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ARTICLE

Mcl-1 and Bcl-x_L sequestration of Bak confers differential resistance to BH3-only proteins

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Abstract

The prosurvival Bcl-2 family proteins Mcl-1 and Bcl-x_L inhibit apoptosis by sequestering BH3-only proteins such as Bid and Bim (MODE 1) or the effector proteins Bak and Bax (MODE 2). To better understand the contributions of MODE 1 and MODE 2 in blocking cell death, and thus how to bypass resistance to cell death, we examined prescribed mixtures of Bcl-2 family proteins. In a Bim and Bak mixture, Bcl-x_L and Mcl-1 each sequestered not only Bim but also Bak as it became activated by Bim. In contrast, in a Bid and Bak mixture, Bcl-x_L preferentially sequestered Bid while Mcl-1 preferentially sequestered Bak. Notably, Bcl-x_L could sequester Bak in response to the BH3 mimetic ABT-737, despite this molecule targeting Bcl-x_L. These findings highlight the importance of Bak sequestration in resistance to anti-cancer treatments, including BH3 mimetics.

Introduction

The mitochondrial pathway of apoptotic cell death is regulated by three subfamilies of Bcl-2 proteins: prosurvival proteins such as Bcl-2, Bcl-x_L and Mcl-1; proapoptotic effectors Bak and Bax; and BH3-only proteins such as Bid and Bim [1, 2]. Defects in the mitochondrial pathway are permissive for tumorigenesis and most chemotherapeutic agents act via this pathway [3]. Small-molecule BH3 mimetics that specifically target Bcl-2 (venetoclax/ABT-199 (ref. [4])) or Mcl-1 (S63845 (ref. [5])) or multiple family members (ABT-737 and navitoclax/ABT-263 (refs. [6–8])) are proving effective as anti-cancer agents. However, chemoresistance can be caused by non-targeted prosurvival proteins, although the mechanisms remain poorly understood [4–9].

Apoptosis can be triggered by damage signals which either upregulate or activate BH3-only proteins, which then convert Bak and Bax into the activated forms capable of homo-oligomerisation and mitochondrial pore formation. The resulting cytochrome *c* release is the 'point of no return' for apoptotic cell death. The mechanism by which Bcl-2 family proteins interact to block cytochrome *c* release has been controversial, but in recent years a consensus has emerged [10–15]. In a unified model (Fig. S1), prosurvival proteins can sequester BH3-only proteins to block apoptosis upstream of Bak and Bax activation, called 'MODE 1 inhibition' [13]. Prosurvival proteins can also act downstream to sequester the activated forms of Bak or Bax, called 'MODE 2 inhibition' [13]. The relative contributions of MODE 1 and 2 to apoptosis resistance will depend on several factors including the Bcl-2 family proteins present and their relative concentrations, the specific binding affinities between them, and the activation status of Bak and Bax [13,16,17].

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Protein interactions involved in MODE 1 inhibition have been widely studied *in vitro*, using BH3-only peptides and recombinant prosurvival proteins [16,18]. Analysis of MODE 2 inhibition has been more limited as the generation of MODE 2 complexes requires the additional components of Bak or Bax as well as membranes to allow Bak and Bax activation [19,20]. In mixtures of Bid or Bim, Bcl-x_L or Mcl-1, and Bak or Bax, most resistance occurred via MODE 1 (refs. [21,22]). However, MODE 2 was enhanced when mutation decreased the affinity of BH3-only protein for prosurvival proteins [13,21]. Moreover, MODE 2 was decreased when mutations decreased the affinity of Bak or Bax for their prosurvival guardians, causing an increase in apoptosis [23–27]. Thus, several studies indicate that both MODE 1 and MODE 2 can contribute to apoptosis resistance, but that the relative contributions differ depending on context.

The present study sought to understand the relative contributions of MODE 1 and MODE 2 to prosurvival protein function by analysing defined mixtures of full-length Bcl-2 proteins at near-physiological concentrations in mitochondrial incubations and intact cells. In most combinations tested, both Mcl-1 and Bcl-x_L functioned in part by sequestering Bak, i.e. MODE 2. Notably, in response to Bid, Mcl-1 caused particularly profound resistance via MODE 2 in mitochondria and in cells. We also found that the BH3 mimetic ABT-737 (that binds Bcl-x_L, Bcl-2 and Bcl-w) was able to disrupt MODE 1 complexes to induce Bak:Bcl-x_L MODE 2 complexes, with higher concentrations required to disrupt those complexes and permeabilise mitochondria. These studies highlight the importance of MODE 2 in the function of Mcl-1 and Bcl-x_L to constrain Bak, and the potential importance of MODE 2 inhibition in chemoresistance.

Results

The concept of prosurvival proteins acting via two MODES to inhibit mitochondrial permeabilisation [13] allows a simplified description of interactions between Bcl-2 family proteins, and is continued here. For clarity, sometimes a distinction is made between the protein complexes (e.g. MODE 1 complexes) and the functional outcome of inhibiting cytochrome *c* release (e.g. MODE 1 inhibition). MODE 1 will mostly be considered in terms of MODE 1 inhibition because complexes are difficult to detect (and because MODE 1 complexes promote cytochrome *c* release if Bak has become activated; see below). MODE 2 will also mostly refer to MODE 2 inhibition, as although MODE 2 complexes are easier to estimate, they may not be sufficient for MODE 2 inhibition.

Mcl-1 blocks Bid signalling by sequestering activated Bak

To compare Mcl-1 binding of BH3-only proteins (MODE 1) with its binding of Bak (MODE 2), the first mixtures we analysed included Mcl-1 and Bak, together with Bid or the Bid^{Bim} chimera which contains the BH3 domain of Bim [20]. To obtain Bak we used mouse liver mitochondria (MLM) which contain Bak but minimal other Bcl-2 family proteins [13,28,29]. We expected that Bid and Bid^{Bim} might compete differently with activated Bak for binding to Mcl-1, as Mcl-1 binds the Bid BH3 peptide weakly compared to that of Bim (Fig. S1B and Fig. 1a). Importantly, however, Bid was only slightly more efficient than Bid^{Bim} in activating Bak and releasing cytochrome *c* in the absence of Mcl-1 (Fig. 1b) [20], allowing us to test how altering MODE 1 affects MODE 2 for Mcl-1.

To test the effect of Mcl-1, MLM were pre-incubated with Mcl-1ΔNC at 35 nM (Fig. 1c). Incubations in which Mcl-1 acted via MODE 1 inhibition (to prevent Bak activation) are shown by green brackets, and were identified as those in which Bid or Bid^{Bim} could trigger Bak activation in the absence, but not in the presence, of Mcl-1 (see proteinase K treatment; Fig. 1b and c). Incubations in which Mcl-1 was acting via MODE 2 inhibition are shown by blue brackets, and were identified in three ways: Bak activation without cytochrome *c* release; Bak cleavage by proteinase K to an ~10 kDa fragment; and Bak co-immunoprecipitation with Mcl-1 (Fig. 1c). A semi-quantitative comparison of MODE 1 and MODE 2 inhibition is provided in Fig. S2A and B.

We note that the ~10 kDa fragment of Bak generated by proteinase K is a new means of identifying Bak when sequestered in MODE 2. The fragment was recognised by antibodies that bind to the Bak BH3 domain (clone 4B5), and was released into the supernatant (Fig. 1c and Fig. S3), consistent with the Bak BH3 domain binding to C-terminally truncated Mcl-1. The strong correlation of the 10 kDa fragment with Bak:Mcl-1 co-immunoprecipitation also indicates that Bak:Mcl-1 heterodimers are stable in digitonin, as previously shown for Bak homodimers [30].

