, suggesting BclX or Bcl-w dependence, as ABT-199 was not reported to show such an effect [117].

A final important aspect is the fact that the Bcl2 family is an important determinant of the outcome of a number of developmental checkpoints, among them immune checkpoints that are still very active in adults. Toxicities and side effects are thus not simply determined by loss of certain cells from a tissue. Immune defects and autoimmunity may be important consequences of altering the BCL2 balance and need to be taken into account [118]. First evidence, again from animal models, is provided by the observation that ABT-737 treatment impacts severely on the persistence of memory B cells, the establishment of bone marrow plasma cells, as well as the induction of a cytotoxic T cell response, and hence adoptive immunity [119]. Which of these effects are mediated by inhibition of BclX, Bcl2 or both remains to be sorted out. Of note, however, lymphocytopenia was also reported in a significant number of patients on the navitoclax phase I study [108].

Taken together the knowledge gained from Bcl2 mouse mutants is clearly of significant value and provides a first guide of what to expect, but will fall short in details in the light of the complexity of the interactions in the Bcl2 family network, precluding reliable and precise predictions of the effects of pharmacological targeting of the family in patients. Thus, the level of sophistication that animal models need to develop, in order to help physicians leave the path of trial and error, still needs to increase significantly. Yet, these models are simply indispensable to create an understanding about the role of these proteins in normal physiology that ultimately is the key to exploring their role in human pathology and their drug-target potential.

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Abbreviations

AML	acute myelois leukaemia
B-ALL	B acute lymhoblastic leukaemia
BCR	B cell receptor
BM	bone marrow

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ConA	concanavalin A
DMBA	7,12-dimethylbenz[α]anthracene
DN	double negative
DP	double positive
LCMV	lymphocytic choriomeningitis virus
NP-KLH	4-Hydroxy-3-nitrophenylacetyl-Keyhole Limpet Hemocyanin
RGC	retinal ganglion cell
SCF	stem cell factor
Treg	regulatory T cell

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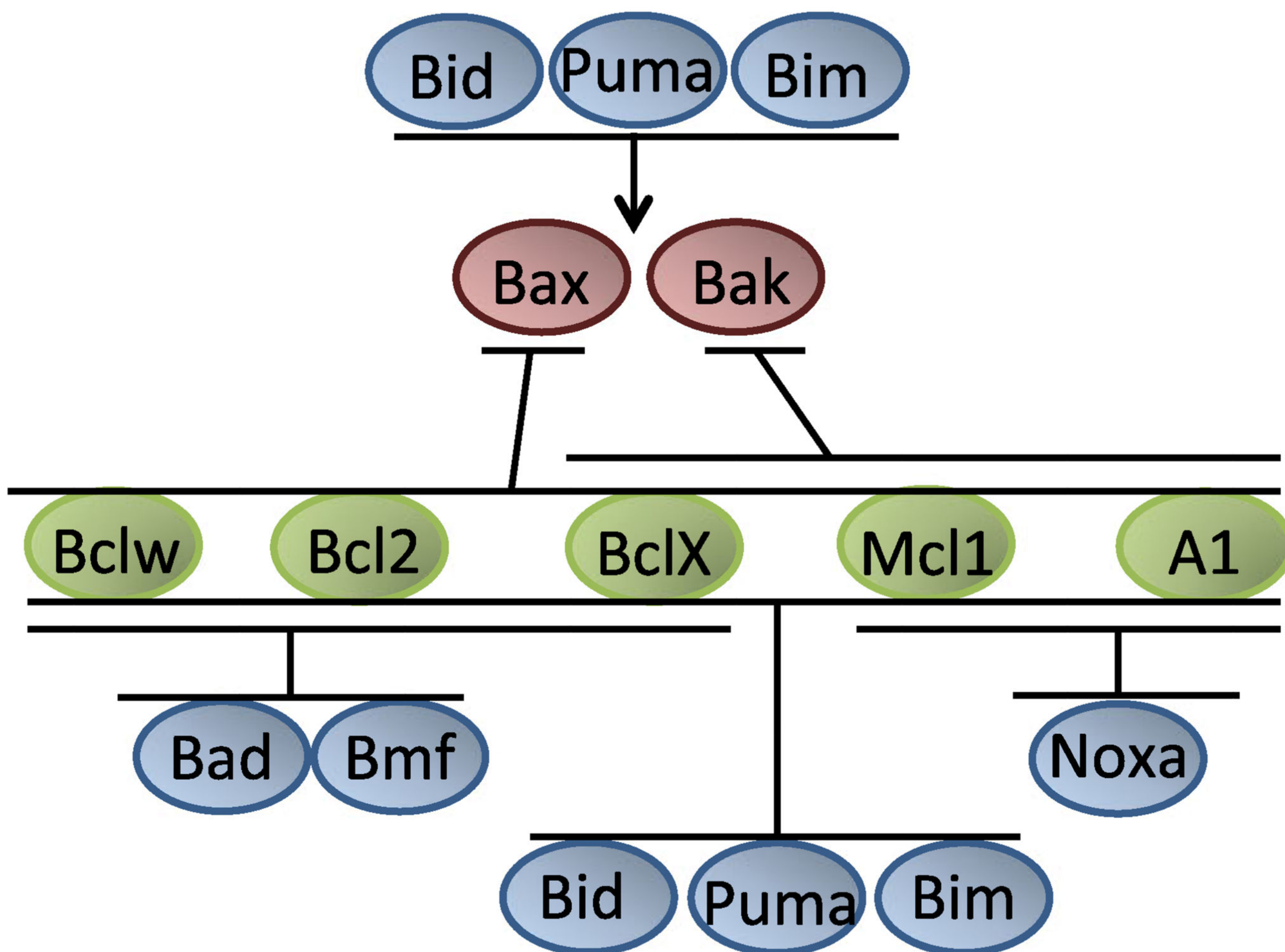

