

1 **The carboxyl-terminal sequence of Bim enables Bax activation and killing of**  
2 **unprimed cells**

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30

31 **Abstract**

32 The Bcl-2 family BH3 protein Bim promotes apoptosis at mitochondria by  
33 activating the pore forming proteins Bax and Bak and by inhibiting the anti-apoptotic  
34 proteins Bcl-XL, Bcl-2 and Mcl-1. Bim binds to these proteins via its BH3 domain and to  
35 the mitochondrial membrane by a carboxyl-terminal sequence (CTS). In cells killed by  
36 Bim, the expression of a Bim mutant in which the CTS was deleted (BimL-dCTS)  
37 triggered variable amounts of apoptosis that correlated with inhibition of anti-apoptotic  
38 proteins being sufficient to permeabilize mitochondria isolated from the same cells.  
39 Detailed analysis of the molecular mechanism demonstrated that BimL-dCTS inhibited  
40 Bcl-XL but did not activate Bax. Our examination of additional point mutants  
41 unexpectedly revealed that the CTS of Bim is required for physiological concentrations  
42 of Bim to activate Bax and that different residues in the CTS enable Bax activation and  
43 binding to membranes.

44 **Keywords**

45 Apoptosis, apoptotic priming, programmed cell death, Bcl-2 family proteins, Bim, Bax,  
46 Bcl-XL, BH3-profiling.

47

## 48 **Introduction**

49

50 Apoptosis is a highly conserved form of programmed cell death that can be  
51 triggered by extrinsic or intrinsic signals. It plays a fundamental role in maintaining  
52 homeostasis by eliminating old, excessive or dysfunctional cells in multi-cellular  
53 organisms (Kerr, Wyllie, and Currie 1972). Defective regulation of apoptosis has been  
54 found in many diseases (Favaloro et al. 2012) and is considered one of the hallmarks of  
55 cancer (Hanahan and Weinberg 2011).

56 Bcl-2 family proteins play a decisive role in apoptosis initiated by intrinsic signaling  
57 by regulating the integrity of the mitochondrial outer membrane (MOM). Commitment to  
58 apoptosis is generally regarded as due to MOM permeabilization (MOMP) releasing  
59 cytochrome c and pro-apoptotic factors from the intermembrane space into the  
60 cytoplasm. These factors activate the executioner caspases that mediate cell death  
61 (Chipuk, Bouchier-Hayes, and Green 2006). Direct interactions between Bcl-2 family  
62 proteins govern both initiation and inhibition of MOMP (Kale, Osterlund, and Andrews  
63 2017). The Bcl-2 family of proteins that regulate apoptosis includes the anti-apoptotic  
64 proteins Bcl-XL, Bcl-2 and Mcl-1 that inhibit the process and share four Bcl-2 homology  
65 domains. These homology domains, referred to as BH domains, are also shared by the  
66 pro-apoptotic proteins Bax and Bak that permeabilize the MOM directly. Both pro- and  
67 anti-apoptotic multi-domain Bcl-2 family proteins are regulated by direct binding  
68 interactions with a group of proteins including Bim, Bid, Puma, Hrk, Bad and Noxa that  
69 contain a single region of homology, the Bcl-2 homology domain number 3, and are  
70 therefore referred to collectively as BH3-proteins. These proteins promote apoptosis by  
71 releasing sequestered activated Bax, Bak and BH3-proteins that activate Bax and Bak  
72 from one or more of the anti-apoptotic proteins. The subset of BH3 proteins that bind to

73 and activate Bax or Bak include Bid, Bim and Puma (Chi et al. 2014). Thus far, the  
74 biochemical basis for the differences between BH3-proteins that inhibit anti-apoptotic  
75 proteins and those that activate Bax and Bak has been attributed entirely to differences  
76 in affinities of the BH3-domain for the BH3-peptide binding sites on multi-domain pro-  
77 and anti-apoptotic proteins. However, static affinities and variations in expression levels  
78 permit only coarse regulation of cell death. Changes in the equilibrium binding of Bcl-2  
79 family proteins on the MOM enable finer control. For example, at physiologic  
80 concentrations the BH3 protein Bid only activates Bax after Bid has bound to a  
81 membrane and undergone a specific conformational change (Lovell et al. 2008;  
82 Shamas-Din, Bindner, et al. 2013). Binding to membranes also enables interaction of Bid  
83 with MCH2 on the MOM to greatly accelerate the Bid conformational change that  
84 results in Bax activation (Shamas-Din, Bindner, et al. 2013). However, it remains unclear  
85 whether membrane interactions by other BH3 proteins like Bim contribute to Bax  
86 activation.

87 The BH3-protein Bim is an important mediator of apoptosis initiated by many  
88 intracellular stressors (Concannon et al. 2010; Mahajan et al. 2014; Puthalakath et al.  
89 2007). Three major isoforms of Bim result from alternative mRNA splicing: BimEL, BimL,  
90 and BimS (O'Connor et al. 1998). All three isoforms include the BH3-domain required for  
91 binding other Bcl-2 family proteins, and a C-terminal sequence (CTS) that binds the  
92 protein to the MOM (Wilfling et al. 2012). BimEL and BimL also share a dynein light  
93 chain binding motif (LC1) that sequesters these isoforms at the cytoskeleton (Lei and  
94 Davis 2003). The absence of this LC1 binding motif in BimS likely accounts for the  
95 constitutive activity of the isoform in cells (Lei and Davis 2003). Accordingly, BimS is  
96 regulated transcriptionally and rarely present in healthy cells, while BimL and BimEL are  
97 present in most tissue types (O'Reilly et al. 2000). Bim has a particularly important

98 function as a regulator of anti-apoptotic proteins, as it binds and thereby inhibits by  
99 mutual sequestration all known anti-apoptotic proteins (Chen et al. 2005; Shamas-Din,  
100 Kale, et al. 2013). Until recently, It was unknown why Bim binds to Bcl-XL with sufficient  
101 affinity to resist displacement by small molecule BH3-mimetics, while other BH3  
102 proteins, such as Bad, are easily displaced (Aranovich et al. 2012). In addition to  
103 interactions via the BH3-domain, residues within the Bim CTS bind to Bcl-XL, and  
104 thereby increase the affinity of the interaction by “double-bolt locking” providing an  
105 explanation for the observations with BH3 mimetic drugs (Liu et al. 2019). Here we  
106 investigated whether the CTS of Bim also contributes to the functional and physical  
107 interactions between Bim and Bax.

108 We demonstrate that both primary cells and cell lines have a range of apoptotic  
109 responses to the expression of a truncated BimL protein lacking the CTS (BimL-dCTS),  
110 while expression of full-length BimL was sufficient to kill all of these cells. To determine  
111 the molecular mechanism that underlies this difference, the two pro-apoptotic functions  
112 of Bim; activation of Bax and inhibition of Bcl-XL, were quantified using purified full-  
113 length BimL protein and cell free assays. Replacing the CTS of Bim with an alternative  
114 tail-anchor that binds the protein to mitochondrial membranes did not fully restore Bax  
115 activation function, demonstrating that sequences within the Bim CTS rather than  
116 membrane binding contribute to Bax activation. Site-directed mutagenesis of the Bim  
117 CTS also revealed residues important for binding to membranes that were not required  
118 for Bax activation (e.g. I125). Furthermore, specific residues within the CTS were  
119 identified that are required for BimL to efficiently activate Bax, but that are not required  
120 for BimL to bind to and inhibit Bcl-XL. Evidence in cell free assays demonstrated that  
121 BimL CTS residues L129 and I132 physically interact with Bax and are required to  
122 activate it. These mutants were used to show that BimL residues L129 and I132 are also

123 required for BimL to efficiently kill cells resistant to BimL-dCTS, demonstrating that it is  
124 necessary to activate Bax to kill these unprimed cells. Together, our data demonstrates  
125 that the unusual sequence of the CTS of Bim separately controls both membrane  
126 binding and Bax activation.

127

## 128 **Results**

129 The CTS of Bim variably contributes to the pro-apoptotic activity of Bim in different cell  
130 lines.

131 Removing the CTS from Bim abrogates pro-apoptotic activity in HEK293 cells  
132 (Weber et al. 2007). While this observation has generally been ascribed to loss of  
133 binding of Bim to MOM our observation that the CTS is also involved in binding BimEL to  
134 Bcl-XL (Liu et al. 2019) suggested that there may be other explanations for the loss of  
135 pro-apoptotic activity for Bim when the CTS is removed. To determine the contribution of  
136 the Bim CTS to pro-apoptotic activity, a BimL mutant was generated in which the  
137 previously characterized membrane binding domain (carboxyl-terminal residues P121-  
138 H140) were deleted (BimL-dCTS) (Wilfling et al. 2012), (Liu et al. 2019). This mutant  
139 was expressed in cells and the effectiveness of induction of cell death was compared to  
140 expression of full-length BimL by confocal microscopy. To detect expression of the  
141 constructs in live cells, they included an N-terminally fused Venus fluorescent protein  
142 (indicated by a superscripted v in the name). Thus a construct in which Venus was fused  
143 to BimL is referred to here as <sup>v</sup>BimL while the mutant lacking the CTS is <sup>v</sup>BimL-dCTS.  
144 As an inactive control we used <sup>v</sup>BimL-4E a mutant in which four conserved hydrophobic  
145 residues in the BH3 domain of BimL were replaced with glutamate, thereby preventing  
146 binding to all other Bcl-2 family proteins (Chen et al. 2005), (Liu et al. 2019).

147 To assay pro-apoptotic activity, the constructs were expressed in primary cells and  
148 cell lines and both expression and cell death were measured using confocal microscopy.  
149 Apoptosis was assessed by detecting externalization of phosphatidylserine by Annexin V  
150 staining in cells expressing detectable levels of  $\Delta$ BimL or the  $\Delta$ BimL mutants as  
151 measured by Venus fluorescence. As expected, expression of  $\Delta$ BimL induced apoptosis  
152 in all cell types tested, while the negative control  $\Delta$ BimL-4E did not (Figure 1A). As  
153 reported previously for Bim-dCTS, the fluorescent version ( $\Delta$ Bim-dCTS) failed to induce  
154 cell death in HEK293 cells (Weber et al. 2007). In contrast, expression of  $\Delta$ BimL-dCTS  
155 induced apoptosis to levels similar to  $\Delta$ BimL in HCT116 and MEF cells but had reduced  
156 potency in BMK and CAMA-1 cells. Thus the CTS of Bim contributed variably to the pro-  
157 apoptotic activity of Bim in different cell lines despite having equal expression across all  
158 cell types (Figure 1 – figure supplement 1).

159 To determine if this difference in response to  $\Delta$ BimL-dCTS expression is a function  
160 of the extent to which the apoptotic machinery is loaded in mitochondrial outer-  
161 membranes, mitochondria were purified from cells resistant (HEK293) and sensitive  
162 (MEF) to  $\Delta$ BimL-dCTS expression and assayed by BH3-profiling (Potter and Letai 2016)  
163 to measure loading of anti-apoptotic proteins with BH3 proteins or active Bax/Bak. Unlike  
164 BH3-profiling experiments conducted with BH3-peptides, in these experiments purified  
165 full-length proteins were used. Thus, purified BimL, BimL-dCTS, Bad and Noxa proteins  
166 were incubated with mitochondria from each of the cell lines and mitochondrial outer  
167 membrane permeabilization (MOMP) was measured by separating supernatant and  
168 pellet fractions for each reaction, and immunoblotting for cytochrome c released from the  
169 intermembrane space as previously described (Pogmore et al. 2016). Immunoblots were  
170 quantified and MOMP assessed as % cytochrome c released (Figure 1B). As expected  
171 from the data in Figure 1A, addition of recombinant BimL was sufficient to induce

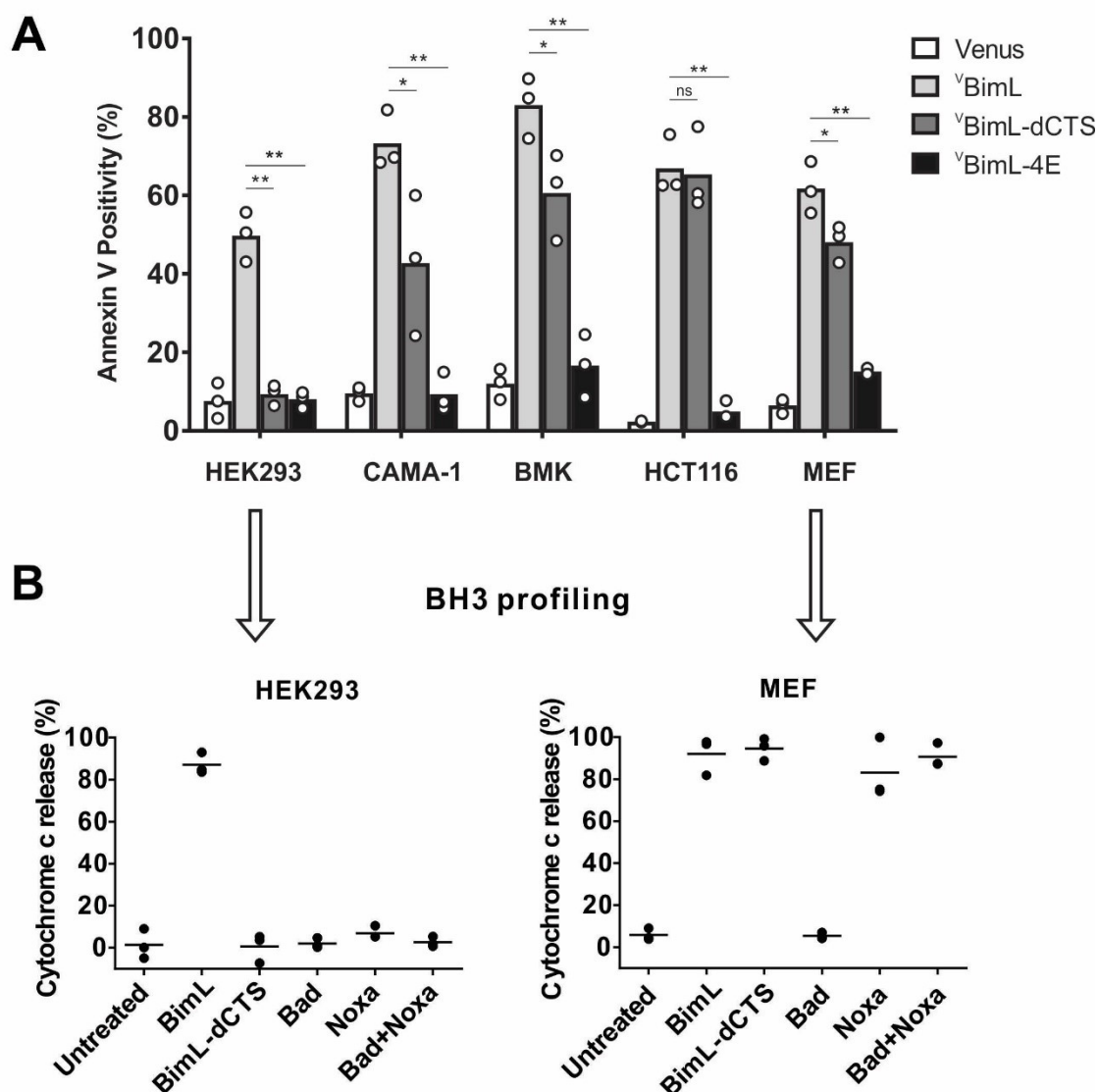
172 cytochrome c release from mitochondria from both HEK293 and MEF cells. However,  
173 addition of BimL-dCTS induced cytochrome c release only in the MEF mitochondria  
174 confirming that resistance to BimL-dCTS in HEK293 cells is manifest at mitochondria.