Mcl-1 was very effective at blocking cytochrome *c* release in response to Bid, even at 300 nM Bid (Fig. 1c, left panels). MODE 1 inhibition was evident at low Bid concentrations (0.03–0.1 nM), while MODE 2 inhibition was evident at higher concentrations (0.3–300 nM). Thus, in isolated mitochondria Mcl-1 caused resistance to a wide range of Bid concentrations, but profound resistance was associated with sequestration of activated Bak.

Mcl-1 was less effective at blocking cytochrome *c* release in response to Bid^{Bim} (Fig. 1b, c, right panels). MODE 1 inhibition was evident up to 1 nM Bid^{Bim}, consistent with the high affinity of Bid^{Bim} for Mcl-1. MODE 2

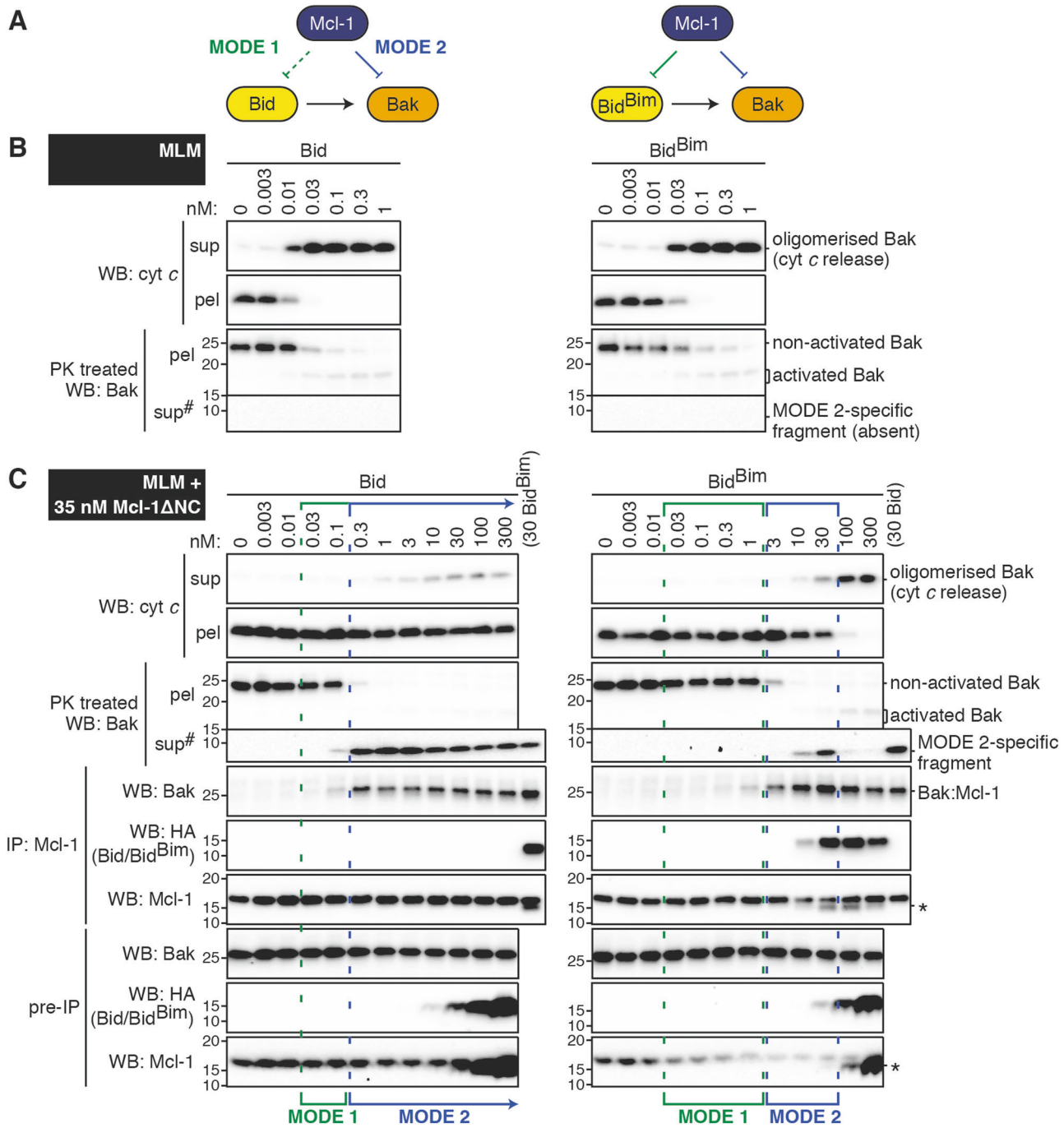

Fig. 1 MODE 2 complexes of Bak:Mcl-1 profoundly inhibit Bid signalling. **a.** Diagram showing interactions between the Bcl-2 family members in the tripartite mixtures tested. The BH3-only proteins Bid and Bid^{Bim} induce similar activation (arrows) of the apoptotic effector Bak. The prosurvival protein Mcl-1 can sequester the BH3-only protein or activated Bak. Note that Bid binds poorly (dotted lines) to Mcl-1 (MODE 1 complex), allowing Mcl-1 to bind activated Bak (MODE 2 complex). **b**, **c** Mouse liver mitochondria (MLM) supplemented with **c** or without **b** Mcl-1 were treated with Bid or Bid^{Bim} at the indicated concentrations. Mitochondrial cytochrome *c* release was assessed by western blot (WB) of supernatant (sup) and pellet (pel) fractions. Bak conformation change (activation) was assessed by incubation with proteinase K (PK) followed by separation to supernatant and pellet. Protein–protein interactions were determined by solubilising samples (pre-IP), immunoprecipitating (IP) for Mcl-1, and western blotting for Bak, HA (Bid or Bid^{Bim}) or Mcl-1. Samples in which Mcl-1 blocked the activation of Bak (MODE 1 inhibition, green) or blocked cytochrome *c* release by binding activated Bak (MODE 2 inhibition, blue) are highlighted. **b** and **c** are from the same experiment, and together with two independent experiments were quantified for MODE 1 and 2 inhibition (Fig. S2B). #Long exposure of supernatant blots compared to pellets. *Bands on Mcl-1 blots are due to previous probing for HA.

inhibition was evident at intermediate levels (~3–30 nM) of Bid^{Bim}, consistent with activated Bak competing with Bid^{Bim} for binding to Mcl-1. Cytochrome *c* was released at higher Bid^{Bim}. Thus, Mcl-1 could function by binding both Bid^{Bim} (MODE 1) and activated Bak (MODE 2), but strong binding of Bid^{Bim} to Mcl-1 (seen by co-immunoprecipitation at ~30 nM Bid^{Bim}) was able to overcome both resistance mechanisms.

In summary, both modes of inhibition contributed to the ability of Mcl-1 to block cytochrome *c* release induced by either Bid or Bid^{Bim}. However, MODE 2 inhibition was dominant in response to Bid due to the low affinity of Bid for Mcl-1, protecting mitochondria from even super-stoichiometric concentrations of Bid.

Mcl-1 transiently blocks Bid^{Bim} signalling by sequestering activated Bak

We next performed time-course experiments to test whether high Bid^{Bim} was able to disrupt Bak:Mcl-1 MODE 2 complexes, or only prevent their formation. As in Fig. 1c, MLM were pre-incubated with or without Mcl-1, but Bid or Bid^{Bim} concentrations were increased over time to mimic their up-regulation in cells during apoptotic signalling (Fig. 2a). In the absence of Mcl-1, ~3 nM of either Bid or Bid^{Bim} activated Bak and released cytochrome *c* at around 30 min (Fig. 2b). In the presence of Mcl-1, Bid also rapidly activated Bak, but this was sequestered by Mcl-1, preventing cytochrome *c* release, even after 110 min at the highest Bid concentration (Fig. 2c). Bid^{Bim} also rapidly activated Bak to generate Bak:Mcl-1 complexes, but cytochrome *c* was released after 80 min at the highest Bid^{Bim} concentration, indicating that Bid^{Bim} had disrupted the Bak:Mcl-1 complexes.

