Bcl-xL acts as an inhibitor of IP₃R channels, thereby antagonizing Ca²⁺-driven apoptosis

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INTRODUCTION
Inositol 1,4,5-trisphosphate receptors (IP₃Rs) are tetrameric Ca²⁺-permeable channels, predominantly located at the endoplasmic reticulum (ER) membrane [1-3]. Ca²⁺ release through IP₃Rs plays fundamental roles in a plethora of cellular processes, including proliferation, gene transcription, protein secretion, neurotransmitter release, fertilization, and apoptosis [4]. To maintain fidelity and specificity of these processes the activity of IP₃Rs is tightly regulated at multiple levels. Among the most common regulatory mechanisms are the modulation of channel expression, post-translational modifications, and interaction with regulatory factors including Ca²⁺ itself, ATP and protein partners [1, 5, 6]. These regulators target different IP₃R regions, which are arranged as globular domains such that the controlled trypsinization of IP₃R generates reproducible fragments [7], which have proven an excellent tool for dissecting the binding sites of different IP₃R partners [8-12].

The B-cell lymphoma 2 (Bcl-2) family of proteins is well known for its role in controlling mitochondrial apoptosis and mitochondrial dynamics [13, 14]. Anti-apoptotic Bcl-2 family members neutralize pro-apoptotic family members, including Bax/Bak and pro-apoptotic BH3-only proteins [15]. At the molecular level,
levels in MDA-MB-231 cells resulted in augmented ATP-induced IP₃R-mediated Ca²⁺ release and in increased sensitivity to staurosporine (STS). Overall, our data challenge the current paradigm that Bcl-xL promotes cell survival by sensitizing IP₃Rs to IP₃. Instead, we demonstrate that Bcl-xL inhibits IP₃R function through a conserved lysine residue in its BH3 domain, thereby protecting cells against IP₃R/Ca²⁺-driven apoptosis.

RESULTS

Bcl-xL inhibits IP₃R-mediated Ca²⁺ release in living cells

Bcl-xL has been reported to sensitize IP₃Rs in living cells [26]. Here, we evaluated the effect of Bcl-xL overexpression on IP₃R function by monitoring agonist-induced Ca²⁺ release (Fig. 1, Fig. S1). First, we performed Ca²⁺ measurements in a population-based assay, using the ratiometric fluorescent Ca²⁺ probe Fura-2 (Fig. S1). We used trypsin, an efficient agonist of protease activated receptors 2 in HEK-293 cells [29], thereby triggering IP₃ formation. We elicited IP₃R-mediated Ca²⁺ release in Fura-2-loaded HEK-3KO cells with reconstituted rIP₃R1 (HEK-rIP₃R1) and we studied the impact of overexpressing Bcl-xL. For this, we transfected the cells with either P2A-mCherry or 3xFLAG-Bcl-xL-P2A-mCherry. The 3xFLAG-Bcl-xL-P2A-mCherry construct generates separate mCherry and 3xFLAG-Bcl-xL proteins due to its P2A self-cleaving sequence. In contrast to previous reports [25, 26], Bcl-xL overexpression significantly reduced the amplitude (Fig. S1a, c) and the area under the curve of the Ca²⁺ signals (Fig. S1b, d) induced by both low (0.1 µM) and high (1 µM) trypsin concentrations. Moreover, similarly to our findings related to Bcl-2 and IP₃R function [24], we noticed that the inhibitory effect of Bcl-xL overexpression was more prominent at low agonist concentrations than at high agonist concentrations.