175         One potential explanation for this difference is that the mitochondria in the cell  
176 lines have different dependencies on multi-domain anti-apoptotic proteins for survival, a  
177 phenomenon known as priming. If BimL-dCTS has lost one of the functions of Bim such  
178 as activating Bax or Bak or inhibiting one of the multi-domain anti-apoptotic proteins Bcl-  
179 2, Bcl-XL and Mcl-1 it would be expected to have different activities on mitochondria with  
180 different priming. Therefore, to better understand why BimL-dCTS can only permeabilize  
181 MEF mitochondria and not mitochondria from HEK293 cells, we compared the sensitivity  
182 of mitochondria from the two cell types to addition of BH3-proteins Bad and Noxa that  
183 inhibit Bcl-2 and Bcl-XL or Mcl-1, respectively, but that do not activate Bax or Bak (Kale,  
184 Osterlund, and Andrews 2017). Incubation of full length Bad and/or Noxa with  
185 mitochondria from HEK293 cells failed to induce cytochrome c release, while the  
186 addition of Noxa was sufficient to permeabilize MEF mitochondria (Figure 1B). This data  
187 suggests that HEK293 cells do not depend on expression of Bcl-2, Bcl-XL or Mcl-1  
188 sequestering active Bax, Bak or their BH3-activators while mitochondria from MEFs  
189 depend on expression of Mcl-1 to prevent apoptosis (Lessene et al. 2013). The results  
190 further suggest that removal of the CTS from BimL results in a mutant protein that only  
191 kills cells dependent on one or more multi-domain anti-apoptotic proteins for survival.  
192 That BimL-dCTS does not kill HEK293 cells further suggests that it does not activate  
193 sufficient Bax or Bak to overcome the unoccupied anti-apoptotic proteins in this cell line.  
194 In this way BimL-dCTS functions as a sensitizer similar to proteins like Bad and Noxa.  
195 However, unlike other relatively specific sensitizer proteins, the known binding activity of  
196 the BH3 region of BimL-dCTS suggests that it inhibits Bcl-2, Bcl-XL and Mcl-1. Indeed



197 we have shown that in live cells BimEL-dCTS binds to Bcl-2 and Bcl-XL but is more  
 198 easily displaced than BimEL by small molecule BH3 mimetics (Liu et al. 2019).

## Figure 1



199

200

201 **Figure 1: Cell lines demonstrate a range of apoptotic response to BimL-dCTS**  
 202 **expression**

203 (A) Venus, <sup>V</sup>BimL, <sup>V</sup>BimL-dCTS or <sup>V</sup>BimL-4E were expressed in the Indicated cell lines  
 204 by transient transfection. The cells were stained with the nuclear dye Draq5 and  
 205 rhodamine labelled Annexin V, and imaged by confocal microscopy to identify cells

206 undergoing apoptosis. At least 400 cells were analyzed for each condition. The Y axis  
207 indicates the percentage of Venus positive cells that stained positive with Annexin V.  
208 Open circles represent the average for each replicate, while the bar height, relative to  
209 the y-axis, represents the average for all three replicates. The means were assessed for  
210 significant differences using a one-way ANOVA within each group followed by a Tukey's  
211 multiple comparisons test. \*p-values less than 0.05, \*\*p-values less than 0.01, ns are  
212 non-significant p-values (>0.05).

213 (B) BH3 profiling of mitochondria isolated from HEK293 and MEF cells. Mitochondria  
214 (1mg/mL) were incubated with 500 nM of the indicated recombinant BH3 protein(s) for 1  
215 hour at 37°C. Cytochrome c release, indicative of MOMP, was quantified by  
216 immunoblotting. Data from three independent experiments are shown as individual  
217 points, with lines representing the average. Some dots are not visible due to overlap.

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### 219 Full-length BimL is required to kill cultures of primary cortical neurons

220 Our data with cell lines and their respective purified mitochondria suggests that  
221 BimL-dCTS does not kill cells that do not depend on anti-apoptotic proteins for survival.  
222 To test this in a more biologically relevant system, we cultured primary murine cortical  
223 neurons and assayed their response to expression of the BimL mutants. For regulated  
224 expression in primary cortical neurons the coding regions for <sup>V</sup>BimL, <sup>V</sup>BimL-4E, and  
225 <sup>V</sup>BimL-dCTS were cloned into a tetracycline-responsive lentiviral vector, and introduced  
226 into primary cortical neuron cultures through lentiviral infection. After culture for 8 days *in*  
227 *vitro*, BimL expression was induced in the neurons by the addition of doxycycline.  
228 Neuronal cell death was assayed using confocal microscopy after staining neurons with  
229 propidium iodide (PI), a dye that exclusively stains the nuclei of dead cells.  
230 Quantification of Venus-expressing neuronal cell bodies revealed that as expected  
231 <sup>V</sup>BimL expression killed cultured primary neurons while <sup>V</sup>BimL-4E did not (Figure 2A-B).  
232 However, the expression of <sup>V</sup>BimL-dCTS was largely ineffective to induce cell death in  
233 cultured primary cortical neurons (Figure 2B). Our data is consistent with previous  
234 reports suggesting that primary murine cultures of hippocampal neurons become  
235 resistant to induction of apoptosis by external stimuli over time in culture. This resistance

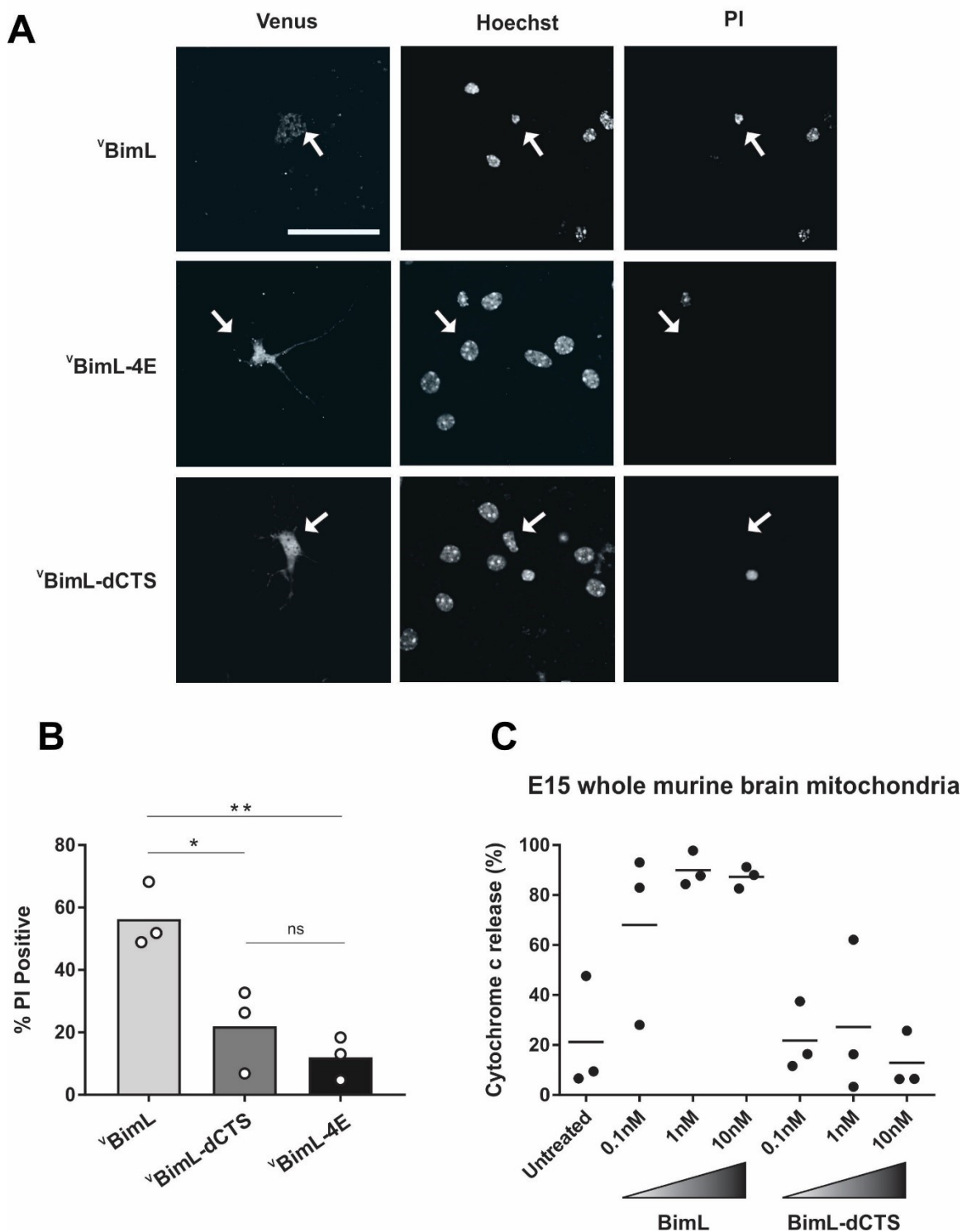
236 has been reported to be due to a difference in Bcl-2 family protein expression that  
237 results in decreased mitochondrial ‘priming’, explaining why our cultures of primary  
238 cortical neurons are resistant to <sup>v</sup>BimL-dCTS (Sarosiek et al. 2016).

239 To determine if resistance to induction of cell death by BimL-dCTS is due to  
240 differential sensitivity of neuronal mitochondria to induction of MOMP by BimL and BimL-  
241 dCTS, mitochondria were isolated from embryonic day 15 (E15) mouse brains, the same  
242 age used to culture primary cortical neurons. Brain mitochondria were used instead of  
243 isolating mitochondria from neuronal cultures due to the low yield from primary cultured  
244 neurons. Untreated mitochondria from day E15 brain released only low levels of  
245 cytochrome c. As expected, addition of 0.1nM recombinant BimL was sufficient to elicit  
246 MOMP as measured by cytochrome c release and detection in the supernatant. In  
247 contrast, 100 times more BimL-dCTS (10nM) failed to induce MOMP (Figure 2C).

248 Taken together our data suggest that BimL-dCTS kills cells in which the  
249 mitochondria are sensitive to inhibition of anti-apoptotic proteins by sensitizers such as  
250 Bad and Noxa. Thus, BimL-dCTS did not permeabilize mitochondria extracted from  
251 HEK293 cells or E15 whole murine brains, and as a result, BimL-dCTS expression did  
252 not kill HEK293 cells or primary cultures of cortical neurons. This finding suggests that  
253 inhibition of anti-apoptotic proteins is not sufficient to kill these cells. Therefore, BimL-  
254 dCTS differs mechanistically from BimL as the latter kills both cell types resistant and  
255 sensitive to BimL-dCTS. Compared to BimL, BimL-dCTS is missing the membrane  
256 binding domain and therefore is not expected to localize at mitochondria (Liu et al.  
257 2019), however, the relationship between Bim binding to membranes and Bim mediated  
258 Bax activation has not been extensively studied. To determine how the molecular

259 mechanism of BimL-dCTS differs from BimL the activities of the proteins were analyzed  
 260 using cell free assays.

## Figure 2



261

262 **Figure 2: Full-length BimL is required to kill cultures of primary cortical neurons.**

263 (A) Representative images of primary cortical neurons infected with lentivirus to  
264 express  $\Delta$ BimL,  $\Delta$ BimL-dCTS or  $\Delta$ BimBH3-4E. White arrows indicate neurons expressing  
265 Venus fluorescence. Scale bar is 80 $\mu$ m.

266 B) Quantified data from Venus expressing primary cortical neurons. Percentage of  
267 Venus expressing cells with nuclei PI intensity scores above threshold (% PI Positive).  
268 Open circles; averages for three biological replicates each representing 90-1000 cells  
269 analyzed. The Bar height; mean. A one-way ANOVA was used followed by a Tukey's  
270 multiple comparisons test to compare the means of each group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; ns  
271  $p > 0.05$ .

272 (C) Mitochondria extracted from embryonic day 15 (E15) mouse brains (0.5 mg/mL)  
273 were incubated with the indicated BH3-only proteins. Cytochrome c release, indicative of  
274 MOMP, was quantified using immunoblotting. Each point (black circle) represents one  
275 independent replicate, with the line representing the average across all three.