Thus, low affinity of Bid for Mcl-1 resulted in durable MODE 2 complexes, while higher affinity of Bid^{Bim} for Mcl-1 allowed it to disrupt MODE 2 complexes (semi-quantitated in Fig. S2C). This disruption required at least 80 min with high levels of Bid^{Bim}, suggesting a slow off-rate for activated Bak bound to Mcl-1. Therefore, MODE 2 sequestration may play a significant role in inhibiting apoptosis triggered by many BH3-only proteins, even for proteins like Bim that have a strong affinity for Mcl-1.

Full-length Mcl-1 also binds more Bak after Bid than after Bid^{Bim}

We next examined how full-length Mcl-1 binds Bak in response to Bid or Bid^{Bim}, since it was possible that Mcl-1 lacking the C-terminus has altered binding specificity, as we and others have recently found for Bcl-x_L [20,31]. We took advantage of *Mcl-1*$^{flox/flox}$ mice which have slightly raised Mcl-1 levels [32]. Liver mitochondria from these mice were incubated with Bid or Bid^{Bim}, as performed in Fig. 1c. As seen with truncated Mcl-1, more Bak:Mcl-1 complexes were detected after treatment with Bid than with Bid^{Bim} (Fig. 3b), indicating that both truncated and endogenous full-length Mcl-1 have higher affinity for Bid^{Bim} than for Bid.

Full-length forms of Bim promote MODE 2 while truncated forms do not

To examine whether Bid^{Bim} was a faithful mimetic of Bim, full-length Bim_S generated by *in vitro* transcription/translation (IVTT) was incubated with mitochondria in the presence of Mcl-1, and examined for MODE 1 and MODE 2 (Fig. 4a). We tested IVTT Bim_S at 5% v/v and lowered the Mcl-1 concentration because >5% v/v IVTT reaction mixture inhibited Bak activation, as reported previously [33]. Bim_S partially activated Bak, which bound to Mcl-1 in MODE 2 (blue brackets, Fig. 4a), indicating that Bim activity is well represented by Bid^{Bim}.

We also tested whether truncated forms of Bim ($Bim_S\Delta C$ and the Bim BH3 peptide) could promote MODE 2 complexes. Bim_S and other BH3-only proteins contain a C-terminal membrane anchor [19,34,35] that is important for Bak activation [19,20]. Accordingly, both truncated forms required higher levels than Bid^{Bim} to activate Bak at mitochondria (Fig. 4a), and only a minor portion of $Bim_S\Delta C$ associated with the membrane fraction (Fig. S4). Notably, there was no evidence of MODE 2 complexes in response to either truncated Bim reagent (Fig. 4a). We conclude that the high levels of $Bim_S\Delta C$ and BH3 peptide required to activate Bak at mitochondria also engaged a large portion of the Mcl-1 in MODE 1, depleting that available for MODE 2 (Fig. 4b).

In summary, Bid^{Bim} is a more accurate mimic of the cellular Bim protein than is $Bim_S\Delta C$ or Bim BH3 peptide. In addition, the contribution of MODE 2 may be grossly underestimated in studies using BH3 peptides or truncated BH3-only proteins.

Bcl-x_L blocks Bid^{Bim} signalling partly via MODE 2

We next examined MODE 1 and MODE 2 in mitochondrial mixtures containing Bcl-x_L rather than Mcl-1. As both Bid and Bid^{Bim} have high affinity for Bcl-x_L [20], we included a Bid mutant (M97A) that has reduced affinity for Bcl-x_L but retains the ability to activate Bak [27] (Fig. 5a). As expected, the Bid variants had similar ability to activate Bak and release cytochrome *c* in the absence of Bcl-x_L (Fig. 5b), allowing us to test how altering MODE 1 affects MODE 2 for this prosurvival protein.

Bcl-x_L efficiently sequestered both Bid and Bid^{Bim} via MODE 1, as 10 nM of Bid or Bid^{Bim} was required to

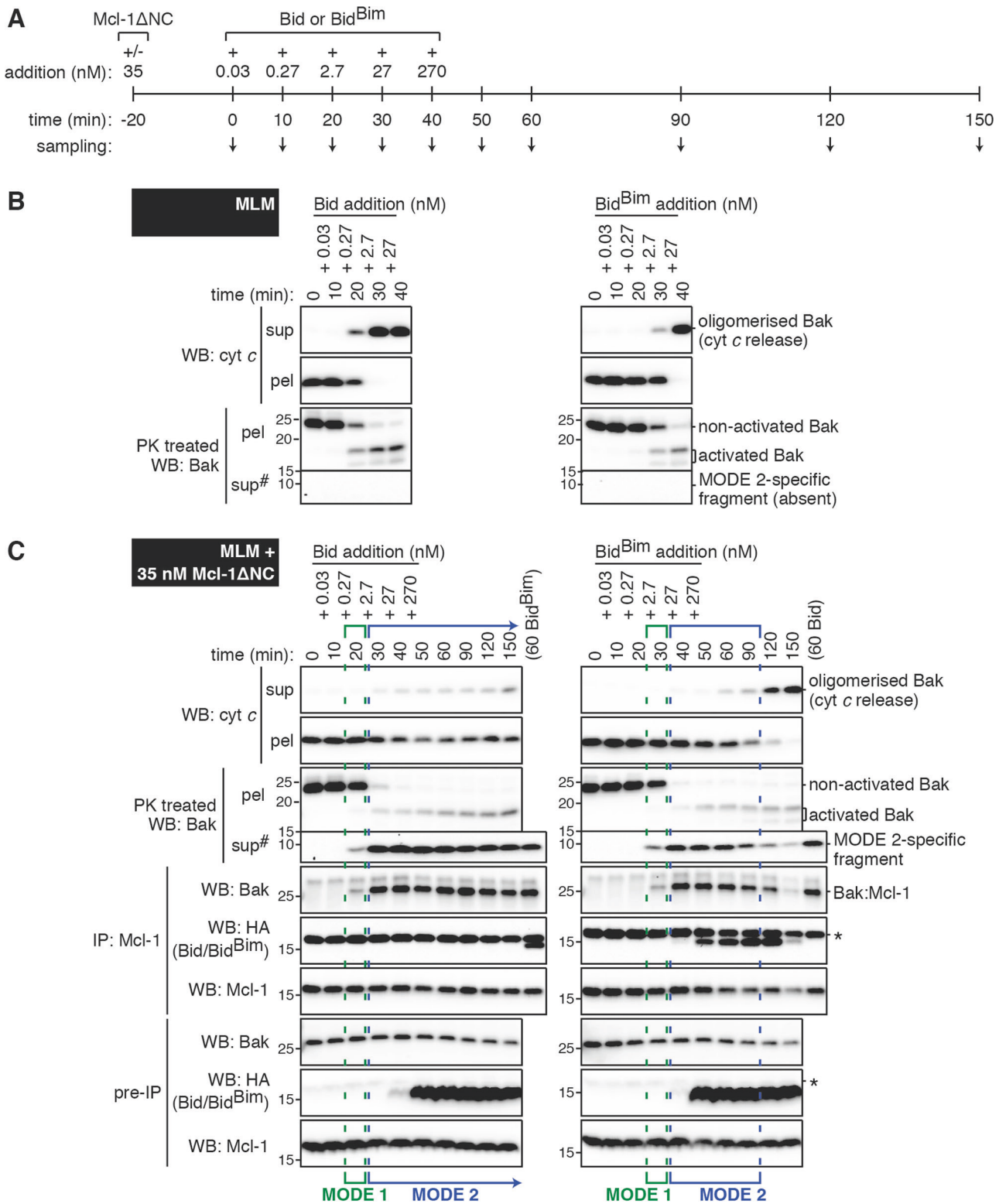

Fig. 2 MODE 2 complexes of Bak:Mcl-1 can be disrupted by Bid^{Bim} to cause cytochrome *c* release. **a** Timeline of protein addition and sampling. **b**, **c** Mouse liver mitochondria (MLM) supplemented with **c** or without **b** Mcl-1 were treated with increasing doses of Bid or Bid^{Bim} according to the timeline in **a**. Samples collected at the indicated time points were assessed as in Fig. 1. **b** and **c** are from the same experiment, and together with two independent experiments were quantified for MODE 1 and MODE 2 inhibition (Fig. S2C). #Long exposure of supernatant blots compared to pellets. *Bands on HA blots are due to previous probing for Mcl-1