This unexpected IP₃R inhibition by Bcl-xL in population-based Ca²⁺ measurements prompted us to validate the effect of Bcl-xL overexpression on Ca²⁺ signals in single cells exposed to other agonists (Fig. 1) and to compare it with Bcl-2 overexpression, an established inhibitory modulator of IP₃Rs [20, 22]. Single cell Ca²⁺ imaging was performed in Fura-2-loaded HEK-293 cells transfected with either P2A-mCherry, 3xFLAG-Bcl-xL-P2A-mCherry or 3xFLAG-Bcl-xL-P2A-mCherry, whereby only mCherry-positive cells were analyzed. Here, we used ATP (10 µM) to trigger IP₃R-mediated Ca²⁺ release (Fig. 1a, b, c). Similarly, we observed that Bcl-xL overexpression reduced the percentage of responding cells (Fig. 1d) and the area under the curve as representative of the extent of Ca²⁺ release (Fig. 1e). Interestingly, Bcl-xL appeared to dampen IP₃R-mediated Ca²⁺ release to a similar extent as Bcl-2. We also measured the effect of Bcl-xL/Bcl-2 overexpression on Ca²⁺ signals elicited by another agonist, namely carbachol (Fig. 1f, g, h). We found that similarly to Bcl-2, Bcl-xL also inhibited carbachol-induced Ca²⁺ signals, although Bcl-xL appeared less potent than Bcl-2 (Fig. 1j). These results indicate that, similarly to Bcl-2, Bcl-xL inhibits IP₃R-mediated Ca²⁺ release, irrespective of the extracellular agonist that is applied.

Full-length Bcl-xL, but not its BH4 domain, targets the LBD of IP₃R1

Next, we elucidated the interaction between Bcl-xL and IP₃R. First, we compared the interaction of Bcl-xL and Bcl-2 with full-length IP₃R. We overexpressed 3xFLAG-tagged Bcl-xL or Bcl-2 in HeLa cells expressing endogenous IP₃Rs, immunoprecipitated Bcl-xL or Bcl-2 using anti-FLAG-coupled beads and immunoblotted for IP₃Rs (Fig. 2a). This co-immunoprecipitation (coIP) analysis revealed that Bcl-xL could immunoprecipitate IP₃Rs to a rather similar extend as Bcl-2, indicating that Bcl-xL and Bcl-2 display quite similar IP₃R-binding properties. Bcl-2 can bind to the central, modulatory region of IP₃R1, specifically to tryptic Fragment 3 [8, 12, 27]. We demonstrated that Bcl-2 binding to this site is associated with inhibition of IP₃R1 activity. In addition to this, we recently discovered that Bcl-2 could also bind to the LBD of IP₃R1,
indicating that multiple regions are involved in IP₃R1/Bcl-2 complex formation and inhibition of channel activity [11]. We also found that Bcl-xL could target this central, modulatory region of IP₃R1 though with lower efficiency than Bcl-2 [12]. However, given the prominent inhibition of IP₃Rs by Bcl-xL and the observation that this inhibition appears dependent on the agonist concentration, we asked whether Bcl-xL could also target the LBD. Thus, we used lysates from COS-7 cells that overexpressed 3xFLAG-Bcl-xL in GST-pull down experiments against purified GST-LBD and GST-Fragment 3 (representing a major part of the central, modulatory region) of IP₃R1 (Fig. 2b). Our analysis revealed that similarly to Bcl-2, Bcl-xL can bind to both regions (Fig. 2c, Fig. S2a). Since GST-pulldowns are only semi-quantitative, we applied microscale thermophoresis (MST), a biophysical approach allowing to measure molecular interactions. This technique is based on detecting a change in fluorescence of a labeled target as a function of the concentration of a non-fluorescent ligand. The change in fluorescence reflects the thermophoretic movement of the fluorescent target subjected to a microscopic temperature gradient. We thus used MST to assess direct binding between purified GST-IP₃R fragments and purified 6xHis-Bcl-xL to determine the binding affinity. Using MST, we demonstrated that both purified GST-LBD and GST-Fragment 3 could bind to wild-type 6xHis-Bcl-xL (Fig. 2d). The specificity of this interaction is underpinned by two negative controls, parental GST and GST-Fragment 5b that lacks the 6th TMD, previously established to be critical for Bcl-xL binding [12]. Indeed, no binding between 6xHis-Bcl-xL and GST or GST-Fragment 5b could be detected. Furthermore, we obtained the dissociation constant for both domains with 6xHis-Bcl-xL, revealing a KD of ~701 nM for 6xHis-Bcl-xL interaction with the GST-LBD and a KD of ~495 nM for 6xHis-Bcl-xL interaction with GST-Fragment 3. This indicates that wild-type 6xHis-Bcl-xL binds to both the LBD and Fragment 3.