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277 The Bim CTS mediates BimL binding to both Bax and membranes

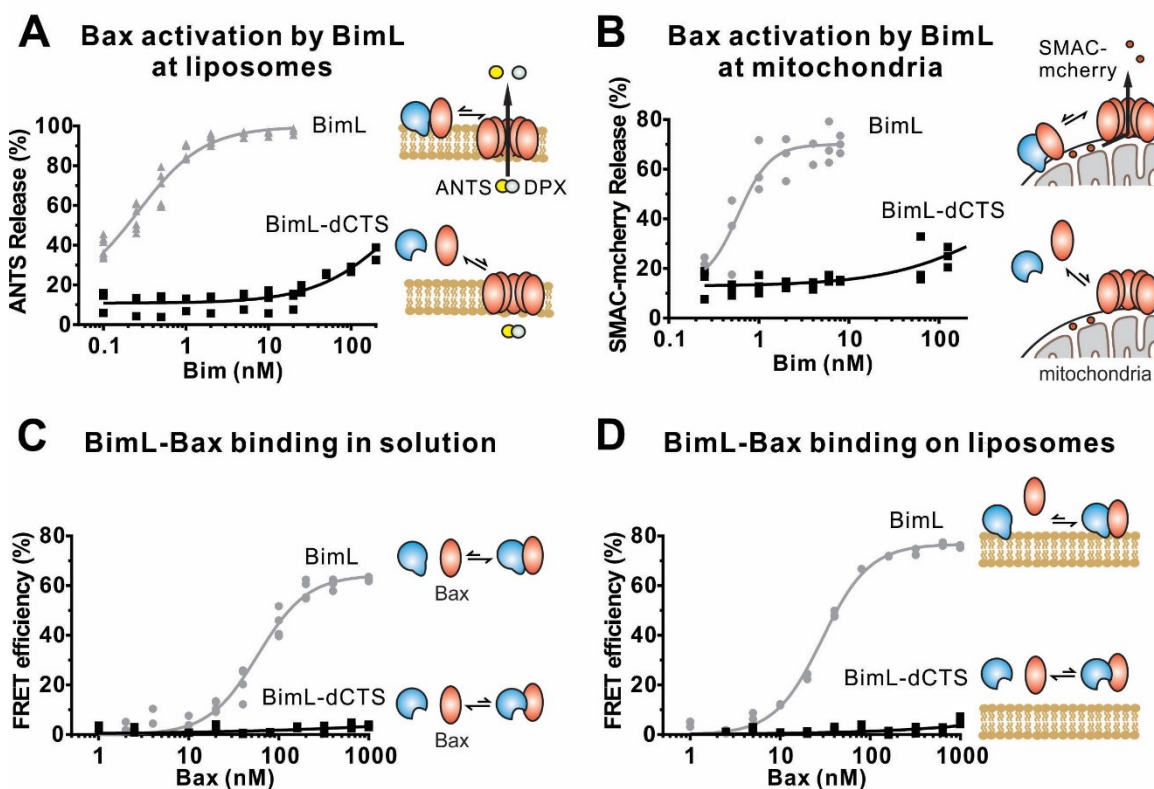
278 To investigate the pro-apoptotic mechanism of BimL and BimL-dCTS without  
279 interference from other cellular components, both were purified as full-length  
280 recombinant proteins and assayed using liposomes and/or isolated mitochondria. To  
281 measure direct-activation of Bax by Bim, either BimL or BimL-dCTS was incubated with  
282 recombinant Bax and liposomes encapsulating the dye and quencher pair: ANTS (8-  
283 Aminonaphthalene-1,3,6-Trisulfonic Acid, Disodium Salt) and DPX (*p*-Xylene-Bis-  
284 Pyridinium Bromide). In this well-established assay (Kale et al. 2014), increasing  
285 amounts of BimL activated Bax resulting in membrane permeabilization measured as an  
286 increase in fluorescence due to the release and separation of encapsulated dye and  
287 quencher (Figure 3A). This result is consistent with previous observations that picomolar  
288 concentrations of BimL induce Bax-mediated membrane permeabilization (Sarosiek et  
289 al. 2013). In contrast, three orders of magnitude higher concentrations of BimL-dCTS  
290 were required to induce Bax-mediated liposome permeabilization (Figure 3A),

291 suggesting that either or both of binding to membranes and the specific CTS of Bim are  
292 required for efficient Bax activation. As expected, similar results were obtained for Bax-  
293 mediated release of mitochondrial intermembrane space proteins (Figure 3B). For these  
294 experiments, MOMP was measured as release of the fluorescent protein mCherry fused  
295 to the N-terminal mitochondrial import signal of SMAC (SMAC-mCherry) from the  
296 intermembrane space of mitochondria (Shamas-Din et al. 2014). Similar to the results  
297 with liposomes (Figure 3A), and mitochondria from cell lines (Figures 1-2) BimL but not  
298 BimL-dCTS triggered Bax mediated SMAC-mCherry release from mitochondria isolated  
299 from Bax  $-/-$  Bak  $-/-$  cells (Figure 3B). In experiments with liposomes and mitochondria,  
300 very small amounts of Bim were sufficient to trigger membrane permeabilization  
301 because once activated, Bax recruits and activates additional Bax molecules (Tan et al.  
302 2006).

303 To assess the impact of the Bim CTS on the interaction between Bim and Bax,  
304 binding was measured using Förster resonance energy transfer (FRET). For these  
305 experiments recombinant BimL proteins were labelled with the donor fluorophore  
306 Alexa568, while Bax was labelled with the acceptor fluorophore Alexa647.  
307 Unexpectedly, and unlike the BH3-only protein tBid (Lovell et al. 2008), BimL bound to  
308 Bax even in the absence of membranes (Figure 3C), while BimL-dCTS had no relevant  
309 Bax binding in the presence or absence of mitochondrial-like liposomes (Figure 3C-D).  
310 Binding of Bim to Bax in solution suggests that the CTS of Bim may be directly involved  
311 in Bim-Bax heterodimerization independent of Bim binding to membranes.

312

## Figure 3



313

### 314 **Figure 3: The Bim CTS is required to activate Bax to permeabilize membranes**

315 Cartoons indicate the binding interactions being measured. Equilibria symbols indicate  
 316 the predicted balance of complexes for BimL (blue), Bax (red) and membranes (tan). For  
 317 each graph, data from three independent experiments are shown as individual points.  
 318 Due to overlap, some points may not be visible.

319 (A) Activation of Bax by BimL assessed by measuring permeabilization of ANTS/DPX  
 320 filled liposomes (0.04 mg/mL) after incubation of Bax (100nM) with the indicated  
 321 concentrations of BimL or BimL-dCTS. Fluorescence intensity, indicative of membrane  
 322 permeabilization, was measured using the Tecan infinite M1000 microplate reader.

323 (B) Permeabilization of the outer mitochondrial membrane by Bax (50 nM) in response  
 324 to activation by the indicated amounts of Bim and BimL-dCTS was assessed by  
 325 measuring SMAC-mCherry release from mitochondria.

326 (C) Bim binding to Bax in solution measured by FRET. Alexa568-labeled BimL or  
 327 BimL-dCTS (4 nM) was incubated with the indicated amounts of Alexa647-labeled Bax  
 328 and FRET was measured from the decrease in Alexa568 fluorescence.

329 (D) Bim binding to Bax measured by FRET in samples containing mitochondrial-like  
 330 liposomes. FRET was measured as in (C) with 4nM Alexa568-labeled BimL or BimL-  
 331 dCTS and the indicated amounts of Alexa647-labeled Bax.



332

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333 To confirm in our system that the labeled BimL proteins bind to membranes via the  
334 CTS sequence, binding of Alexa568-labeled recombinant BimL and BimL-dCTS to DiD  
335 labeled liposomes was measured by FRET (Figure 4A). In these experiments DiD  
336 serves as an acceptor for energy transfer from Alexa568 labeled BimL. The same  
337 approach was used to quantify BimL binding to mitochondrial outer membranes with  
338 mitochondria isolated from BAK<sup>-/-</sup> mouse liver (Figure 4B), which lack Bax and Bak  
339 (Shamas-Din, Bindner, et al. 2013). In both cases, BimL spontaneously bound to  
340 membranes with picomolar affinity, while stable binding of BimL-dCTS to liposomes and  
341 mitochondria was not-detectable (Figure 4A-B). Furthermore, BimL-dCTS again had no  
342 relevant binding to Bax even in the presence of purified mitochondria (Figure 4C).

343 Taken together, our data strongly suggest that the CTS of Bim is required for both  
344 BimL to bind to membranes *in vitro* and for binding Bax with or without membranes.  
345 Alternatively purified BimL-dCTS may be completely non-functional. To demonstrate that  
346 purified BimL-dCTS binds to and inhibits Bcl-XL as shown for <sup>V</sup>BimL-dCTS expressed in  
347 cells (Figure 1) and in (Liu et al. 2019), inhibition of Bcl-XL was measured using  
348 liposomes and mitochondria.

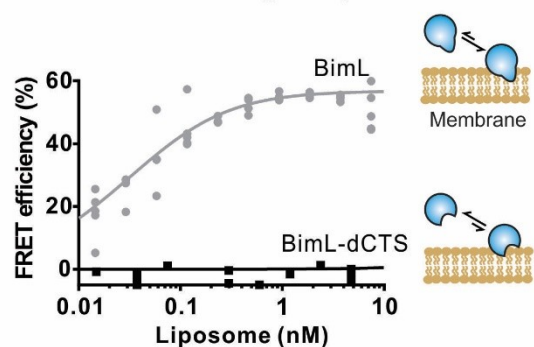
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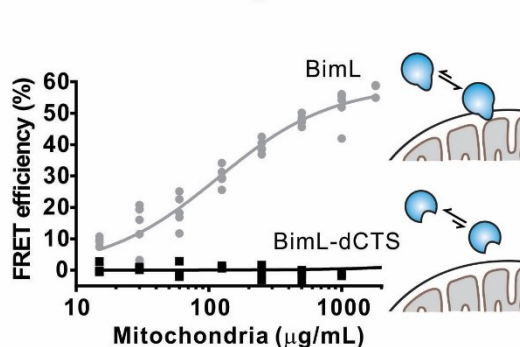


## Figure 4

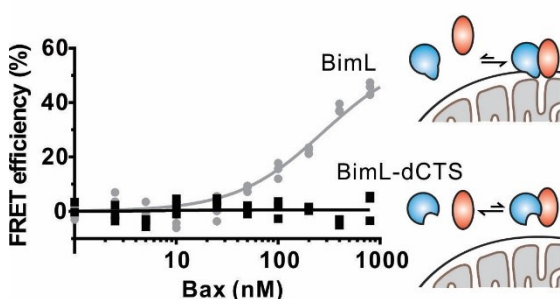
### A BimL binding to liposomes



### B BimL binding to mitochondria



### C BimL-Bax binding at mitochondria



351

352

### 353 Figure 4: The Bim CTS is required to bind BimL to membranes *in vitro*

354 Cartoons indicate the binding interactions being measured. Equilibria symbols indicate  
355 the predicted balance of complexes for Bim (blue), Bax (red) and membranes (tan). For  
356 each graph, data from three independent experiments are shown as individual points.  
357 Due to overlap, some points may not be visible.

358 (A) Bim binding to mitochondrial-like liposomes assessed by measuring FRET between  
359 20nM Alexa568-labeled BimL or BimL-dCTS and the indicated amounts of DiD-labeled  
360 liposomes.

361 (B) The CTS of Bim is necessary for Bim to bind to mitochondria. Binding of 4nM  
362 Alexa568-labeled BimL (n=5) or BimL-dCTS (n=3) to the indicated amounts of DiD  
363 labeled mouse liver mitochondria was assessed by measuring FRET.

364 (C) Deletion of the CTS prevented Bim binding to Bax at mitochondria. Bim binding to  
365 Bax was measured by FRET in samples containing mouse liver mitochondria, 4nM  
366 Alexa568-labeled BimL (grey) or BimL-dCTS (black) and the indicated amounts of  
367 Alexa647-labeled Bax (n=3).

368

369 The CTS is not required for BimL to inhibit Bcl-XL

370 In addition to direct Bax activation, Bim promotes apoptosis by binding to Bcl-XL  
371 and displacing either activator BH3-proteins (Mode 1) or activated Bax or Bak (Mode 2)  
372 (Llambi et al. 2011). In the ANTS/DPX liposome dye release assay, BimL-dCTS was  
373 functionally comparable to the well-established Bcl-XL inhibitory BH3-protein Bad in  
374 reversing Bcl-XL mediated inhibition of cBid (Figure 5A) or Bax (Figure 5B). Consistent  
375 with the observation that BimL-dCTS was less resistant to displacement by BH3  
376 mimetics in live cells, in cell free assays BimL-dCTS was also less effective than BimL at  
377 displacing cBid or Bax from Bcl-XL (Liu et al. 2019). Nevertheless, when assayed with  
378 mitochondria BimL-dCTS disrupted the interaction between tBid and Bcl-XL resulting in  
379 Bax activation and permeabilization of mitochondria as measured by cytochrome c  
380 release (Figure 5C, solid black line). This activity is due to inhibition of Bcl-XL function,  
381 as in controls without Bcl-XL the same concentration of BimL-dCTS did not directly  
382 activate sufficient Bax to mediate MOMP (Figure 5C, dashed black line). Thus, purified  
383 BimL-dCTS is functional and can initiate MOMP by displacing direct-activators (Mode 1)  
384 or activated Bax (Mode 2) from Bcl-XL (Figures 5A-C). Finally, BimL-dCTS labelled with  
385 Alexa568 retained high affinity binding for Bcl-XL labelled with Alexa647 both in solution  
386 ( $K_d < 16$  nM) and on membranes (~35 nM apparent  $K_d$  on liposomes and on  
387 mitochondria) as measured by FRET (Figure 6B).

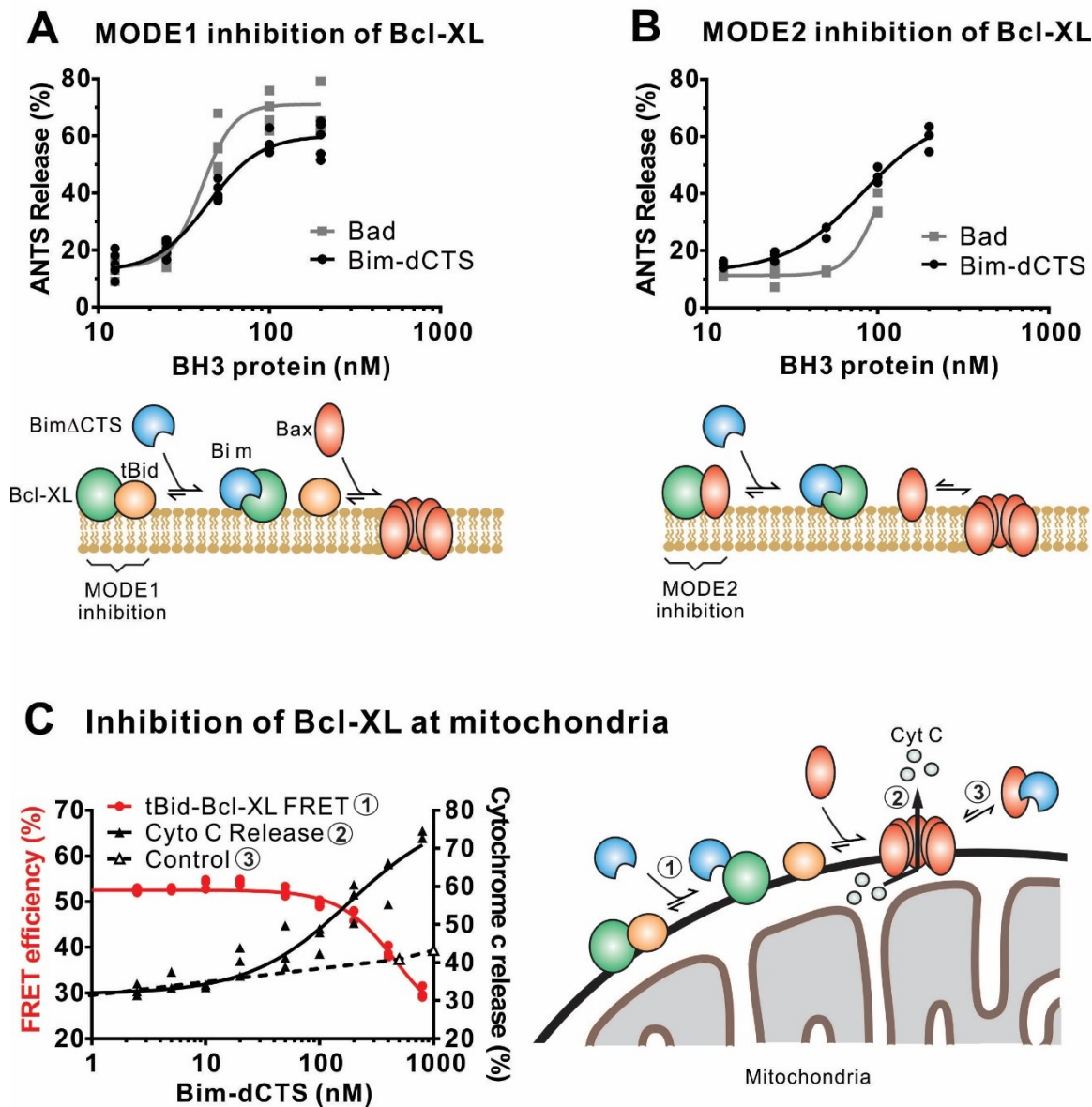
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389