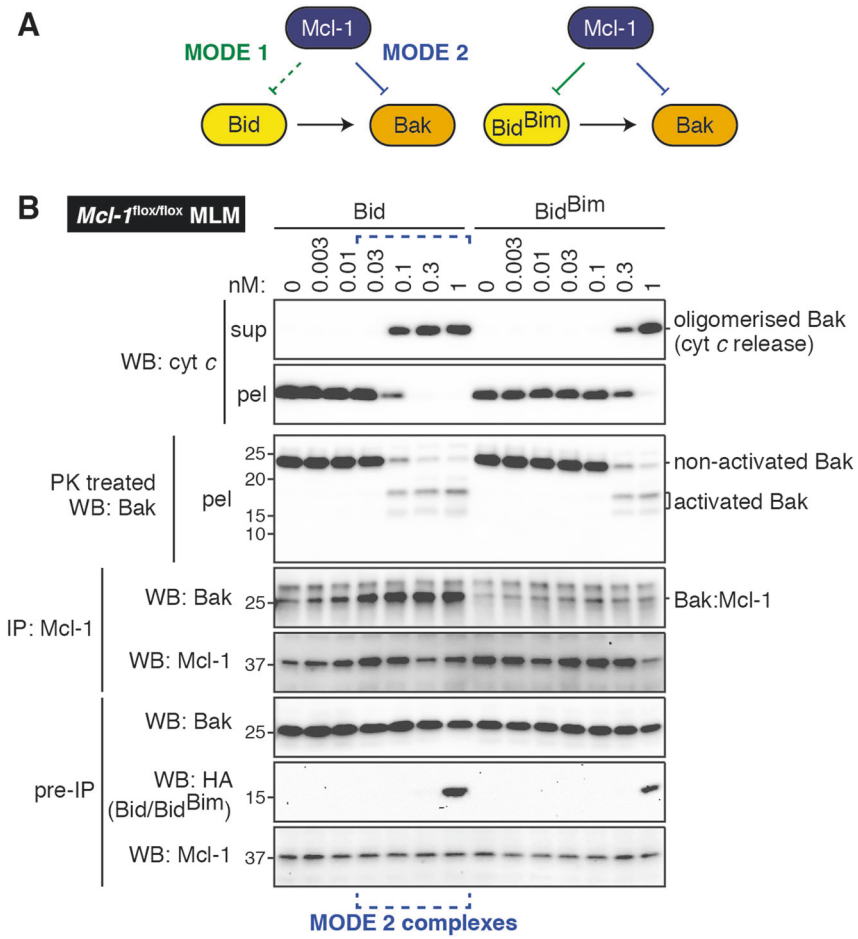

Fig. 3 Full-length Mcl-1 also binds more Bak after Bid than after Bid^{Bim}. **a** Diagram showing interactions between the Bcl-2 family members in the tripartite mixtures tested. **b** Mouse liver mitochondria (MLM) from *Mcl-1*$^{flox/flox}$ mice were treated with Bid or Bid^{Bim} at the indicated concentrations. Samples were assessed as in Fig. 1. Blots are from the same experiment, representative of two independent experiments. Note that any Bid or Bid^{Bim} co-immunoprecipitated with Mcl-1 (MODE 1) was below the limits of detection and therefore not shown.

activate Bak in the presence of 10 nM Bcl-x_L (Fig. 5c and Fig. S2D) compared to 0.03 nM in its absence. In fact, Bcl-x_L blocked Bid only via MODE 1 inhibition, as no dose of Bid caused Bak activation without cytochrome *c* release, consistent with a previous report that Bcl-x_L acted only via MODE 1 in a Bid and Bax mixture [21]. Bcl-x_L did block one concentration of Bid^{Bim} (10 nM) via MODE 2 inhibition. Thus, in these conditions Bcl-x_L caused resistance to Bid- and Bid^{Bim}-induced cytochrome *c* release predominantly by directly sequestering these proteins (MODE 1) rather than by sequestering activated Bak (MODE 2).

In the Bid M97A incubations, Bcl-x_L could still act via MODE 1 to block low concentrations of the M97A variant (green brackets, Fig. 5c), indicating some affinity. However, MODE 2 inhibition was prominent, persisting even at the highest doses of Bid M97A (blue brackets, Fig. 5c) (semi-quantitated in Fig. S2D). Thus, Bcl-x_L may be able to robustly inhibit apoptosis via MODE 2 if the upregulated BH3-only proteins can activate Bak but bind only poorly to Bcl-x_L.

We note that in these Bcl-x_L experiments the MODE 2-specific fragment of Bak generated by proteinase K was in the pellet fraction (Fig 5C and S3), rather than in the supernatant as in the Mcl-1 experiments (Fig 1C and S3). This is likely due to greater membrane-insertion of full-length Bcl-x_L compared to C-terminally truncated Mcl-1.

In cells, endogenous Mcl-1 and Bcl-x_L bind Bak in response to tBid or Bim$_S$ signalling

To study the role of MODE 2 complexes formed by Mcl-1 and Bcl-x_L in a cellular context, we used the DU145 prostate carcinoma cell line. These cells are expected to mimic the mitochondrial system used in Figs. 1 and 5, as apoptosis is dependent on Bak due to a frameshift mutation in Bax [36], and the cells have sufficient Mcl-1 and Bcl-x_L to cause resistance to TRAIL [37]. To obtain rapid expression of tBid (p15 fragment of Bid) or Bim$_S$, DU145 cells were transduced with doxycycline-inducible expression constructs. Upon doxycycline treatment, Bak activation was evident at 2 h as indicated by immunoprecipitation (Fig. 6) [38]. Notably, the activated Bak formed MODE 2 complexes as indicated by its co-immunoprecipitation with Mcl-1 and Bcl-x_L at 2 and 4 h. Moreover, the Bak:Mcl-1 and Bak:Bcl-x_L complexes appeared transient in the Bim$_S$-expressing cells, as observed for those complexes formed in the Bid^{Bim} mitochondria experiments (Figs. 1c and 5c). We