We previously characterized the binding characteristics of the BH4 domain of Bcl-2 and Bcl-xL with Fragment 3 in detail via surface plasmon resonance (SPR) [27]. This study showed that the BH4 domain of Bcl-2 but not the one of Bcl-xL could interact with the Fragment 3. Recently, we also identified a novel binding site for Bcl-2's BH4 domain in the LBD, but had not yet characterized its ability to interact with Bcl-xL's BH4 domain [24]. Thus, we examined the importance of the BH4 domain of Bcl-xL for binding to the LBD using SPR. Biotin coupled to a peptide encompassing BH4-Bcl-xL was immobilized on streptavidin chips and different concentrations of purified LBD were applied as an analyte. Background binding was determined using a peptide with a scrambled sequence and subtracted. Biotin-BH4-Bcl-xL was used as a positive control for detecting LBD binding. The association curves for 1.1 µM GST-LBD show prominent binding to BH4-Bcl-xL while its binding to BH4-Bcl-xL is much lower (Fig. 2e). Similarly to what was observed for the binding of Fragment 3 to BH4-Bcl-2 versus BH4-Bcl-xL [27], GST-LBD displayed a strong concentration-dependent binding to immobilized BH4-Bcl-xL [11], while its binding to immobilized BH4-Bcl-xL appeared much weaker (Fig. 2f).

Taken together, these data reveal that while both Bcl-2 and Bcl-xL target the same regions in IP₃R, they employ different binding determinants for these interactions. In contrast to Bcl-2, which exploits its BH4 domain for binding to LBD [24] and Fragment 3 [27], Bcl-xL seems to interact with the same IP₃R regions but via motifs located outside of the BH4 domain.

Residue K87 of Bcl-xL contributes to the interaction with IP₃R and particularly to the binding to LBD and Fragment 3

Our previously published results [27] and the data reported here (Fig. 2f) indicate that in contrast to the BH4-Bcl-2, the BH4-Bcl-xL could not be responsible for targeting LBD and Fragment 3 of IP₃R1. We therefore aimed to elucidate the molecular determinants in Bcl-xL responsible for its interaction with IP₃R. Since Bcl-xL targets the same IP₃R regions as Bcl-2, we envisioned that a similar interaction surface could underlie this phenomenon. We previously showed that K17 located in the middle of the BH4-Bcl-2 was critical for binding and inhibiting IP₃Rs [27]. In the BH4-Bcl-xL, the corresponding residue is not a lysine but an asparagine, preventing its ability to bind to IP₃R. However, a previously performed in silico Bcl-2/Bcl-xL structure superposition revealed that K87, located in the BH3 domain of Bcl-xL (B3-Bcl-xL), likely is spatially constrained in a similar manner to K17 of BH4-Bcl-2 (Fig. 3a) [28]. Moreover, sequence analysis of Bcl-xL orthologs among main vertebrate lineages revealed that K87 is highly
conserved (Fig. 3b) and thus the importance of this residue was examined further. Interestingly, this lysine is located on the opposite side of the binding pocket involved in the interaction with Bak and Bax. First, we used confocal microscopy to assess whether altering K87 into an aspartate affected Bcl-xL’s subcellular localization. We transfected HeLa cells to express a mitochondria- or an ER-targeted RFP and compared the localization of 3xFLAG-Bcl-xL versus 3xFLAG-Bcl-xLK87D using anti-FLAG-based immunofluorescence (Fig. S3a–d). We calculated average Pearson’s coefficients above 0.75 for all conditions (Fig. S3e, f), indicating a high colocalization of Bcl-xL and Bcl-xLK87D with both the mitochondria and the ER. We also calculated average Manders’ M1 coefficient to quantify the fraction of Bcl-xL or Bcl-xLK87D overlapping with the mitochondria, being 0.8 for both Bcl-xL and Bcl-xLK87D, or with the ER, being 0.6 for both Bcl-xL and Bcl-xLK87D (Fig. S3g, h). The similar coefficients calculated for the wild-type Bcl-xL and the Bcl-xLK87D indicate that the K87D mutation in Bcl-xL did not alter its subcellular localization.