390

391

## Figure 5



392

393

394 **Figure 5: The Bim CTS is not required to inhibit Bcl-XL**

395 (A-B) BimL-dCTS and Bad release tBid (A) or Bax (B) from Bcl-XL. 20nM tBid (A) or  
 396 tBidmt1 (B), which activates Bax but does not bind Bcl-XL, were incubated with 100nM  
 397 Bax, 40nM Bcl-XL, 0.04mg/mL ANTS/DPX liposomes, and the indicated amounts of  
 398 either Bad or BimL-dCTS. Liposome permeabilization was assessed after incubation at  
 399 37°C for three hours by measuring the increase in fluorescence due to ANTS/DPX  
 400 release. Cartoons indicate the interactions being measured, BimL (blue), Bax (red), tBid  
 401 (orange), Bcl-XL (green), membranes (tan).

402 (C) BimL-dCTS displaced tBid from Bcl-XL and permeabilized mitochondria.  
403 Mitochondria were incubated with Bcl-XL (40 nM), tBid (20 nM), Bax (100 nM) and  
404 mitochondria. Increasing concentrations of BimL-dCTS were added and displacement of  
405 tBid from Bcl-XL was measured by FRET. Mitochondria were then pelleted and  
406 cytochrome c release measured by western blotting. Control reactions containing only  
407 Bax and BimL-dCTS did not result in cytochrome c release (dotted line). Individual points  
408 are shown for three independent replicates. Not all points are visible due to overlap. The  
409 adjacent cartoon diagrams the interactions measured.

410

---

411

#### 412 Different residues in the Bim CTS regulate membrane binding and Bax activation

413 To identify which residues in the Bim CTS mediate binding to membranes and/or  
414 Bax we generated a series of point mutations. Sequence analysis using HeliQuest  
415 software (Gautier et al. 2008) predicts that the Bim CTS forms an amphipathic  $\alpha$ -helix  
416 (Figure 6A). Two arginine residues (R130&134) are predicted to be on the same  
417 hydrophilic side of the helix, whereas hydrophobic residues (e.g. I125, L129, I132) face  
418 the other side (Figure 6A). To determine the functional importance of these residues,  
419 Bim CTS mutants were created including: BimL-CTS2A in which R130 and R134 were  
420 mutated to alanine; and a series of single hydrophobic residue substitutions by  
421 glutamate (V124E, I125E, L129E, and I132E) (Figure 6A). To compare the effects of the  
422 CTS mutations on BimL binding interactions and function, we measured by FRET the  
423 Kds for the various binding interactions and the activities for the mutants to promote Bax  
424 mediated liposome permeabilization as EC50's for ANTS release (Figure 6B and Figure  
425 6 – Figure supplement 1A-E).

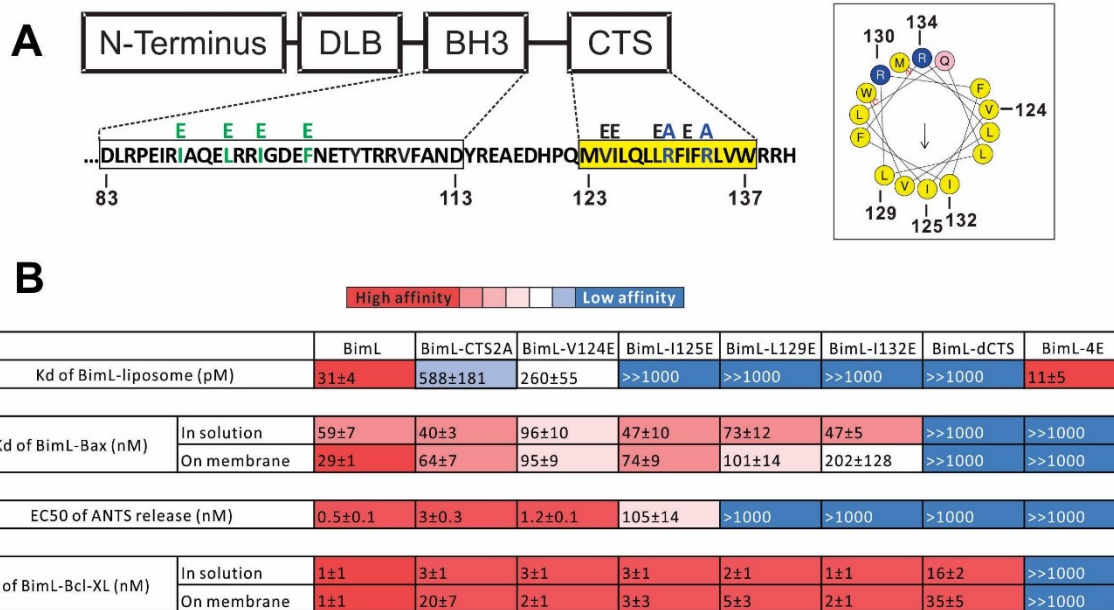
426 Mutation of individual hydrophobic residues on the hydrophobic side of the Bim  
427 CTS (BimL-I125E, BimL-L129E or BimL-I132E) abolished binding to membranes (Figure  
428 6B). In contrast, mutations on the other side of the helix including BimL-V124E and  
429 BimL-CTS2A had less effect on Bim binding to membranes (Figure 6B). Despite the

430 dramatic changes in affinity for membranes among Bim CTS mutants, the mutations did  
431 not abolish binding to Bax both in the presence and absence of membranes (Figure 6B).  
432 Indeed most of the mutants had Kd values for binding to Bax of less than 100 nM and to  
433 our surprise many of them bound to Bax better in solution than on membranes. This data  
434 further confirms that binding to membranes and Bax are independent functions of the  
435 Bim CTS. In the case of BimL-I125E, a mutant that activates Bax to permeabilize  
436 liposomes, the initial interaction with Bax must occur in solution as neither protein  
437 spontaneously binds to membranes (Figure 6B).

438         Unexpectedly, there was not a good correlation between BimL binding to  
439 membranes and Bax activation. For example, while BimL bound to membranes with a  
440 Kd of 31 pM, BimL-CTS2A and BimL-I125E bound to membranes very poorly (Kds of  
441 ~600 and >1000 pM, respectively) yet both mutants triggered Bax mediated membrane  
442 permeabilization, demonstrating that specific residues in the CTS rather than binding to  
443 membranes enabled BimL to mediate Bax activation. Moreover, BimL binding to Bax  
444 was also not sufficient to activate Bax efficiently. BimL-L129E and BimL-I132E are two  
445 Bim mutants that do not bind membranes, retain reasonable affinities for Bax in the  
446 presence of membranes (Kds ~100-200nM), but were unable to activate Bax (Figure  
447 6B). These results indicate that these two residues play a key function in Bax activation.  
448 As expected, the negative control BimL-4E mutant does not bind to nor activate Bax  
449 even though its CTS is intact and the protein binds membranes (Figure 6B). This result  
450 confirms the essential role of the BH3 domain and suggests that the Bim CTS provides a  
451 secondary role in Bax binding rather than providing an independent high affinity binding  
452 site.

453

## Figure 6



454

455 **Figure 6: Residues within the Bim CTS distinctly regulate membrane binding and**  
 456 **Bax activation.**

457 (A) Diagram of BimL depicting the various domains (DLB: dynein light chain binding  
 458 motif) and the sequences of the BH3-domain and CTS. The four essential hydrophobic  
 459 residues in BH3 domain that were mutated to glutamic acid are colored green. Two  
 460 positive charged residues in the CTS mutated to alanine are colored blue. Glutamic acid  
 461 mutations for individual hydrophobic residues in the CTS are indicated in black on top of  
 462 the original sequence. A predicted alpha helix structure generated via HeliQuest  
 463 software is shown on the right, indicating the amphipathic nature of the CTS. The arrow  
 464 central to the helix shows the polarity direction for hydrophobicity. The Q indicated in  
 465 pink is the last amino acid before the CTS. Other residues are colored as in the linear  
 466 sequence.

467 (B) Binding of BimL mutants to liposomes, Bax and Bcl-XL expressed as apparent  
 468 dissociation constants (Kd) measured from raw data as in Figure 6 – figure supplement  
 469 1 for each binary interaction. Activation of Bax (EC50) measured from ANTS/DPX  
 470 assays in Figure 6 – figure supplement 1. Values are mean ± SEM (n=3). The table is  
 471 colour-coded in a heat map fashion as follows: red 0-40; light red 40-80; light pink 80-  
 472 120; white 120-500; light blue 500-1000; Dark blue >1000. All values are nM except for  
 473 binding to liposomes which is in pM. The Kds for ‘on membrane’ measurements are  
 474 apparent values since diffusion for the protein fraction bound to membranes is in two  
 475 dimensions while for the fraction of protein in solution diffusion is in three dimensions  
 476 and several of the binary interactions take place in both locations. Apparent Kd values  
 477 may also be affected by competing interactions with membranes.

478



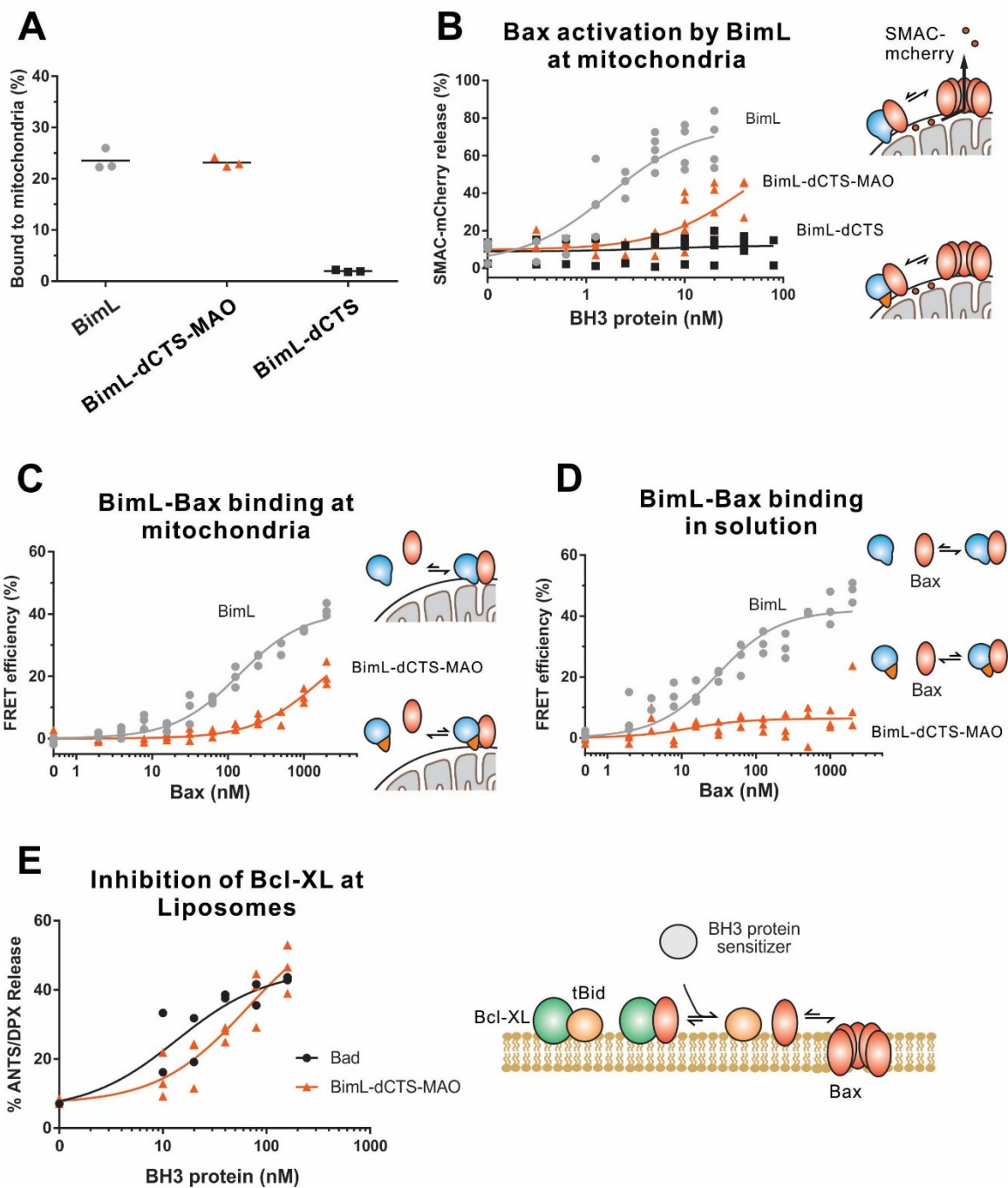
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480 Both functional and binding assays for the various point mutants suggest that  
481 specific residues in the Bim CTS participate in Bim-Bax protein interactions that lead to  
482 Bax activation, however, these mutants did not clearly separate the membrane binding  
483 function of the CTS of Bim from a potential function in Bax activation. Thus, it remains  
484 possible that restoring membrane binding to BimL-dCTS would be sufficient to restore  
485 Bax activation function. To address this, we fused the mitochondrial tail-anchor from  
486 mono-amine oxidase (MAO residues 490-527, UniProt: P21397-1) to the C-terminus of  
487 BimL-dCTS to restore membrane binding with a sequence unlikely to contribute to Bax  
488 activation directly. This protein, BimL-dCTS-MAO, bound to purified mitochondria equally  
489 efficiently as full-length BimL (Figure 7A). However, while low nanomolar concentrations  
490 of BimL induced Bax-mediated SMAC-mCherry release from mitochondria, much higher  
491 concentrations of BimL-dCTS-MAO were required to trigger MOMP (Figure 7B). To  
492 analyze BimL-dCTS-MAO binding to Bax, Alexa568 labelled BimL-dCTS-MAO was  
493 incubated with Alexa647 labelled Bax in the presence (Figure 7C) or absence (Figure  
494 7D) of purified mitochondria. The FRET results demonstrate that BimL-dCTS-MAO does  
495 not bind to Bax in solution (Figure 7D), however, restoring membrane binding to BimL-  
496 dCTS by adding the MAO sequence increased BimL-dCTS binding to Bax in the  
497 mitochondria marginally (Figure 7C, BimL-dCTS-MAO). Nevertheless, compared to  
498 BimL the binding of Bax by BimL-dCTS-MAO was at least an order of magnitude less  
499 efficient, explaining the reduced Bax activation function for BimL-dCTS-MAO (Figure  
500 7B). Thus, similar to what was seen for Bim binding to Bcl-XL (Liu et al. 2019), specific  
501 sequences within the CTS of Bim increase Bim binding to Bax. However, consistent with  
502 sequences in the Bim CTS increasing the affinity of BimEL binding such that the  
503 heterodimer is resistant to disruption by BH3-mimetics (Liu et al. 2019), BimL-dCTS-

504 MAO retained sufficient binding to Bcl-XL to functionally inhibit its sequestration of  
505 activated Bax (Figure 7E). By this definition BimL-dCTS-MAO functions as a sensitizer  
506 similar to the canonical sensitizer, Bad (Figure 7E).