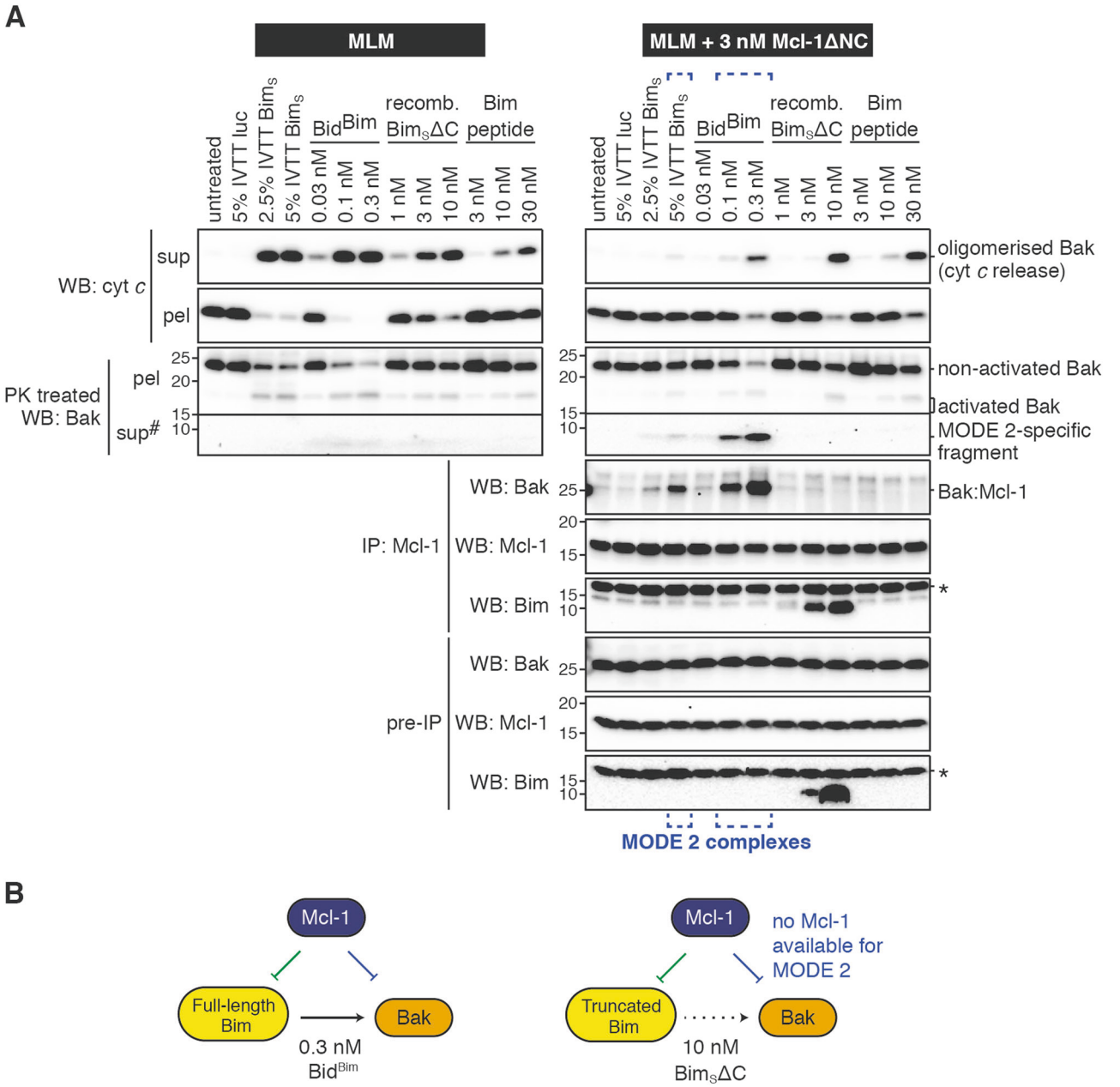

Fig. 4 Membrane-targeted Bim variants but not truncated Bim or Bim peptide cause MODE 2 complexes. **a** Mouse liver mitochondria (MLM) supplemented with or without Mcl-1 were treated with four variants of Bim at the indicated concentrations (IVTT reagents were added at the indicated percentage v/v). Samples were assessed as in Fig. 1. Blots are from the same experiment, representative of at least two independent experiments. #Long exposure of supernatant blots compared to pellets. *Bands on Bim blots are due to previous probing for Mcl-1. **b** Diagram showing interactions between the Bcl-2 family members in the tripartite mixtures tested. Note that activators (e.g. Bim$_S$ΔC) that do not target to mitochondria are not potent activators of Bak (broken arrow) and so underestimate the potential for MODE 2.

also note that tBid preferentially generated Bak:Mcl-1 complexes while Bim$_S$ generated both Bak:Mcl-1 and Bak:Bcl-x$_L$ complexes (Fig. 6). Although the levels of tBid and Bim$_S$ were not equivalent, this finding reflects the distinct binding profiles of tBid and Bim observed in the mitochondria experiments comparing Bid and Bid^{Bim} (Figs. 1c and 5c). In summary, MODE 2 complexes can form in cells without artificially over-expressing prosurvival proteins, and the complexes appear to be disrupted as Bim$_S$ levels increase (Fig. 6, last lane).

BH3 mimetics must overcome MODE 2 to cause cytochrome c release, and can also induce MODE 2

Having established robust conditions for generating MODE 1 and MODE 2 complexes containing Bcl-x$_L$ (Fig. 5c), we