Second, we tested the effect of the K87D mutation on the interaction of Bcl-xL with full-length IP3R. We therefore overexpressed 3xFLAG-tagged Bcl-xL or Bcl-xLK87D in HeLa cells expressing endogenous IP3Rs (Fig. 3c), immunoprecipitated Bcl-xL or Bcl-xLK87D using anti-FLAG-coupled beads and immunoblotted for IP3Rs. This co-immunoprecipitation analysis revealed that Bcl-xLK87D binding to the IP3R channel is severely impaired compared to wild-type Bcl-xL (Fig. 3c, d). We also immunoblotted for Bax to determine Bax binding to Bcl-xL or Bcl-xLK87D. We found that both Bcl-xL and Bcl-xLK87D could bind Bax, though Bax binding to Bcl-xLK87D appeared slightly reduced compared to its binding to Bcl-xL (Fig. 3c, e).

Third, we performed GST-pull down experiments with lysates from COS-7 cells overexpressing 3xFLAG-Bcl-xL or 3xFLAG-Bcl-xLK87D (Fig. 3f, Fig. S2b). We compared their binding to purified

Fig. 2 Bcl-xL, but not its BH4 domain, binds to IP3R1 involving LBD and Fragment 3. a Representative co-immunoprecipitation experiments using anti-FLAG performed in lysates from HeLa cells transiently overexpressing 3xFLAG-Bcl-2 or 3xFLAG-Bcl-xL. This experiment was performed three times using each time independently transfected and freshly prepared cell lysates. The samples were analyzed via western blot using antibodies against IP3R1 and FLAG. Total HeLa lysates were used as input (20 µg). PD: pull down; IB: immunoblot. b Linear representation of a mouse IP3R1 (mIP3R1) monomer. The three functional domains, including the ligand-binding domain (LBD), and the five tryptic fragments, including Fragment 3, are represented. Respective amino acids are indicated by numbers. TMDs: transmembrane domains. c Representative GST-pull down experiment for assessing the binding of 3xFLAG-Bcl-xL from COS-7 cell lysate to GST-fused IP3R1 fragments. The samples were analyzed via western blot. Total COS-7 lysate was used as input (0.1 µg). This experiment was performed four times utilizing each time independently transfected and freshly prepared cell lysates. PD: pull down; IB: immunoblot. The corresponding western blot for the GST-LBD, GST-Fragment 3, GST-Fragment 5b and parental GST proteins were titrated down from 15 µM to 5 nM. The unit of the left axis ($\Delta F_{\text{norm}}$) is a ratio of normalized fluorescence. Data points represent mean ± SD from triplicate measurements. e Representative sensorgrams of SPR experiments showing the binding properties of GST-fused IP3R-LBD, applied at 1.1 µM, to biotin-BH4-Bcl-xL and biotin-BH4-Bcl-2 peptides. The biotin-BH4 peptides, immobilized on a streptavidin-coated sensor chip. Sensorgrams were obtained after background correction for binding to the scrambled peptides. Data are expressed in resonance units (R.U.) as a function of time. f Quantitative analysis of the binding properties of biotin-BH4-Bcl-2 and biotin-BH4-Bcl-xL peptides to GST-LBD measured by SPR. Values obtained from independent experiments were plotted as mean ± SEM (N = 4).
GST-LBD and GST-Fragment 3 of IP$_3$R1. In comparison to wild-type Bcl-xL, the ability of Bcl-xL$_{K87D}$ to bind the LBD and the Fragment 3 appears significantly reduced (Fig. 3f, g).