507

## Figure 7



508



509

510 **Figure 7: BimL-dCTS-MAO binds to mitochondria and Bax but activates Bax**  
511 **poorly.**

512

513 Cartoons at the side indicate the measurements being made with equilibria arrows  
514 representing the results obtained. Blue objects, Bim. Red ovals, Bax. Blue and orange  
515 objects, BimL-dCTS-MAO.

516 (A) BimL-dCTS-MAO targets to mitochondria as efficiently as BimL. Alexa568 labelled  
517 single cysteine (Q41C) recombinant BimL, BimL-dCTS, and BimL-dCTS-MAO (20 nM)  
518 were incubated with 0.2 mg/ml of mitochondria purified from BMK Bax<sup>-/-</sup> Bak<sup>-/-</sup> cells for  
519 40 minutes at 37°C. Mitochondria were pelleted by centrifugation for 10 minutes at  
520 13000 x g. After correcting for background fluorescence binding to membranes was  
521 calculated from fluorescence signals in the supernatant and pellet fractions.

522 (B) Restoring membrane binding to BimL-dCTS does not fully restore Bax activation  
523 function. Bax activation was measured by SMAC-mCherry release from purified  
524 mitochondria (n=5 for BimL and Bim $\Delta$ CTS-MAO; n=3 for BimL-dCTS). The indicated  
525 amounts of BH3-only proteins were incubated with 20 nM of Bax in the presence of 0.2  
526 mg/ml mitochondria. Reactions were incubated for 40 minutes at 37°C.

527 (C-D) BimL-dCTS-MAO has reduced binding affinity to Bax compared to BimL in the  
528 presence (C) or absence (D) of mitochondria. 10 nM of Alexa 568-labeled BimL, BimL-  
529 dCTS or BimL-dCTS-MAO was incubated with the indicated amounts of Alexa 647-  
530 labelled Bax with or without of 0.2 mg/ml mitochondria. FRET was measured from the  
531 decrease in A568 fluorescence signal.

532 (E) BimL-dCTS-MAO released activated Bax from sequestration by Bcl-XL.  
533 ANTS/DPX filled liposomes were incubated with Bcl-XL (40 nM), cBid (20 nM), Bax (100  
534 nM) to load Bcl-XL with activated Bax. Increasing concentrations of BimL-dCTS-MAO  
535 (orange line) or Bad (black line) were added and Bax-mediated liposome  
536 permeabilization was measured as an increase in ANTS fluorescence. Thus, BimL-  
537 dCTS-MAO functions as a sensitizer as illustrated in the adjacent cartoon.

538

539

540 Residues within the Bim CTS are proximal to Bax in solution and on mitochondrial  
541 membranes

542 Our binding and mutagenesis data suggest that the Bim CTS binds to and  
543 activates Bax in solution and on membranes. To detect this binding interaction we used  
544 a photocrosslinking approach, in which a BimL protein was synthesized with a  
545 photoreactive probe attached to a single lysine residue positioned in the CTS using an *in*

546 *vitro* translation system containing 5-azido-2-nitrobenzoyl-labeled Lys-tRNA<sup>Lys</sup> that  
547 incorporates the lysine analog ( $\epsilon$ ANB-Lys) into the polypeptide when a lysine codon in  
548 the BimL mRNA is encountered by the ribosome. The BimL synthesized *in vitro* was also  
549 labeled by <sup>35</sup>S via methionine residues enabling detection of BimL monomers and  
550 photoadducts by phosphor-imaging.

551 The radioactive, photoreactive BimL protein was incubated with a recombinant  
552 His<sub>6</sub>-tagged Bax protein in the presence of mitochondria isolated from BAK<sup>-/-</sup> mouse liver  
553 lacking endogenous Bax and Bak to prevent competition and increase BimL-Bax protein  
554 interactions. Mitochondrial proteins were then separated from the soluble ones by  
555 centrifugation. Both soluble and mitochondrial fractions were photolyzed to activate the  
556 ANB probe generating a nitrene that can react with atoms in close proximity ( $\leq 12$  Å from  
557 the C $\alpha$  of the lysine residue). Thus, for photoadducts to form, the atoms of the bound  
558 Bax molecule are likely to be located in or near the binding site for the Bim CTS. The  
559 resulting photoadduct between the BimL and the His<sub>6</sub>-tagged Bax was enriched by  
560 Ni<sup>2+</sup>-chelating agarose resin and separated from the unreacted BimL and Bax  
561 monomers using SDS-PAGE. The <sup>35</sup>S-labeled BimL in the photoadduct with His<sub>6</sub>-tagged  
562 Bax and BimL monomer bound to the Ni<sup>2+</sup>-beads specifically via the His<sub>6</sub>-tagged Bax or  
563 nonspecifically were detected by phosphor-imaging. A BimL-Bax specific photoadduct  
564 was detected when the ANB probe was located at four different positions in the Bim CTS  
565 on both hydrophobic and hydrophilic surfaces of the potential  $\alpha$ -helix (Figure 8A). These  
566 photoadducts have the expected molecular weight for the Bim-Bax dimer, and were not  
567 detected or greatly reduced when the ANB probe, the light, or the His<sub>6</sub>-tagged Bax  
568 protein was omitted (Figure 8A). Consistent with the FRET-detected BimL-Bax  
569 interaction in both solution and membranes, the BimL-Bax photocrosslink occurred in  
570 both soluble and mitochondrial fractions. Less photocrosslinking occurred in the

571 mitochondrial fraction likely due to the fact that in membranes homo-oligomerization of  
572 activated Bax competes with hetero-dimerization between BimL and Bax.

573 As expected, BimL-Bax photocrosslinking was detected in both soluble and  
574 mitochondrial fractions when the ANB probe was positioned in the Bim BH3 domain as a  
575 positive control (Figure 8B). Crosslinking with the Bim BH3 domain is consistent with the  
576 canonical BH3 interaction well supported by experimental evidence including co-crystal  
577 structures and NMR models ((Walensky et al. 2008; Robin et al. 2015). Furthermore,  
578 loss of photocrosslinking for BimL mutants with the BH3 4E mutation that abolished  
579 binding to Bax demonstrates that direct binding between the proteins is required for  
580 crosslinking to be detectable (Figure 8C). Therefore, the crosslinking data suggests that  
581 similar to the BH3 domain, the Bim CTS binds to Bax. To further demonstrate that the  
582 CTS of Bim binds to Bax independent of both membrane binding and Bax activation the  
583 experiment was repeated with BimL-L129E, a mutant that binds Bax without activating it  
584 and that does not bind membranes (Figure 6B. As shown in Figure 8C, the L129E  
585 mutation in the CTS did not inhibit photocrosslinking of BimL to Bax in either the soluble  
586 or mitochondrial fractions. Furthermore, this mutant also resulted in photocrosslinking to  
587 Bcl-XL, consistent with data demonstrating that the Bim CTS also binds to this anti-  
588 apoptotic protein (Figure 8C and [Liu et al. 2019](#)).

589

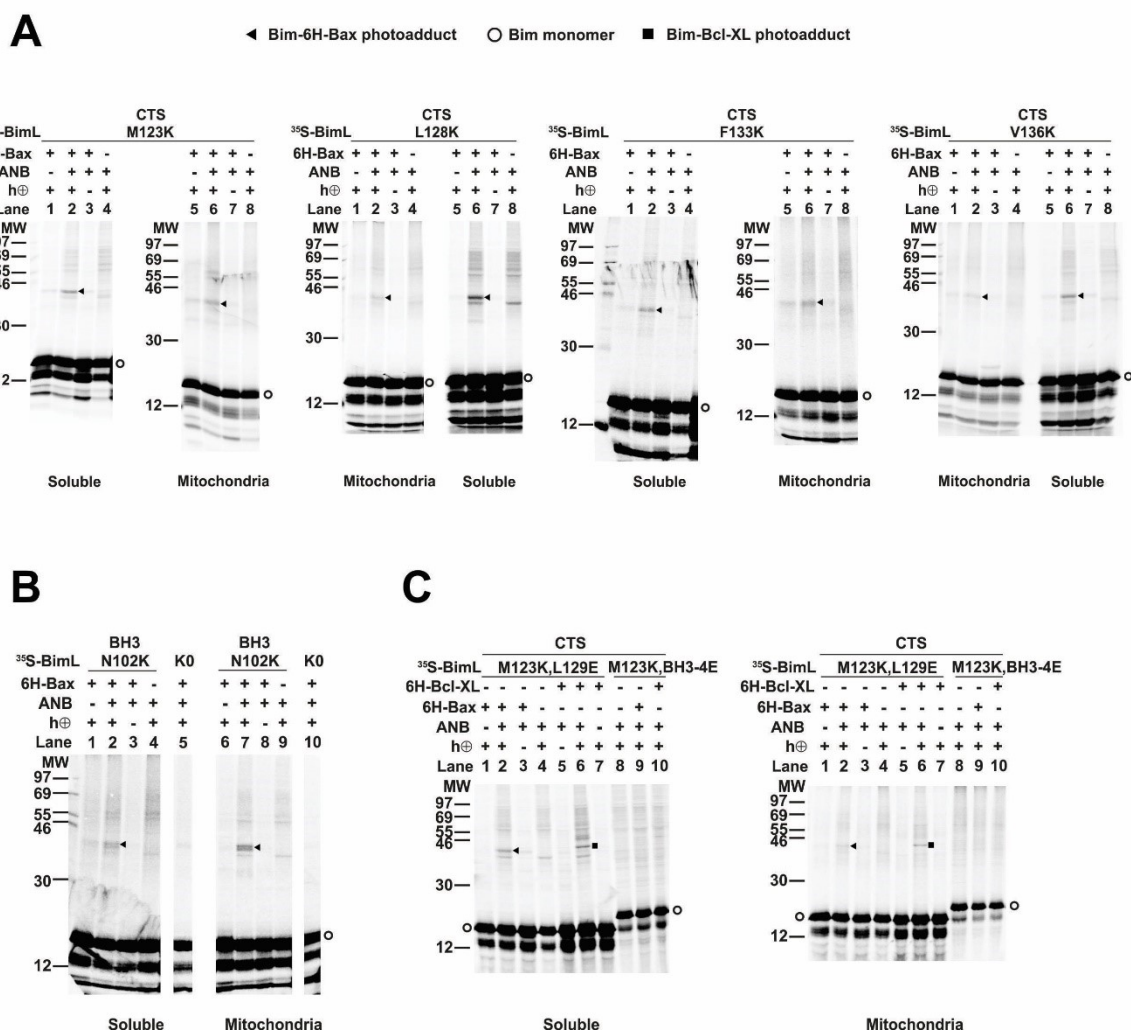
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593

## Figure 8



594

### 595 Figure 8: Residues within the Bim CTS interact with Bax

596 (A) Interaction of the Bim CTS with Bax in both soluble and mitochondrial fractions  
 597 detected by photocrosslinking. The BimL proteins, each with a single  $\epsilon$ ANB-lysine  
 598 residue located at the position in the CTS indicated at the top of the panels and  $^{35}\text{S}$ -  
 599 labeled methionine residues, were synthesized in vitro, and incubated with His<sub>6</sub>-tagged  
 600 Bax protein (6H-Bax) in the presence of mitochondria lacking endogenous Bax since  
 601 Bak. The mitochondria were then separated from the soluble proteins by centrifugation  
 602 and both fractions were photolyzed. The resulting radioactive BimL/6H-Bax  
 603 photoadducts were enriched with Ni<sup>2+</sup>-beads, and analyzed by SDS-PAGE and  
 604 phosphor-imaging. BimL/6H-Bax dimer specific photoadducts were detected in both  
 605 mitochondrial and soluble fractions and indicated by arrowheads. They were of reduced  
 606 intensity or not detected in control incubations in which the ANB probe, light (h $\nu$ ) or 6H-  
 607 Bax protein was omitted, as indicated. The radioactive BimL monomers are indicated by

608 circles. The migration positions of protein standards are indicated by molecular weight  
609 (MW) in kDa.

610 (B) Interaction of the Bim BH3 domain with Bax in both soluble and mitochondrial  
611 fractions detected by photocrosslinking. As a positive control illustrating the expected  
612 efficiency for the photocrosslinking experiments BimL protein with a single  $\epsilon$ ANB-lysine  
613 located at the indicated position in the BH3 domain was used to photocrosslink 6H-Bax  
614 protein. In another control experiment, a lysine-null BimL protein that does not contain  
615 any  $\epsilon$ ANB-Lys (K0) was used. As expected, a BimL/6H-Bax specific photoadduct was  
616 detected in the former but not the latter experiment.