Fig. 1.

Liaisons in the Bcl2 family. The Bcl2 family can be categorized into three classes of proteins that control mitochondrial cell death by complex protein–protein interactions, indicated here. These interactions are based on different affinities between individual family members and are influenced by various post-translational protein modifications. Anti-apoptotic Bcl2, BclX, Bclw, Mcl1 and A1/Bfl1 show only partially overlapping binding preference for pro-apoptotic Bax and Bak. Selective binding is also intricate to individual BH3-only proteins that act as sensors of cell stress and bind selectively to one or more anti-apoptotic Bcl2 proteins while some can also interact directly with Bax or Bak to promote mitochondrial outer membrane permeabilization. For detailed information on the molecular mode of action, see [1,2].

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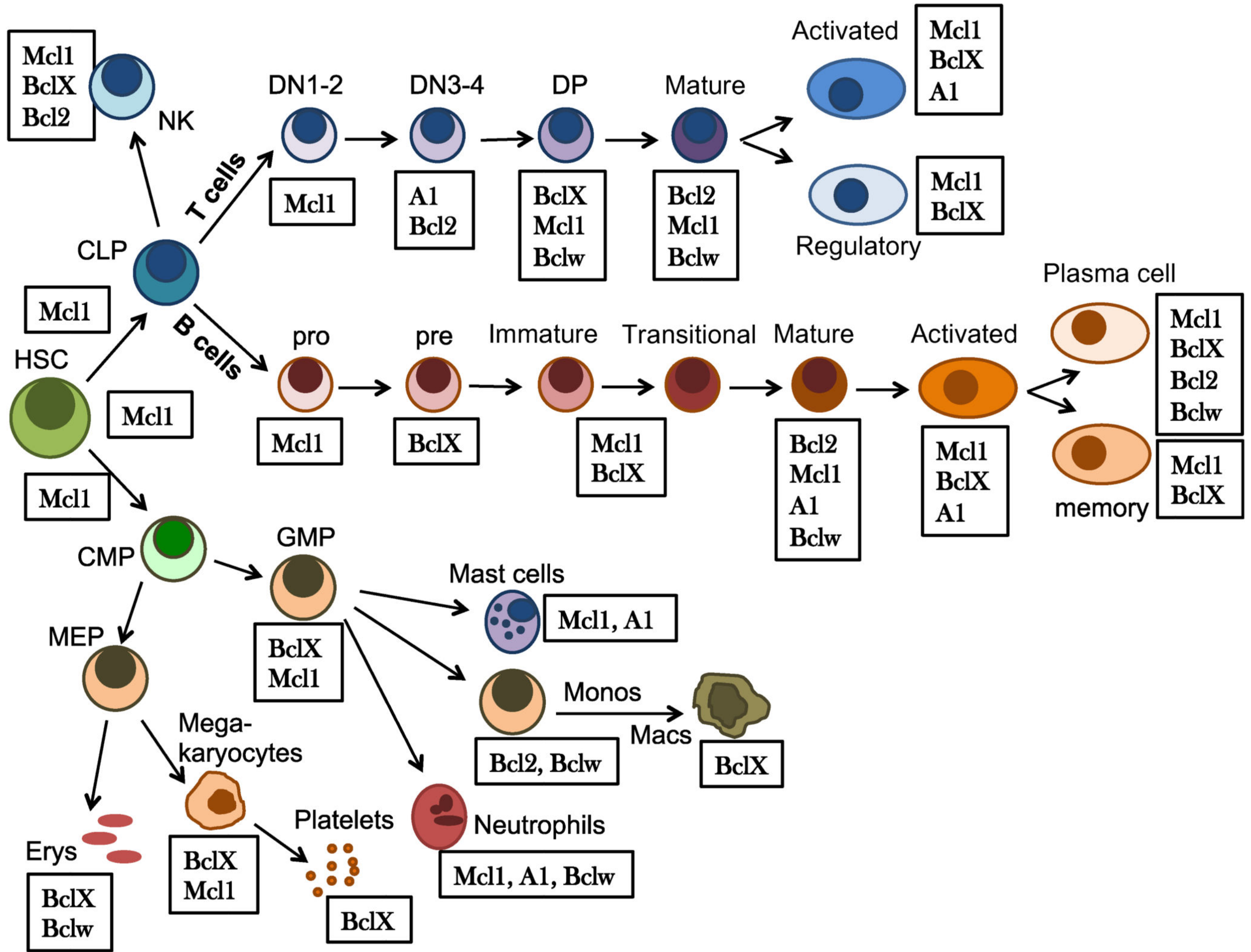