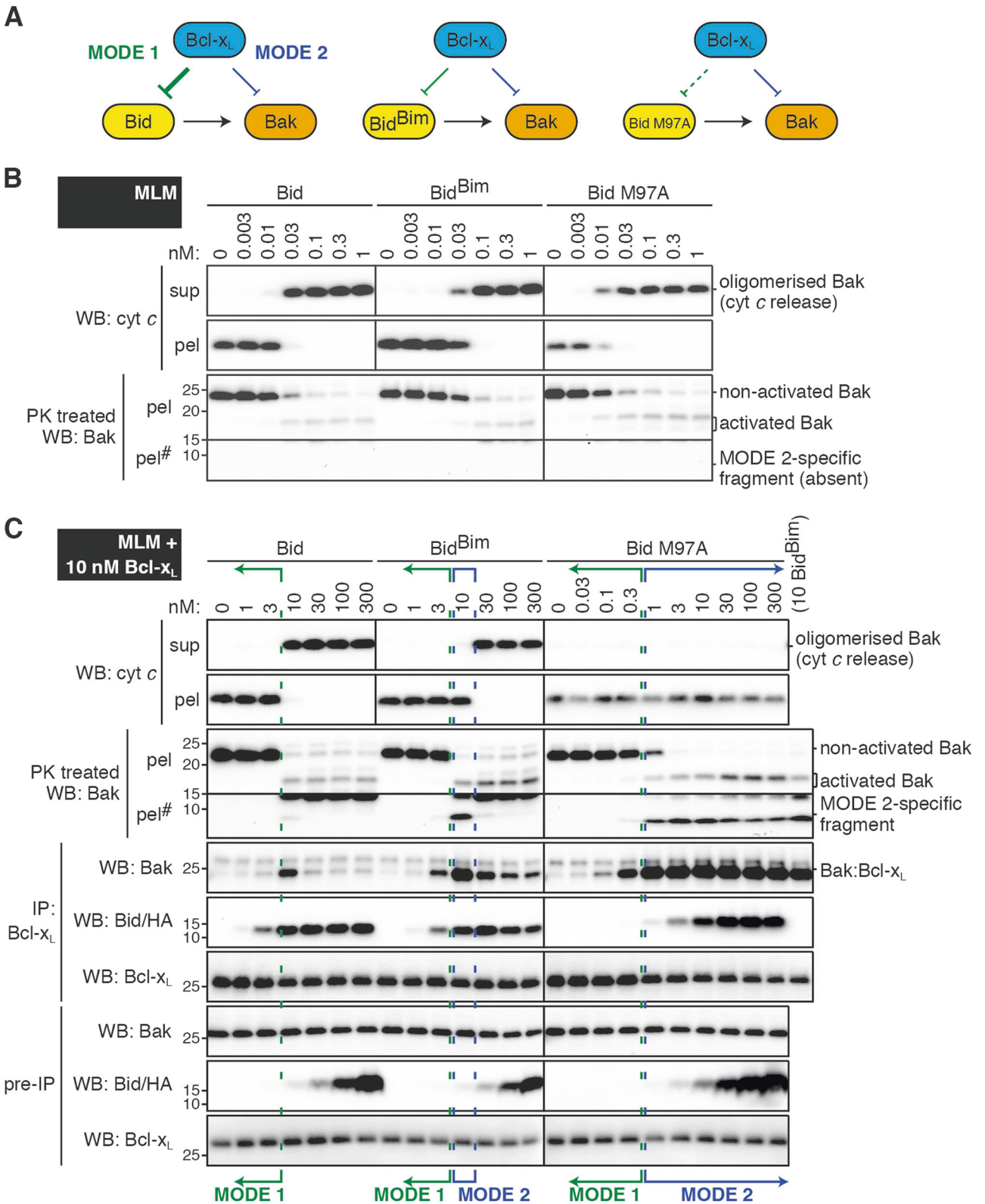

Fig. 5 Bcl-x_L blocks Bid^{Bim} signalling partly via MODE 2. **a** Diagram showing interactions between the Bcl-2 family members in the tripartite mixtures tested. **b**, **c** Mouse liver mitochondria (MLM) supplemented with **c** or without **b** full-length Bcl-x_L were treated with Bid, Bid^{Bim} or Bid M97A at the indicated concentrations. Samples were analysed as in Fig. 1, except that solubilised samples were immunoprecipitated for Bcl-x_L rather than Mcl-1, and Bid M97A was detected by probing for Bid rather than HA. Blots from **b** and **c** are from the same experiment, and together with two independent experiments were quantified for MODE 1 and MODE 2 inhibition (Fig. S2D). #Long exposure of pellet fraction to observe the 'MODE 2-specific fragment' (unlike Mcl-1 experiments, Fig. S3)

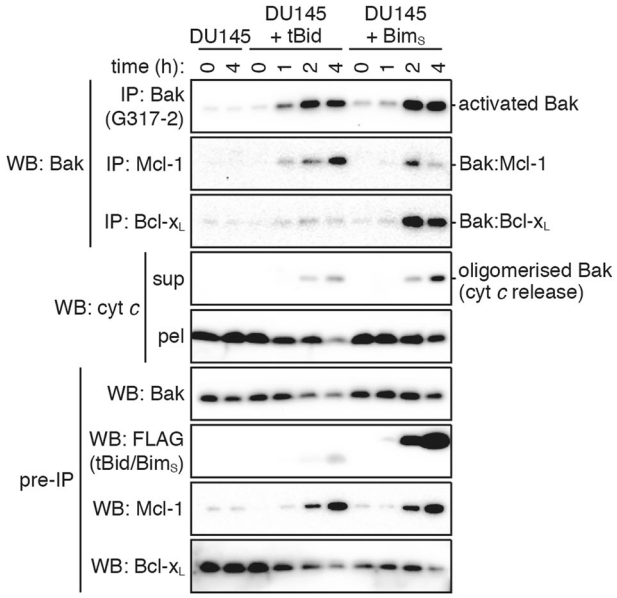

Fig. 6 In cells, endogenous Mcl-1 and Bcl-x_L bind Bak in response to either tBid or Bim_S signalling.DU145 cells or cells infected with tBid or Bim_S lentivirus were treated with doxycycline for up to 4 h. Bak activation and protein–protein interactions were determined by solubilising samples with 1% digitonin (pre-IP) and immunoprecipitating (IP) with antibodies against Mcl-1, Bcl-x_L or activated Bak (G317-2). Cytochrome *c* release was determined by permeabilising samples with 0.025% digitonin and centrifuging to separate supernatant (sup) and pellet (pel). Blots are from the same experiment, representative of three independent experiments

next compared their disruption by ABT-737, a BH3 mimetic that targets Bcl-x_L [6]. As illustrated in Fig. 7a, the incubations involved a 1 h incubation with Bid or Bid M97A to generate MODE 1 or MODE 2 complexes, respectively, followed by a 60 min incubation with increasing concentrations of ABT-737 to disrupt the complexes. The 1 h samples were run in lane 2 (Fig. 7b) and show MODE 1 inhibition (left panel) and MODE 2 inhibition (middle panel). The 2 h samples in the left panel (Fig. 7b) show that MODE 2 inhibition could be induced by low levels of ABT-737 (blue brackets). For example, 0.3 μM ABT-737 activated Bak (presumably by displacing Bid from MODE 1 complexes), which then became sequestered by Bcl-x_L. Much higher levels of ABT-737 (10 μM) could prevent MODE 2 and release cytochrome *c*. A similarly high level of ABT-737 (30 μM) could also disrupt preformed MODE 2 complexes in the Bid M97A incubations (Fig. 7b, middle panel). Thus, in this mixture of Bcl-2 family members, cytochrome *c* release occurred when ABT-737 could circumvent (i.e. either prevent or disrupt) both MODE 1 and MODE 2 complexes. In addition, the concentrations of ABT-737 that either prevent or disrupt MODE 2 complexes appear similar.

We next used the Bid^{Bak} chimera [20] to activate Bak, and create a system in which the MODE 1 and MODE 2 complexes both involved the Bak BH3 domain binding to Bcl-x_L. A 1 h incubation with Bid^{Bak} resulted in MODE 1 inhibition (Fig. 7b, right panel), consistent with significant affinity between Bid^{Bak} and Bcl-x_L [20]. Subsequent ABT-737 addition had the same effect as in the Bid incubations, with intermediate ABT-737 causing MODE 2 and higher levels releasing cytochrome *c*. Therefore, even when the same BH3 domain (from Bak) mediated MODE 1 and MODE 2 complexes with Bcl-x_L, ABT-737 was more efficient at overcoming MODE 1 inhibition.

Thus, regions other than the Bak BH3 domain may be involved in binding to Bcl-x_L resulting in higher affinity binding in the MODE 2 complex, as suggested previously [13]. However, an alternative explanation is that only a fraction of the activator needs to be displaced from MODE 1 to activate all of Bak (e.g. 0.03 nM Bid in Fig., 1a,c and 5b,c), whereas around half of Bak needs to be displaced from the Bak:Bcl-x_L complexes to allow cytochrome *c* release (Fig. 7b).

In summary, in each of the three types of experiments in Fig. 7, ABT-737 needed to circumvent MODE 2 to allow cytochrome *c* release. Indeed, intermediate levels of ABT-737 (0.3–3 μM) induced MODE 2 in the Bid and Bid^{Bak} incubations. Thus, resistance to ABT-737 and other BH3 mimetics may involve the targeted Bcl-2 prosurvival protein (i.e. Bcl-x_L) sequestering Bak or Bax in MODE 2, especially if intermediate levels of drug are used.

Discussion

Our studies clarify how specific binding of prosurvival proteins to their proapoptotic relatives directs their ability to block apoptosis. Titration experiments showed that MODE 1 inhibition predominates at low levels of BH3-only reagents, while MODE 2 inhibition predominates at higher levels. If sufficient Mcl-1 or Bcl-x_L is present, all Bak can be engaged in MODE 2 complexes, especially if the Bak activator competes poorly with activated Bak for binding to prosurvival proteins. In addition, Bcl-x_L sequestration of Bak could block ABT-737 signalling, despite ABT-737 having significant affinity for Bcl-x_L. As proposed previously [39], time-course experiments confirm that neither the MODE 1 or MODE 2 means of sequestration involved 'dead-end' complexes, as BH3-only proteins released from MODE 1 sequestration could activate Bak, and Bak released from MODE 2 sequestration could release cytochrome *c*.