617 (C) The 4E mutation in the BH3 domain but not the L129E mutation in the CTS of Bim  
618 inhibited photocrosslinking of the Bim CTS to Bax. The BimL protein with either the BH3-  
619 4E or the L129E mutation and the  $\epsilon$ ANB-Lys in the CTS was used in the  
620 photocrosslinking reaction with either 6H-Bax or 6H-Bcl-XL protein in both soluble and  
621 mitochondrial fractions. While the L129E mutation did not inhibit photocrosslinking of  
622 BimL to either 6H-protein, the BH3-4E mutation did. The BimL/6H-Bcl-XL photoadducts  
623 are indicated by squares.

624

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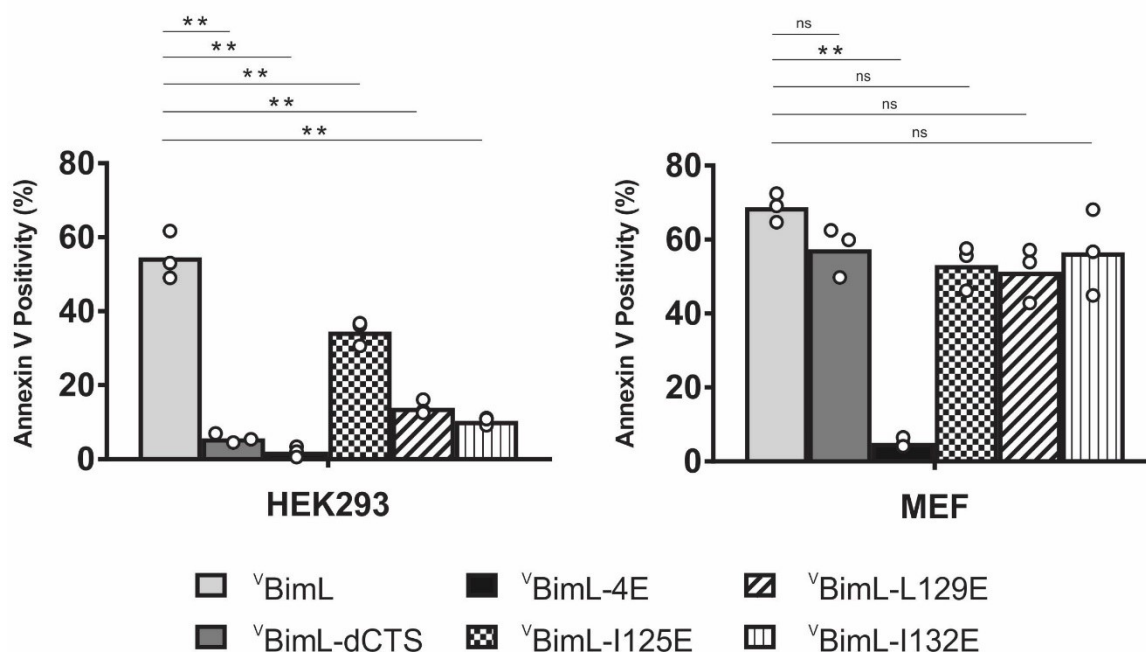
#### 626 Bim CTS mutants that cannot activate Bax *in vitro* do not kill HEK293 cells

627 Together, our data suggests that specific residues within the Bim CTS are involved in  
628 different aspects of BimL functioning to activate Bax. Residue I125 is required for Bim to  
629 bind to mitochondria but is of lesser importance in activating Bax. In contrast, residues  
630 L129 and I132 are not required for BimL to bind Bax but are important for it to efficiently  
631 activate Bax. Finally BimL-dCTS functions only to bind and inhibit Bcl-XL. The defined  
632 mechanism(s) of these mutants makes them useful for probing the differential sensitivity  
633 of HEK293 and MEF cells to expression of  $\Delta$ BimL-dCTS as seen in Figure 1. Expression  
634 of the mutants in HEK293 cells by transient transfection revealed that similar to  $\Delta$ BimL-  
635 dCTS, expression of either  $\Delta$ BimL-L129E or  $\Delta$ BimL-I132E was not sufficient to kill  
636 HEK293 cells, despite expression of either mutant being sufficient to kill the primed MEF  
637 cell line (Figure 9). In contrast, HEK293 cells were killed by expression of  $\Delta$ BimL-I125E,  
638 albeit to a lesser extent than by  $\Delta$ BimL (Figure 9). This result is consistent with our

639 findings with purified proteins showing that the EC50 for liposome permeabilization by  
 640 BimL-I125E was 100 nM compared to ~ 1nM for BimL (Figure 6B). The activity of  $\nabla$ BimL-  
 641 I125E also demonstrates that BimL binding to membranes is not required to kill HEK293  
 642 cells as BimL-I125E does not bind membranes (Figure 6B). Together, this data suggests  
 643 that unlike MEF cells, only mutants of BimL that can efficiently activate Bax kill HEK293  
 644 cells.

645

## Figure 9



646

647

648 **Figure 9: Bim CTS mutants that cannot activate Bax *in vitro* cannot kill HEK293**  
 649 **cells.**

650 The indicated cell lines were transiently transfected with DNA to express  $\nabla$ BimL, and the  
 651 indicated  $\nabla$ BimL mutant proteins. Cells expressing Venus fusion proteins were stained  
 652 with the nuclear dye Draq5 and rhodamine labelled Annexin V, and apoptosis was  
 653 assessed by confocal microscopy as in Figure 1. The y-axis indicates Annexin V  
 654 Positivity (%), which was calculated based on the total number of Venus expressing cells  
 655 that also score positive for Annexin V rhodamine fluorescence. A minimum of 400 cells  
 656 were imaged for each condition. Individual points (open circles) represent the average

657 for each replicate, while the bar heights, relative to the y-axis, represent the average for  
658 all three replicates. A one-way ANOVA was used within each cell line followed by a  
659 Tukey's multiple comparisons test to compare the means of each transfection group. \*p-  
660 values less than 0.05, \*\*p-values less than .01, ns, non-significant p-values (>0.05).

661

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662

## 663 **Discussion**

664 The apoptotic activity of Bim in live cells is likely mediated by a combination of  
665 functions that result in both activation of Bax and inhibition of anti-apoptotic proteins.  
666 Unlike any of the known BH3-proteins or small molecule inhibitors, BimL-dCTS inhibits  
667 all of the major multi-domain Bcl-2 family anti-apoptotic proteins without activating Bax or  
668 Bak. Thus expression of this protein in cells enables new insight into the importance of  
669 the extent to which a cell depends on the expression of anti-apoptotic proteins for  
670 survival (Figure 1A). Our results strongly suggest that the varying levels of apoptotic  
671 response of cell lines to BimL-dCTS reflect the extent to which that particular cell type is  
672 primed. Thus, HEK293 cells and adult neurons that are resistant to inhibition of Bcl-2,  
673 Bcl-XL and Mcl-1 but sensitive to activation of Bax, are functionally unprimed. Our data  
674 also reveal that intermediate states exist in which cells like CAMA-1 and BMK are  
675 partially resistant to inhibition of the multi-domain anti-apoptotic proteins (Figure 1A).

676 Partial resistance to expression of BimL-dCTS suggests that the flow of Bcl-2  
677 family proteins between different binding partners leads to differential levels of  
678 dependency on the activation of Bax to trigger apoptosis. To illustrate this we have  
679 created a schema illustrating protein flow at the two extremes represented by fully  
680 unprimed and primed cells and the effects of mutations in the Bim CTS on regulating  
681 apoptosis (Figure 10). In the schema, flow is indicated by the different lengths of the  
682 equilibria arrows and illustrates the consequences of the various dissociation constants



683 displayed in Figure 6B. BH3-proteins that do not efficiently activate BAX, such as BimL-  
684 L129E or BimL-I132E, interact primarily with anti-apoptotic proteins (illustrated here as  
685 Bcl-XL since it was possible to measure binding with purified proteins). The binding  
686 measurements in Figure 6B allow prediction of the outcome of more subtle differences in  
687 interactions for BimL and its mutants. For example, even though BimL-I125E activates  
688 Bax the concentration required is around 100nM while the dissociation constant for Bcl-  
689 XL is less than 3nM (Figure 6B) such that in cells BimL-I125E would preferentially bind  
690 and inhibit Bcl-XL rather than activate Bax (Figure 10B). While the CTS is necessary for  
691 Bim to activate Bax at physiologically relevant concentrations, membrane binding  
692 mediated by the CTS is not a prerequisite for interaction with Bax. Rather, binding to  
693 membranes increases subsequent Bax activation possibly through facilitating Bax  
694 conformational changes on the membrane (Figure 7B; compare BimL-dCTS-MAO and  
695 BimL-dCTS; and Figure 6B compare BimL, BimL-CTS2A and BimL-I125E). Thus it is  
696 likely that in cells expressing endogenous Bim, binding to membranes contributes to the  
697 efficiency with which the protein kills cells. Nevertheless, there exists the distinction in  
698 mechanism between Bim and tBid, as tBid requires membrane binding and a  
699 subsequent conformational change in order to bind and efficiently activate Bax (Lovell et  
700 al. 2008), while BimL can do so in solution via dual interactions by the Bim BH3 and CTS  
701 regions (Figure 3C and 10).

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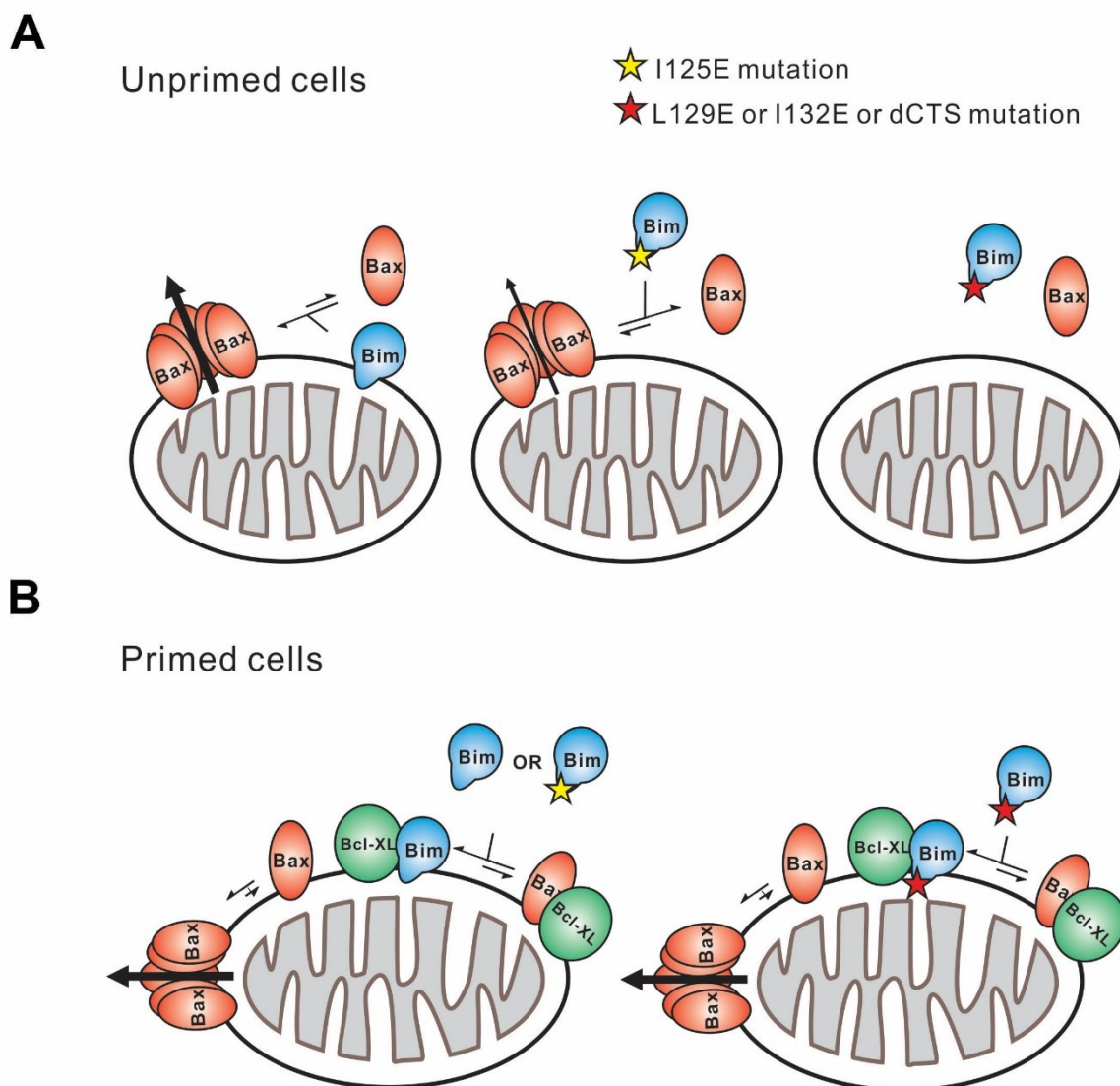
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## Figure 10



707

### 708 **Figure 10: Schematic overview for the Bim CTS pro-apoptotic function**

709 Interactions between BimL (blue), effector protein Bax (red) and Bcl-XL (green) are  
 710 shown at mitochondria of unprimed and primed cells as indicated. Mutations of the Bim  
 711 CTS are shown as a red or yellow star. Direction of protein flow into complexes indicated  
 712 by lengths of the equilibria arrows is based on the Kds measured for the binding  
 713 interactions (Figure 6B), the approximate cellular concentrations of the various proteins  
 714 and activity assays with liposomes and mitochondria.

715 (A) In unprimed cells the direct activation of Bax is the main function of Bim for  
 716 inducing apoptosis. Comparison of BimL125E with BimL-L129E, BimL-I132E, and BimL-  
 717 dCTS shows that the CTS, not membrane binding controls the activation of Bax by BimL  
 718 (Figure 6B). BimL binds membranes and can activate Bax. BimL-I125E (yellow star) has

719 no detectable membrane binding activity but still binds to and activates Bax, albeit with  
720 reduced activity compared to BimL (Figure 6B). At physiologically relevant  
721 concentrations BimL-L129E, BimL-I132E, and BimL-dCTS do not activate Bax.  
722 However, BimL-L129E and BimL-I132E binding to Bax is not reduced enough to account  
723 for the loss in Bax activation and membrane permeabilization suggesting these two  
724 residues are involved in activating Bax.