Fig. 2.

Dynamic expression changes of Bcl2 family proteins in haematopoiesis. Expression of individual Bcl2 family proteins is dynamically regulated in the developing hematopoietic system in response to a broad range of developmental cues, including cytokines, interactions of blood cells with stroma or antigen receptor expression and reactivity. Despite the fact that usually more than one anti-apoptotic Bcl2 family member is expressed at a given developmental stage, loss of function usually affects only a subset of blood cells. For example, why loss of Mcl1 is usually fatal, despite the fact that Bcl2 or BclX is still present, is poorly understood. Similarly, the need to change the panel of pro-survival proteins expressed during, for example, lymphocyte development is in many instances still unclear. In this figure we rank the expressed proteins according to the severity of the reported knockout phenotypes reported. Note also that the knockout of many of the Bcl2 proteins listed as expressed in a certain cell type has no effect on the survival of these cells, at least under homeostatic conditions (see also Table 1).

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Table 1

Summary of Bcl2 family protein knockout lines generated and phenotypes observed. A more detailed description of the major phenotypic changes and possible implications for targeted therapy can be found in the text.

Bcl2 $^{-/-}$	• Postnatal growth retardation, hypopigmentation, early postnatal death due to fatal polycystic kidney disease • Involution of the spleen and thymus with severe loss of mature lymphocytes; normal mitogenic activation • Increased death of intestinal epithelium • Progressive postnatal degeneration of motoneurons, sympathetic and sensory neurons • Myeloid lineage development favoured upon HSC transfer • Normal T cell activation and dispensable for Treg cell function	[7-9,29,31-33]
Bcl2 $^{fl/fl}$ *LysM-Cre*	• Bcl2 deficiency in granulocytes and macrophages; no effect on macrophages in steady state, but only on advanced macrophage apoptosis in atherosclerotic lesions	[35]
BclX $^{-/-}$	• Embryonic lethality at E13 • Extensive apoptosis in the developing CNS, loss of erythroblasts and mature erythrocytes • Reduced numbers of pre-B and immature B cells in bone marrow; and enhanced cell death of lymphocyte progenitors • Reduced formation of T and B cells in blastocyst reconstitution experiments	[5,6,53]
Bcl-x $^{fl/fl}$ *Lck-Cre*	• Number of thymocytes reduced by half but normal subset distribution • Naïve T cells in the spleen reduced by half due to reduced thymic output	[46]
BclX $^{fl/fl}$ *CD4-Cre*	• Minor reduction in T cell number due to functional redundancy with Mcl1 during development	[46]
BclX $^{fl/fl}$ *Rag1-Cre*	• B cell development arrested at the pre-B cell stage	[47]
BclX $^{fl/fl}$ *Aicda-Cre*	• Reduced bone marrow plasma cell number	[48]
BclX $^{fl/fl}$ *MMTV-Cre*	• Reduced platelet numbers • Megakaryocyte and erythroblast amplification • Haemolytic anaemia • Accelerated involution of mammary epithelium	[50,55]
BclX $^{fl/fl}$ *Pf4-Cre*	• Defective platelet shedding, but normal maturation of megakaryocytes • Redundancy with Mcl1	[52,120]
BclX $^{fl/fl}$ *K5-Cre*	• Normal skin development • Increased spontaneous apoptosis of keratinocytes and higher sensitivity to UVB • Reduced susceptibility to skin cancer	[56,57]
BclX $^{fl/fl}$ *Sftpc–Cre*	• Reduced protection of respiratory epithelium against oxygen-induced toxicity • Early postnatal death in about 50% of offspring	[60]
BclX $^{fl/fl}$ *Alb-Cre*	• Increased spontaneous hepatocyte apoptosis, liver fibrosis	[58,59]