Our findings support the proposition that MODE 2 is more efficient and less easily derepressed than MODE 1 (ref. [13]). One possible explanation is that activated Bak has high affinity for Mcl-1, perhaps due to interactions beyond the BH3 domain. An alternative argument is that

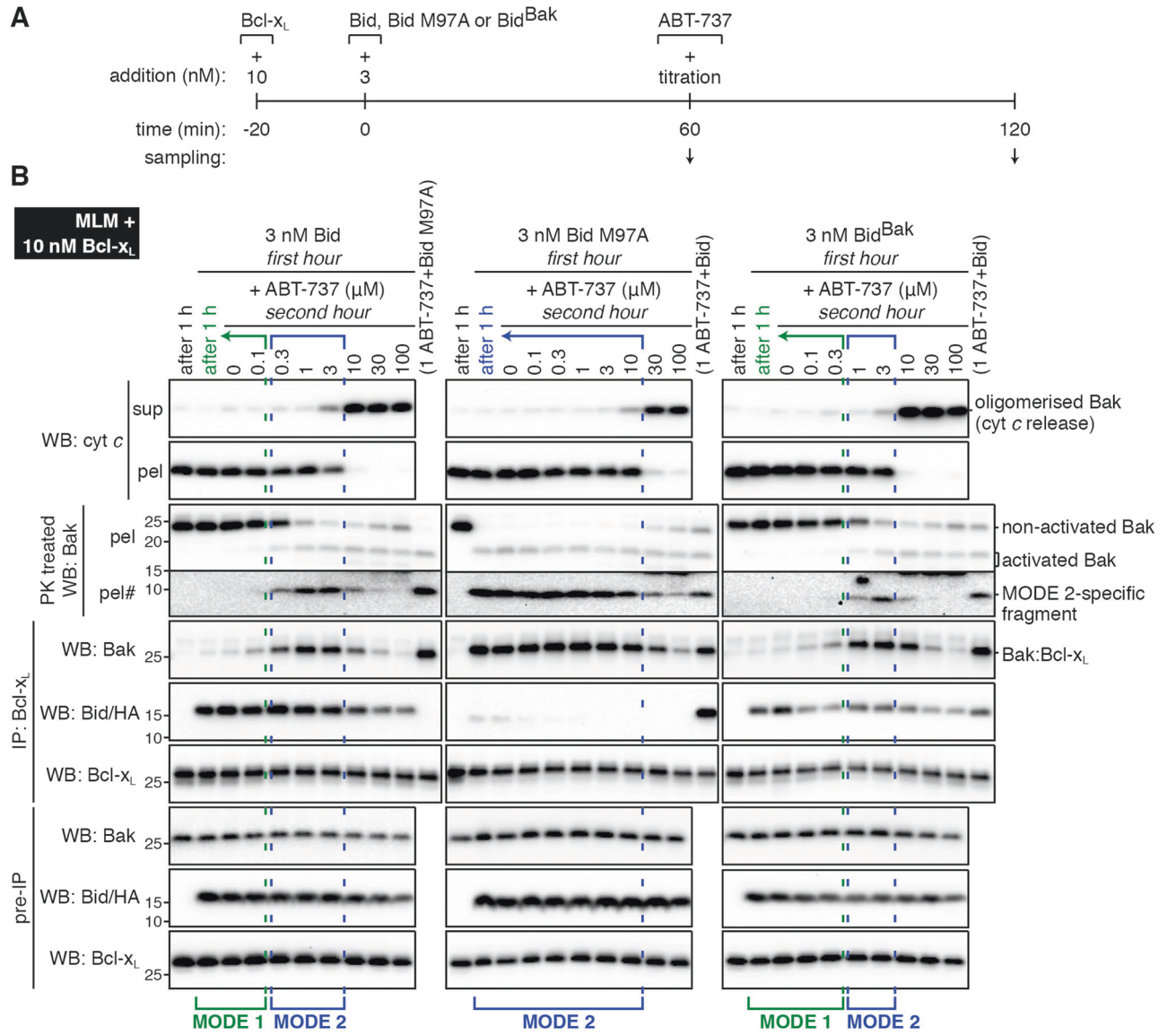

Fig. 7 ABT-737 can both induce and disrupt MODE 2 complexes of Bak:Bcl-x_L. **a** Timeline of protein addition and sampling. Note that prior to the addition of ABT-737 at 60 min, MODE 1 inhibition was generated by incubation with Bid or Bid^{Bak}, or MODE 2 inhibition was generated by incubation with Bid M97A (also see 1 h samples in lane 2 in **b**). **b** Mouse liver mitochondria (MLM) supplemented with full-length Bcl-x_L were treated as in **a**, and samples analysed as in Fig. 5. Blots are from the same experiment and representative of at least two independent experiments. #Longer exposure

different thresholds apply for circumventing MODE 1 and MODE 2. For example, circumventing MODE 1 inhibition required displacement of only a fraction of the activator from Mcl-1 to 'catalytically' activate Bak (e.g. 0.03 nM compared to 0.3 nM Bid in Fig. 1b, c). In contrast, circumventing MODE 2 inhibition required much more of Bak to be freed from Mcl-1 to mediate cytochrome *c* release (e.g. the 100 nM Bid^{Bim} sample in Fig. 1c).

We found that MODE 2 failed to occur when BH3 peptide or truncated BH3-only protein was used to trigger apoptotic signalling. Of four Bim reagents that could bind to Mcl-1 (MODE 1), only the membrane-targeted reagents (IVTT Bim_S and Bid^{Bim}) could cause Bak activation and still allow its binding to Mcl-1 (MODE 2). Most BH3-only proteins have hydrophobic membrane anchors that target them to mitochondria [34] and enhance their ability to activate Bak and Bax [19,20,40]. As BH3 peptides are sometimes used to assess responsiveness to anti-cancer therapies, replacement with BH3 reagents that target to mitochondria may improve their accuracy.

MODE 2 sequestration contributed significantly to blockade of Bid signalling by both truncated and full-length Mcl-1, in mitochondrial and cell-based assays, and was explained by the low affinity of Bid for Mcl-1. The low

affinity of Bid for Mcl-1 is consistent with several studies using peptides or recombinant or cellular proteins [13,16,18,20,41,42], although not all [26,43]. Thus, it is possible that Bid-induced MODE 2 may contribute to resistance to TRAIL signalling, granzyme B or other Bid-dependent stimuli [44–46], with resistance more likely where Bax is low or absent [47,48]. A recent study concluded that Bid has a preference for activating Bak while Bim has a preference for activating Bax [49]. As those activation preferences were not apparent in the mitochondrial system used in the present study [20] or a related model system [50], it remains possible that Bid-related MODE 2 explains the reduced effect on mitochondria.

Notably, MODE 2 inhibition occurred even when the BH3 reagent bound strongly to a prosurvival protein, but was applied at intermediate concentrations. For example, Bid^{Bim} and ABT-737 each have high affinity for Bcl-x_L, but generated MODE 2 resistance when applied at intermediate concentrations. Thus, MODE 2 complexes may form regularly in both physiological and pathological settings. In keeping with this, we recently reported defects in T-cell and blood platelet survival in mice expressing a variant of Bak that bound poorly to Bcl-x_L [27]. In addition, MODE 2 complexes may contribute to resistance to chemotherapies, including BH3 mimetics, that target Bcl-2 and Mcl-1 (refs. [4,5,13,51]). In our model systems, the complexes were only generated after treatment. However, MODE 2 complexes have been reported in untreated cells, including certain cancer cell lines [24], possibly related to the ability of Bak to become activated in the absence of known activators [52,53].

In conclusion, our studies validate the importance of MODE 2 in the ability of prosurvival proteins to inhibit apoptosis, and provide important insight into targeting these proteins for therapeutic benefit in cancer and other diseases. As MODE 2 complexes become better characterised, ready detection in patient samples may help tailor therapy in a way that minimises resistance due to MODE 2.