725 (B) In primed cells, one or more pro-apoptotic proteins (activated Bax/Bak and/or a  
726 Bax-activating BH3-protein) are sequestered by anti-apoptotic proteins at the MOM. For  
727 simplicity only active Bax is shown. Depending on the amount of active pro-apoptotic  
728 protein sequestered and the amount of free inactive Bax and or Bak in the cell, BimL  
729 may initiate apoptosis primarily by inhibiting anti-apoptotic proteins or by activating Bax  
730 and inhibiting anti-apoptotic proteins. The Bim CTS is not required for binding to and  
731 inhibiting anti-apoptotic proteins as BimL-L129E, BimL-I132E, and BimL-dCTS bind to  
732 anti-apoptotic proteins such as Bcl-XL and release both pro-apoptotic BH3-proteins and  
733 Bax (Figure 5 and Figure 6B), thus enabling killing of primed cells.

734

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735

736 The activities of the various Bim mutants analyzed here further suggest that  
737 specific residues in the Bim CTS enable physiological concentrations of Bim to activate  
738 Bax. That BimLV124E, BimLI125E and BimLL129E all bind Bax in solution and on  
739 membranes with similar affinities yet vary functionally to trigger Bax mediated liposome  
740 permeabilization by three orders of magnitude, suggests a specific role for this region in  
741 activation of Bax (Figure 6B) rather than the region simply increasing overall binding  
742 affinity. The situation is further complicated by another major role of the CTS of Bim in  
743 binding the protein to membranes. BimL-dCTS-MAO binds to mitochondria yet is  
744 defective in activating Bax to induce MOMP further suggesting a role for specific  
745 residues in the CTS binding to and activating Bax (Figure 7B). Such a role is consistent  
746 with our crosslinking data suggesting direct binding between these positions in the CTS  
747 of Bim and Bax (Figure 8) and that these residues particularly L129 (which corresponds  
748 to L185 in BimEL) increased the affinity for Bim binding to Bcl-XL such that it conferred  
749 resistance to BH3 mimetic drugs (Liu et al. 2019). Nevertheless, it remains formally

750 possible that changes in binding affinity coupled with alterations in effective off-rate due  
751 to membrane binding may also contribute to the activation of Bax by Bim.

752         Currently, BH3-profiling is the technique used to assay the state of apoptotic  
753 priming for different tissue types, however, this technique requires the addition of BH3  
754 peptides at high concentrations, and can only be performed on cells/tissues after  
755 permeabilization of the plasma membrane (Potter and Letai 2016). As an alternative, we  
756 propose lentiviral delivery and expression of BimL-dCTS be performed on living cells (or  
757 tissue samples), with readouts currently being used to assay cell death such as Annexin  
758 V staining, condensed nuclei, PI staining of nuclei, etc. Recently, it was reported in  
759 adults that most tissues are unprimed (Sarosiek et al. 2016), however the status of  
760 priming for different cell types that make up a single tissue may differ. In contrast, in  
761 tissue culture most cells are at least partially primed (Figure 1). We speculate that stress  
762 responses that result when fully or partially transformed cell lines are grown under non-  
763 physiological conditions (high glucose and oxygen, in the presence of serum and on  
764 plastic with abnormal stromal interactions) generally account for the dependence of  
765 these cell lines on continued expression of anti-apoptotic proteins. BH3-profiling can only  
766 provide an answer at the tissue level or for cell populations that can be isolated in  
767 sufficient quantities or easily cultured (Sarosiek et al. 2016). However, lentiviral delivery  
768 and expression of <sup>V</sup>BimL-dCTS to specific cells in cultures, tissue slices and *in vivo* can  
769 provide the means to assay the level of dependence of individual cells on expression of  
770 anti-apoptotic Bcl-2 family proteins. This information could prove valuable to  
771 understanding which cell types may be most affected by small molecule BH3 mimetics  
772 used or in trial as chemotherapeutics and to better predict and prevent off-target  
773 toxicities that result in cell priming.

774 Overall, our data suggests a model in which the unusual CTS of Bim is not only  
775 required for binding to membranes but is directly involved in the activation of Bax. This  
776 function is crucial for BimL in killing unprimed cells. The CTS also increases the affinity  
777 of Bim for binding to Bcl-XL and Bcl-2 that is sufficient to induce apoptosis in primed  
778 cells (Figure 10). The very much higher affinity of Bim for Bcl-XL and Bcl-2 compared to  
779 Bax also ensures that in cells with excess anti-apoptotic proteins Bim is effectively  
780 sequestered and neutralized. In separate studies, we demonstrate that the additional  
781 affinity of the interaction of Bim with Bcl-XL and Bcl-2 provided by the Bim CTS is  
782 sufficient to dramatically reduce displacement of Bim by small molecule BH3 mimetics  
783 (Liu et al. 2019). Thus, regulation of apoptosis by Bcl-2 proteins is more complicated  
784 than presented in most current models. Moreover, the mutants and binding affinities  
785 described here provide the tools necessary for future studies of the relative importance  
786 of activation of Bax compared to inhibition of anti-apoptotic proteins in intact cells and in  
787 animals.

788

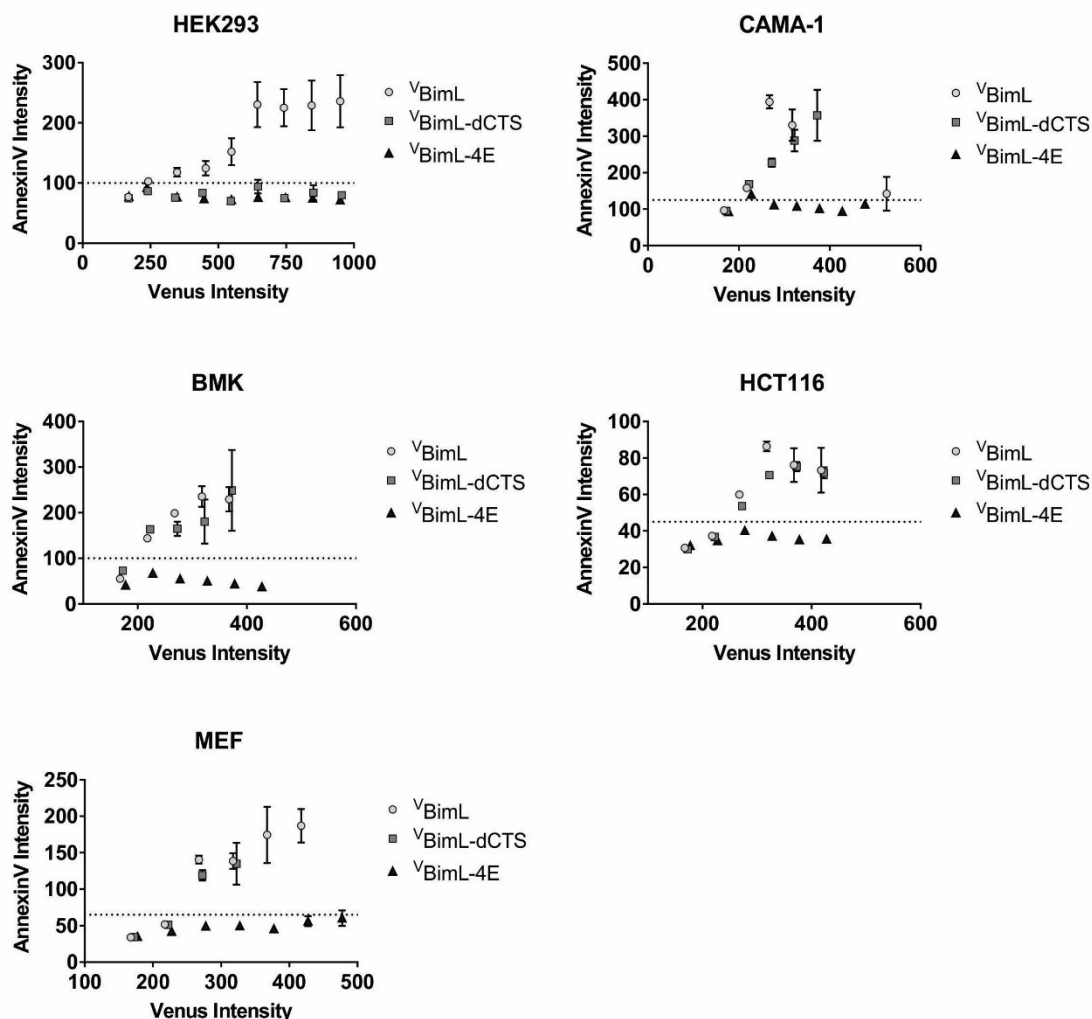
789

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## Figure 1- figure supplement 1



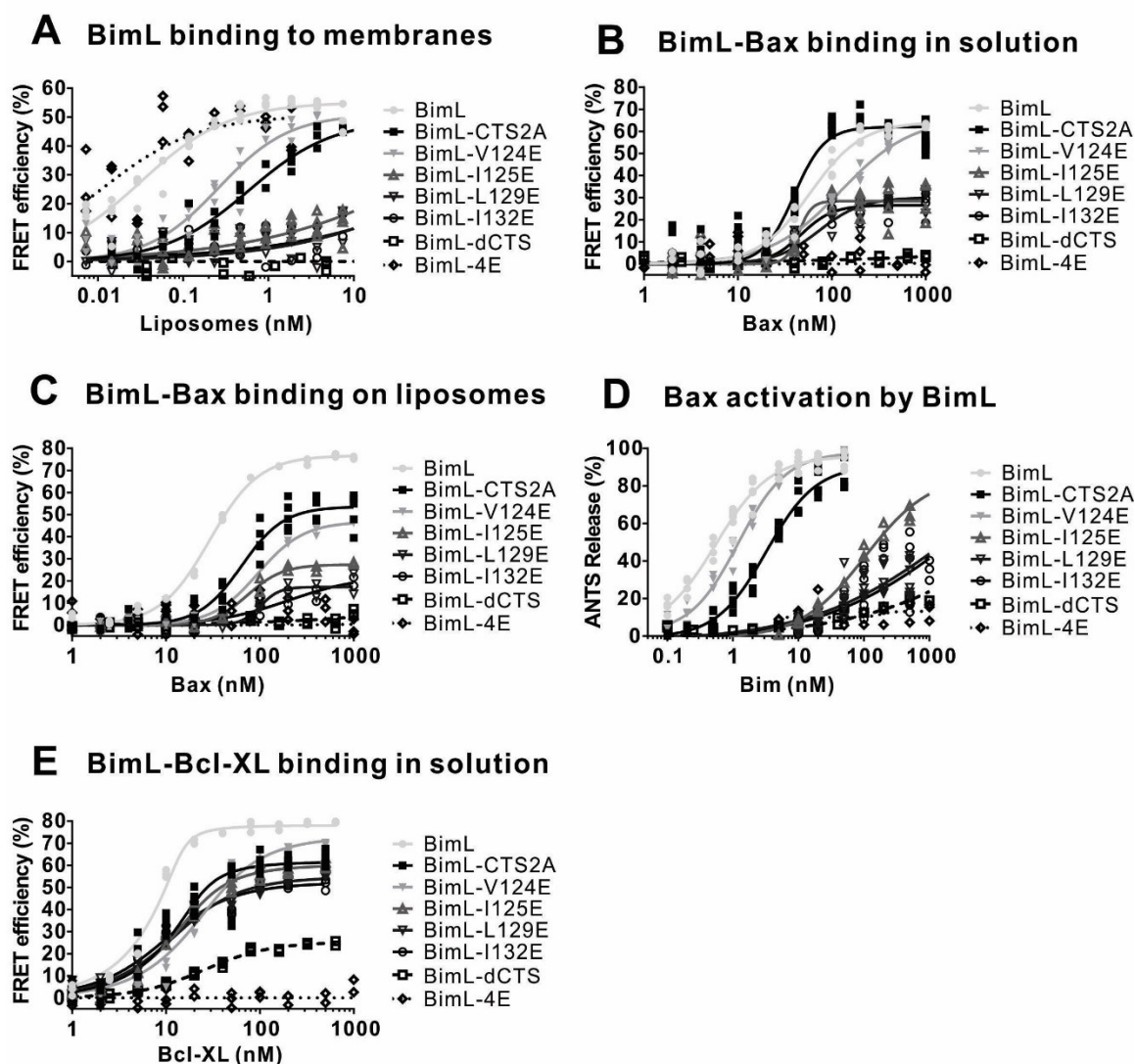
793

794 **Figure 1 – figure supplement 1**

795 Correlation between expression of V<sup>BH3</sup>-proteins and apoptosis measured as Annexin V  
796 labeling. For each cell line the Annexin V Rhodamine intensity was plotted against the  
797 Venus intensity. The Venus intensity acts as a surrogate for the relative amount of each  
798 of the BimL mutant proteins being expressed. Cells with intensity in the Venus channel  
799 equal to or less than the mean signal from untransfected cells were pooled as the first  
800 point, the subsequent points are data from intensity bins with 50 arbitrary unit  
801 increments. The horizontal dotted line indicates two standard deviations above the signal  
802 from untransfected cells in the AnnexinV channel. The cell line is indicated at the top of  
803 each panel.

804

## Figure 6- figure supplement 1



805

806

807 **Figure 6 – Figure supplement 1**

808 (A) BimL binding to membranes.

809 (B) BimL binding to Bax in solution.

810 (C) BimL binding to Bax with 2.9 nM liposomes.

811 (D) Bax (100 nM) activation by BimL.

812 (E) BimL binding to Bcl-XL in solution.



813 For FRET experiments in (A-C) and (E), 20 nM of the indicated Alexa568-labeled BimL  
814 mutants (FRET donor) were incubated with the indicated concentrations of the Alexa647  
815 labeled FRET acceptor labeled proteins. For each panel data from three independent  
816 experiments are shown as individual points, some points are not visible due to overlap.  
817 The mutants analyzed are indicated to the right of the graphs. To permit accurate  
818 estimation of the binding constants presented in Figure 6, data was collected to  
819 saturation for all mutants (for some curves 1600nM or 3200nM acceptor concentrations  
820 were required). For presentation purposes all curves were truncated at 1000 nM.

821

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822

## 823 **Experimental Procedures**

### 824 Protein Purification

825 Wild type and single cysteine mutants of Bax, Bcl-XL, and cBid were purified as  
826 described previously (Kale et al. 2014). cBid mutant 1 (cBidmt1) was purified with the  
827 same protocol used for cBid (Kale et al. 2014). Bad was purified as described previously  
828 (Lovell et al. 2008).

829 His-tagged Noxa was expressed in E. coli strain BL21DE3 (Life Tech, Carlsbad, CA). E.  
830 coli cells were lysed by mechanical disruption with a French press. The cell lysate was  
831 diluted in lysis buffer (10mM HEPES (7.2), 500mM NaCl, 5mM MgCl<sub>2</sub>, 0.5% CHAPS,  
832 1mM DTT, 5% glycerol, 20mM Imidazole) and Noxa was purified by affinity  
833 chromatography on a Nickel-NTA column (Qiagen, Valencia, CA). Noxa was eluted with  
834 a buffer containing 10mM HEPES (7.2), 300mM NaCl, 0.3% CHAPS, 20% glycerol,  
835 100mM imidazole, dialyzed against 10mM HEPES 7.2, 300mM NaCl, 10% glycerol,  
836 flash-frozen and stored at -80 °C.