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	• Hypersensitivity to inflammatory signals	
BclX$^{fl/fl}$ *Six3-Cre*	• Loss of retinal ganglion cells (RGC) in the embryo • Reduced retinal thickness • RGC maintenance after axon injury in the adult	[61,63]
BclX$^{fl/fl}$ *TH-Cre*	• Reduction in catecholaminergic neurons • Deficiency in formation of substantia nigra	[62]
BclX$^{fl/fl}$ *CathepsinK-Cre*	• Increased bone resorption • Osteopaenia	[62]
Mcl1$^{-/-}$	• Peri-implantation embryonic lethality • Null blastocysts unable to hatch or attach *in vitro*	[4]
Mcl1$^{fl/null}$ *Mx-Cre*	• Loss of B and T cells in Rag2 transfer experiments • Bone marrow failure after systemic deletion • Loss of antigen-specific effector T cells upon LCMV infection	[73,76,77]
Mcl1$^{fl/fl}$ *Cre*^{ER}	• Rapid death of T cells *in vitro*, not rescued by IL-7 • Reduced viability upon T cell activation • Impaired plasma cell survival	[46,79]
Mcl1$^{fl/fl}$ *Rag1-Cre*	• B cell development arrested at the pro-B cell stage	[47]
Mcl1$^{fl/null}$ *CD19-Cre*	• Pre-pro-B ($CD43^+B220^+CD19^-$) fraction unaffected • Pro-B and pre-B cell population markedly reduced; arrest of B cell development	[76]
Mcl1$^{fl/fl}$ *Aicda-Cre*	• Impaired generation of antigen-specific B cells • Loss of germinal centres and memory B cells • IgG1 but not IgM titre reduced	[48]
Mcl1$^{fl/null}$ *Lck-Cre*	• Thymocytes reduced 5-fold • Accumulation of DN2 stage thymocytes	[76]
Mcl1$^{fl/fl}$ *CD4-Cre*	• Drastic reduction of DP thymocytes > 80%; low level expression of TCR-β • Mature SP thymocyte populations and mature T cells decreased	[46]
Mcl1$^{fl/fl}$ *Ncr-1-Cre*	• Complete loss of NK cells in all tissues	[49]
Mcl1$^{fl/fl}$ *LysM-Cre*	• Impaired neutrophil maturation • Accumulation of immature myeloid precursors in the BM • No effect on resting monocytes and macrophages	[74,75]
Mcl1$^{fl/fl}$ *Cpa3-Cre*	• Impaired mast cell survival • Resistance to systemic anaphylaxis	[80]
Mcl1$^{fl/fl}$ *Pf4-Cre*	• Normal megakaryocyte development and platelet production • Redundancy with BclX	[52,120]

Mcl1^fl/fl^ Alb-Cre	• Severe liver damage and compensating proliferation • Hepatocellular carcinoma • Redundancy with BclX	[59,81]
Mcl1^fl/fl^ Ckmm-Cre	• Thinning of the heart muscle, cardiac dilatation • Thrombus deposition, severe cardiomyopathy and fibrosis • Early postnatal death	[85]
*Myh-Cre^ER^*	• Impaired heart muscle contractility • Heart failure	[85]
Mcl1^fl/fl^ Nestin-Cre	• Massive cell death of newly committed neurons • Embryonic lethality before E15	[86]
Mcl1^fl/fl^ Foxg1-Cre	• Reduced brain mass, impaired telencephalic development • Embryonic lethality between E16 and E17	[86]
Mcl1^fl/fl^ CamKIIα Cre	• Loss of cortical neurons associated with autophagic stress • Early postnatal mortality	[88]
$A1a^{-/-}$	• Reduced survival of neutrophils *ex vivo* • Impaired response of $A1a^{-/-}$ macrophages to microbial patterns • Impaired survival of activated mast cells	[10] [99,100]
A1 RNAi	• Reduced number of follicular B cells • Enhanced B cell apoptosis upon BCR ligation • Reduced colony formation of granulocyte progenitors • Impaired mast cell survival upon activation	[101,102]

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