Materials and methods

Materials

Thrombin-cleaved HA-tagged human Bid and Bid^{Bim} chimera [20], mouse Mcl-1ΔN151C23 (ref. [16]) and human Bcl-x_L (ref. [20]) were prepared as previously described. Caspase 8-cleaved human Bid M97A was prepared as described for wild-type Bid (ref. [27]). Recombinant human Bim_SΔC27 was expressed as an N-terminal hexaHis fusion protein in BL21 DE3 *Escherichia coli* cells following induction with isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. The cell pellet was resuspended in Tris-buffered saline (TBS) pH 8, and lysed by addition of lysozyme (8 mg/g cell pellet) and deoxycholic acid (4 mg/g cell pellet) for ~30 min at room temperature. After the removal of cell debris by centrifugation, Bim_SΔC27 was purified from the supernatant by nickel-affinity chromatography using a HiTrap chelating column (Amersham Biosciences, Buckinghamshire, UK) charged with nickel chloride. The eluate was further purified by gel-filtration chromatography on a Superdex 75 16/600 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) pre-equilibrated and run in TBS. The pooled peak fractions used for all experiments were >90% pure as assessed by Coomassie Blue staining. To help accurate dilution of recombinant proteins, stock solutions were prepared in buffer containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), which minimises binding of proteins to tubes [20]. Human Bim peptide (DMRPEIWIAQELRRIGDEFNAYYARR) was synthesised by Mimotopes (Notting Hill, VIC, Australia) [16]. Stock solutions of Bim peptide and ABT-737 (a gift from D.C.S. Huang) were prepared with dimethyl sulfoxide.

In vitro transcription/translation was performed with the TNT T7 Coupled Wheat Germ Extract System (Promega, Madison, WI, USA). Reaction mixtures with luciferase control DNA (supplied in kit) or mouse Bim_S cDNA cloned into pBluescript II (+) linearised with ScaI were prepared according to the manufacturer's recommendation, using complete amino acid mixture (Promega). Reactions were incubated at 30 °C for 45 min before being added to isolated mitochondria.

Mitochondrial assays

MLM were prepared from C57BL/6 wild-type, $Bak^{-/-}$ or $Mcl\text{-}1^{flox/flox}$ mice as described [20]. MLM were diluted to 1 mg/ml in MELB (100 mM KCl, 2.5 mM $MgCl_2$, 100 mM sucrose, 20 mM HEPES/KOH pH 7.5) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and 4 mg/ml pepstatin A (Sigma-Aldrich). MLM were pre-incubated for 20 min at 37 °C with or without Mcl-1 or Bcl-x_L as indicated (except $Mcl\text{-}1^{flox/flox}$ MLM which had no pre-incubation step). BH3-only stimuli were added as indicated and incubated at 37 °C for 2 h except where otherwise indicated. In the time-course experiments, samples were collected just prior to Bid or Bid^{Bim} addition at each time point.

To assess cytochrome *c* release, supernatant and pellet were separated by centrifugation at 16,000 × *g* for 5 min. To measure Bak activation, pre-chilled samples were incubated with proteinase K (30 µg/ml; Roche) for 20 min on ice. Proteinase K was quenched with 2 mM PMSF (Sigma-Aldrich) and supernatant and pellet fractions obtained as described above. To immunoprecipitate Bcl-2 proteins, pre-chilled samples were solubilised by the

addition of 1% w/v digitonin (Biosynth AG, Staad, Switzerland) and incubated on ice for 30 min before pelleting debris by centrifugation at 16,000 × *g* for 5 min. The resulting clarified lysates were immunoprecipitated with antibodies as described [30] using anti-Mcl-1 rat monoclonal 14C11 (WEHI mAb Facility, Bundoora, VIC, Australia [54]), anti-Bcl-x rat monoclonal IC2 (WEHI mAb Facility [16]) or anti-Bak mouse monoclonal G317-2 (BD Biosciences, San Jose, CA, USA) and Protein G Sepharose 4 Fast Flow resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting were performed as described [55], except that for immunoprecipitated samples the nitrocellulose membranes were blocked with 5–20% (v/v) horse serum (SAFC Biosciences, St Louis, MO, USA) in addition to the usual 5% (w/v) skimmed milk powder in TBS with 0.1% (v/v) Tween-20 (Sigma-Aldrich). Primary antibodies used were anti-cytochrome *c* mouse monoclonal 7H8.2C12 (BD Biosciences); anti-HA rat monoclonal 3F10 (Roche); anti-Mcl-1 rat monoclonal 19C4–15 (WEHI mAb Facility [56]), mouse monoclonal clone 22 (BD Transduction Laboratories) or rabbit polyclonal (Rockland Immunochemicals, Pottstown, PA, USA); anti-Bcl-x rabbit polyclonal (BD Biosciences) or mouse monoclonal clone 44 (BD Transduction Laboratories); anti-Bak rabbit polyclonal a23–38 (Sigma) or rat monoclonal 4B5 (WEHI mAb Facility [30]); anti-Bid rat monoclonal 2D1-3 (WEHI mAb Facility [57]); anti-FLAG rat monoclonal 11F3 (WEHI mAb Facility); and anti-Bim rat monoclonal 3C5 (WEHI mAb Facility [58]). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse, anti-rabbit and anti-rat secondary antibodies (Southern Biotech, Birmingham, AL, USA).

Western blots were quantitated by densitometry using Image Lab 4.1 software (Bio-Rad, Hercules, CA, USA). Percentage cytochrome *c* release was calculated independently from the supernatant and the pellet as described in ref. [20]. Percentage Bak activation was calculated as percentage reduction in the band labelled 'non-activated Bak' on the 'PK treated' blots. Relative Bak:Mcl-1 or Bak:Bcl-x_L IP was calculated relative to the brightest Bak:Mcl-1 band on the blot, and then normalised across blots by samples run on both blots.

DU145 cell experiments

N-terminally FLAG-tagged tBid and Bim_S were cloned into the Tetracycline Response Element (TRE)-tight vector, kindly provided by Marco Herold. TRE-linked tBid and Bim_S were then cloned into a modified version of the lentiviral vector FUGW [59]. Lentiviral particles were produced by transfecting HEK293T cells with lentiviral vector (10 µg), pMDL (5 µg), RSV-REV Eco (2.5 µg) and Eco (5 µg), using calcium phosphate co-precipitation [59]. After 48 h, each filtered (0.45 µm) viral supernatant was mixed with polybrene (4 µg/ml) and used to transduce DU145 cells by spin-infection (2000 rpm, 45 min, 21–26 °C). Fresh media was added to the remaining transfected HEK293T and after a further 24 h, viral supernatants were used to infect DU145 cells again. Transduced (mCherry positive) DU145 cells were selected by FACS. HEK293T and DU145 cells were maintained in Dulbecco's modified Eagle's medium (Thermo-Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo-Fisher Scientific).

DU145 cells (parental or cells transfected with tBid or Bim_S constructs) were pre-treated for at least 1 h with 50 µM Q-VD.oph (MP Biomedicals, Santa Ana, CA, USA) to block caspase activation, and then incubated with 5 µg/ml doxycycline (Sigma-Aldrich) for up to 8 h as indicated. Floating cells and trypsinised attached cells were collected, washed in PBS and pelleted by centrifugation. Cells were resuspended in MELB supplemented with protease inhibitor cocktail (Roche) and 4 mg/ml pepstatin A. To assess cytochrome *c* release, part of the cell sample was permeabilised by the addition of 0.025% digtonin for 10 min on ice, and the supernatant and pellet fractions tested as above. The remainder of the sample was solubilised by the addition of 1% digitonin and immunoprecipitated for Mcl-1, Bcl-x_L and activated Bak as described above.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

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