837 Purification of BimL and single cysteine mutants of BimL was carried out as previously  
838 described (Liu et al. 2019). Briefly, cDNA encoding full-length wild-type murine BimL was  
839 introduced into pBluescript II KS(+) vector (Stratagene, Santa Clara, CA). Sequences

840 encoding a polyhistidine tag followed by a TEV protease recognition site  
841 (MHHHHHHGGSGGTGGSENLVYFQGT) were added to create an in frame fusion to the  
842 N-terminus of BimL. All of the purified BimL proteins used here retained this tag at the  
843 amino-terminus. However, control experiments demonstrated equivalent activity of the  
844 proteins before and after cleavage with TEV protease. Mutations as specified in the text  
845 were introduced into this sequence using site-directed mutagenesis.

846 Bim was expressed in Arabinose Induced (AI) *E. coli* strain (Life Tech, Carlsbad, CA). *E.*  
847 *coli* were lysed by mechanical disruption with a French press. Proteins were purified  
848 from the cell lysate by affinity chromatography using a Nickel-NTA column (Qiagen,  
849 Valencia, CA). A solution containing 20mM HEPES pH7.2, 10mM NaCl, 0.3% CHAPS,  
850 300mM imidazole, 20% Glycerol was used to elute the proteins. The eluate was  
851 adjusted to 150 mM NaCl and applied to a High Performance Phenyl Sepharose (HPPS)  
852 column. Bim was eluted with a no salt buffer and dialyzed against 10mM HEPES pH7.0,  
853 20% glycerol, flash-frozen and stored at -80 °C.

#### 854 Protein labeling

855 Single cysteine mutants of Bax, Bcl-XL, cBid and Bad were labeled with the  
856 indicated maleimide-linked fluorescent dyes as described previously (Pogmore et al.  
857 2016; Lovell et al. 2008; Kale et al. 2014). Single cysteine mutants of Bim were labeled  
858 with the same protocol as cBid with the exception that the labeling buffer also contained  
859 4M urea.

#### 860 Bim binding to membranes

861 Liposomes (100 nm diameter) with a lipid composition resembling MOM were  
862 prepared as described previously (Kale et al. 2014). Mouse liver mitochondria were  
863 isolated from Bak<sup>-/-</sup> mice as previously described (Pogmore et al. 2016). Liposomes and



864 mitochondria were labeled with 0.5% and 2% mass ratios DiD, respectively (Life Tech,  
865 Carlsbad, CA). The single-cysteine mutant of Bim, BimQ41C, was labeled with  
866 Alexa568-maleimide and incubated with the indicated amount of unlabeled or DiD-  
867 labeled mitochondria or liposomes at 37° C for 1h. Intensities of Alexa568 fluorescence  
868 were measured in both samples as  $F_{\text{unlabeled}}$  and  $F_{\text{labeled}}$  respectively using the Tecan  
869 infinite M1000 microplate reader. FRET, indicating protein-membrane interaction, was  
870 observed by the decrease of Alexa568 fluorescence when Bim bound to DiD labeled  
871 membranes compared to unlabeled membranes. FRET efficiency was calculated as  
872 described previously (Shamas-Din, Bindner, et al. 2013). The data was fit to a binding  
873 model as described below. Lines of best fit were calculated using least squares in  
874 Graphpad Prism software.

#### 875 Membrane permeabilization

876 Membrane permeabilization assays with liposomes encapsulating ANTS and DPX were  
877 performed as described previously (Kale et al. 2014). To measure permeabilization of  
878 BMK mitochondria, the indicated amounts of proteins were incubated with mitochondria  
879 (1mg/mL) purified from BMK cells genetically deficient for Bax and Bak expressing  
880 mCherry fluorescent protein fused to the SMAC import peptide responsible for  
881 localization in the inter-membrane space. After incubation for 45 min at 37° C samples  
882 were centrifuged at 13000g for 10min to separate the pellet and supernatant fractions  
883 and membrane permeabilization was calculated based on the mCherry fluorescence in  
884 each fraction (Shamas-Din, Bindner, et al. 2013). For mouse liver mitochondria,  
885 cytochrome c release was measured by immunoblotting as described previously  
886 (Pogmore et al. 2016; Sarosiek et al. 2013).

#### 887 BH3 profiling

888 Heavy membranes enriched in mitochondria were isolated as described previously  
889 (Pogmore et al. 2016; Brahmhatt et al. 2016). Membrane fractions (1mg/ml) were  
890 incubated with 500nM of the specified BH3 proteins (Bim, Bad and/or Noxa). For E15  
891 brain mitochondria, 0.5mg/mL of membrane fractions were used and incubated with the  
892 indicated amounts of BH3-only proteins for 30 min at 37°C. Membranes were pelleted by  
893 centrifugation at 13000g for 10 min and cytochrome c release was analyzed by  
894 immunoblotting using a sheep anti-cytochrome c antibody (Capralogics). Mitochondria  
895 from embryonic mouse brains for BH3 profiling experiments were prepared from ~20  
896 mouse embryos, E15 in age, following the same protocol used for liver mitochondria  
897 (Pogmore et al. 2016).

#### 898 Protein-protein binding

899 For FRET experiments, single cysteine mutants of cBid (126C), Bcl-XL (152C), Bax  
900 (126C), BimL (41C) and BimL mutants were purified and labeled with either Alexa 568-  
901 maleimide (donor) or Alexa 647-maleimide (acceptor) as specified. To determine binding  
902 constants donor protein was incubated with the indicated range of acceptor proteins and  
903 where specified liposomes or mitochondria. The intensity of Alexa568 fluorescence with  
904 unlabeled or Alexa647-labeled Bcl-XL was measured as  $F_{\text{unlabeled}}$  or  $F_{\text{labeled}}$  respectively  
905 and FRET was calculated as described in (Pogmore et al. 2016). All measurements  
906 were collected using the Tecan infinite M1000 microplate reader. Lines of best fit were  
907 calculated using least squares in Graphpad Prism software.

908 For each pair of proteins a dissociation constant ( $K_d$ ) was measured in solution  
909 and with liposomes. Curves were fit to an advanced function taking into account the  
910 concentration of acceptor ( $[A]$ ) change when  $[A]$  is close to  $K_d$ :

911 
$$F = (F_{\max}) \left( \frac{([D] + [A] + K_d) - \sqrt{([D] + [A] + K_d)^2 - 4[D][A]}}{2[D]} \right)$$

912 [D] is the concentration of donor, F indicates the FRET efficiency with the concentration  
913 of acceptor as [A],  $F_{\max}$  is the maximum FRET efficiency in the curve (Pogmore et al.  
914 2016).

### 915 Photocrosslinking of Bim to Bax

916 The photocrosslinking method for studying interactions among the Bcl-2 family proteins  
917 has been described in detail (Lin, Johnson, and Zhang 2018). Briefly, [<sup>35</sup>S]Met-labeled  
918 BimL proteins with a single εANB-Lys incorporated at specific locations were  
919 synthesized using an in vitro translation system. 10 μl of the resulting BimL proteins  
920 were incubated at 37°C for 1 h with 1 μM of 6H-Bax or 6H-Bcl-XL protein and Bak<sup>-/-</sup>  
921 mouse liver mitochondria (0.5 mg/ml total protein) in a 21-μl reaction adjusted by buffer  
922 A (110 mM KOAc, 1 mM Mg(OAc)<sub>2</sub>, 25 mM HEPES, pH 7.5). The mitochondrial and  
923 soluble fractions were separated by centrifugation at 13,000 x g and 4°C for 5 min, and  
924 the mitochondria were resuspended in 21 μl of buffer A. Both mitochondrial and soluble  
925 fractions were photolyzed to induce crosslinking via the ANB probe. The resulting  
926 samples were adjusted to 250 μl with buffer B (buffer A with 1% Triton X-100 and 10 mM  
927 imidazole) and incubated with 10 μl of Ni<sup>2+</sup>-chelating agarose at 4°C for overnight. After  
928 washing the Ni<sup>2+</sup>-beads three times with 350 μl of buffer B and one time with 400 μl of  
929 PBS, the photoadducts of the radioactive BimL protein and the 6H-tagged Bax or Bcl-XL  
930 protein and other proteins bound to the Ni<sup>2+</sup>-beads were eluted and analyzed by  
931 reducing SDS-PAGE and phosphor-imaging.

### 932 Measurement of cell death in response to expression of <sup>V</sup>BimL constructs

933 HEK293, CAMA-1, BMK, MEF, and HCT116 cells were maintained at 37°C (5%  
934 v/v CO<sub>2</sub>) in dMEM complete [dMEM, 10% Fetal Bovine Serum, 1% essential amino acids  
935 (Gibco, Grand Island, NY)]. Cells were seeded in CellCarrier-Ultra 384-well plates (1000  
936 cells/well for BMK and MEF, 2000 cells/well for HEK293 and HCT116, 3000 cells/well for  
937 CAMA-1). One day later, cells were transfected using FugeneHD (Promega, Madison,  
938 WI) with plasmids encoding Venus, or Venus-fused BimL constructs in an EGFP-C3  
939 backbone. Cell culture medium was added to each reaction (50µl/0.05µg DNA) and the  
940 whole mix added to each well (50µl/well) of a pre-aspirated 384-well plate of cells. After  
941 24 hours, cells were stained with Draq5 and Rhodamine-labeled Annexin V and image  
942 acquisition was performed using the Opera QEHS confocal microscope (Perkin Elmer,  
943 Woodbridge, ON) with a 20x air objective. Untransfected cells and cells treated with  
944 1µg/mL staurosporine were used as negative and positive controls for Annexin V  
945 staining. Cells were identified automatically using software as described previously  
946 (Shamas-Din, Bindner, et al. 2013). Intensity features were extracted using a script  
947 (dwalab.ca) written for Acapella high content imaging and analysis software (Perkin  
948 Elmer, Woodbridge, ON). Cells were scored as Venus or Annexin V positive if the Venus  
949 or Annexin V intensity was greater than the average intensity plus two standard  
950 deviations for the Venus or Annexin V channels in images of non-transfected cells. Cell  
951 death ascribed to the <sup>V</sup>BimL fusion proteins was quantified as the percentage of Venus  
952 positive cells that were also Annexin V positive. For neuron cultures, cell segmentation  
953 using conventional methods could not be achieved due to complex cellular  
954 morphologies. Therefore, nuclei were first identified, then a ring region ~10% of nuclear  
955 area was drawn around each nuclei. Venus intensity was calculated for this ring region,  
956 representing the neuronal cell body, to determine if the neuron was expressing the  
957 Venus fluorescent protein.

958 Primary brain cortical neuron cultures were prepared from embryonic day 15, C57BL/6J  
959 mouse embryos as previously described (Mergenthaler et al. 2012). All animal breeding  
960 and handling was performed in accordance with local regulations and after approval by  
961 the Animal Care Committee at Sunnybrook Research Institute, Toronto. Briefly, after  
962 separation from hippocampus and subcortical structures, cortices were washed twice  
963 with ice-cold PBS, digested with 1x trypsin for 15 minutes at 37°C, washed twice with  
964 ice-cold PBS and then resuspended with a flame-treated glass pipette in N-Medium  
965 (DMEM, 10% v/v FBS, 2 mM L-glutamine, 10 mM Hepes, 45 µM glucose). The  
966 dissociated cortices were gently pelleted by centrifugation (200g for 5 minutes), N-media  
967 was removed, and neurons were resuspended and cultured in Neurobasal-Plus medium  
968 (ThermoFisher Scientific) supplemented with B27-Plus (ThermoFisher Scientific) and 1X  
969 Glutamax (ThermoFisher Scientific). Neurons were seeded at 5000 cells per well in a  
970 384 well plate (Greiner µclear) after coating with poly-d-lysine (Cultrex). The medium  
971 was partially replaced on day five in culture with Neurobasal-Plus supplemented with  
972 B27-Plus and 1X Glutamax.

973 Lentivirus to express <sup>V</sup>BimL and other BimL mutants were cloned into the pTet-O-Ngn2-  
974 Puro construct with the Ngn2 gene cut out. This construct was a kind gift from Dr. Philipp  
975 Mergenthaler, Charité Universitätsmedizin Berlin. Primary neuron cultures were infected  
976 with both <sup>V</sup>BimL and rtTA lentiviral particles (~3µL of each concentrated stock) on the  
977 day of seeding. 24 hours later, Neurobasal-Plus medium containing lentiviral particles  
978 was removed and replaced with fresh Neurobasal-Plus medium.

979 Doxycycline (ThermoFisher Scientific) was added to 8 day *in vitro* old cultures of neurons  
980 at a concentration of 1µg/mL to induce <sup>V</sup>BimL protein expression. 20 hours later,  
981 neurons were stained with 1 µg/mL Hoechst 33342 (Cell Signaling Technologies) and

982 1µg/mL propidium iodide (Bioshop), then incubated for 30 min at 37°C. Confocal

983 microscopy was performed immediately after.

#### 984 Lentiviral Production

985 Each lentivirus was made using the following protocol adhering to biosafety level 2

986 procedures. On day 0, lentiviral vectors psPax2 (10µg) and pMD2.G (1.25µg) were

987 mixed with 10µg of desired <sup>V</sup>BimL lentiviral construct in 1000µL of Opti-MEM media

988 (ThermoFisher Scientific). Next, 42µL of polyethylenimine (PEI) solution [1mg/mL] was

989 added, the mixture vortexed, then allowed to settle for 15 minutes at room temperature.

990 After 15 minutes, 1.5x10<sup>7</sup> of resuspended HEK293 cells and the transfection solution

991 were mixed and seeded onto a 100mm culture dish with 10mL of dMEM complete plus

992 10µM of the caspase inhibitor Q-VD-Oph (Selleckchem), and left to incubate at 37°C

993 (5% v/v CO<sub>2</sub>) for 72 hours. On day 3, media containing lentiviral particles was filter

994 sterilized using a 0.45µm polyethersulfone filter, and mixed with polyethylene glycol

995 (Bioshop) to achieve a final concentration of 10% (w/v). This was left to mix and

996 precipitate the virus overnight at 4°C. On day 4, the media was centrifuged for 1 hour at

997 1600g, supernatant was then removed and the pellet was resuspended with 200µL of

998 phosphate buffered saline. Resuspended virus was then stored at -80°C until needed.

999

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1001

1002

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1008

1009



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