Fourth, we used MST to quantitatively assess the interaction of purified GST-LBD and GST-Fragment 3 with purified 6xHis-Bcl-xL$_{K87D}$, similarly to the experiment performed using wild-type 6xHis-Bcl-xL (Fig. 2d). We showed that although 6xHis-Bcl-xL$_{K87D}$ could interact with both IP$_3$R domains, it was with lower affinity than wild-type 6xHis-Bcl-xL (Fig. 3h). Of note, 6xHis-Bcl-xL$_{K87D}$ did not interact with GST (Fig. S4). Indeed, 6xHis-Bcl-xL$_{K87D}$ displayed...
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representations of Bcl-2 and Bcl-xL three-dimensional structures. The lysine residues of interest (K17 in Bcl-2 and K87 in Bcl-xL) are indicated. Image taken from our previously published work [28]; this work is licensed under a Creative Commons Attribution 4.0 International License. B Alignment of the conserved amino acid motifs for Bcl-xL-BH3 domain in vertebrates. The conserved lysine (K87 in human) is highlighted (red rectangle). The number of species used for each motif construction is shown in parentheses. *Z* means glutamic acid or glutamine. Top numbers represent amino acid numbers in human Bcl-xL sequence. c Representative co-immunoprecipitation (coIP) experiments using anti-FLAG performed on lysates from HeLa cells transiently overexpressing 3xFLAG-Bcl-xL or 3xFLAG-Bcl-xLK87D. The samples were analyzed via western blot using antibodies against IP3R1, FLAG and Bax. Total HeLa lysates were used as input (10 µg). PD: pull down; IB: immunoblot. The immunoreactive bands from independent coIP experiments, using each time independently transfected cells and freshly prepared lysates, were quantified and normalized to the binding of IP3R1 (d) and Bax (e) to 3xFLAG-Bcl-xL. Data represent mean ± SD (N = 5). Statistically significant differences were determined using a one-way ANOVA (*P < 0.05). f Representative GST-pull down experiment comparing the binding of 3xFLAG-Bcl-xL vs. 3xFLAG-Bcl-xLK87D from COS-7 cell lysate to purified GST-fused IP3R1 fragments and parental GST control. The samples were analyzed via western blot using anti-FLAG. Total COS-7 lysates were used as input (0.1 µg). The corresponding western blot for the GST-IP3R1 fragments is shown in Fig. 5b. g The immunoreactive bands from independent GST-pull down experiments, using each time independently transfected cells and freshly prepared lysates, were quantified and normalized to the binding of 3xFLAG-Bcl-xL and 3xFLAG-Bcl-xLK87D to parental GST control, which was set as 1 for each experiment. The data are plotted as mean ± SD (N = 5). Statistically significant differences were determined using paired t test (*P < 0.05). h Binding curves showing the interaction of purified 6xHis-Bcl-xL and 6xHis-Bcl-xLK87D with titrated GST-fused IP3R domains generated by MST. Concentration of the 6xHis-Bcl-xL and 6xHis-Bcl-xLK87D targets was kept constant at 50 nM, whereas the GST-LBD and GST-Fragment 3 were titrated down from 15 µM to 5 nM. The unit of the left axis (ΔFnorm) is a ratio of normalized fluorescence. The binding curves of wild-type 6xHis-Bcl-xL with GST-fused proteins are represented from Fig. 2d and are shown as reference. The binding curve of 6xHis-Bcl-xLK87D with parental GST is shown in Fig. 5a. Data points represent mean ± SD from triplicate measurements. i Left: Representative example of a MAPPIT experiment. The binding is shown as fold induction, calculated by dividing the average luciferase activity of erythropoietin-stimulated cells by the average obtained in non-stimulated cells. Binding of Bcl-xL, the Bcl-xLK87D mutant or irrelevant prey control (SV40 large T antigen) to the IP3R Fragment 3 and as negative control the bait vector without Fragment 3 are shown. Fold induction values at least four times higher than the irrelevant prey control are considered as bona fide protein-protein interactions. Values are represented as the mean of triplicates ± SEM within one representative experiment. The experiment was independently performed four times (N = 4). Statistically significant differences were determined using a one-way ANOVA (*P < 0.05). Right: Odyssey western blot analyses for the FLAG tag of the prey vector containing Bcl-xL or the Bcl-xLK87D mutant fusion proteins (green) or for β-actin (red).

higher dissociation constants than wild-type 6xHis-Bcl-xL for the interaction with the IP3R fragments. GST-LBD: Kd ~1166 nM (with 6xHis-Bcl-xL-K87D) versus Kd ~701 nM (with 6xHis-Bcl-xL). GST-Fragment 3: Kd ~990 nM (with 6xHis-Bcl-xL-K87D) versus Kd ~495 nM (with 6xHis-Bcl-xL). Thus, the GST-pull down assays and MST analysis indicate that, compared to wild-type Bcl-xL, Bcl-xLK87D is impaired in binding to LBD and Fragment 3.

Finally, we applied an in cellulo mammalian protein–protein interaction trap (MAPPIT) assay, which is based on the functional complementation of cytokine receptor signaling [30]. The MAPPIT data confirmed that Bcl-xL is able to interact with Fragment 3 and that the interaction was impaired by the introduction of the K87D mutation (Fig. 3). No binding was detected with the negative control, indicating that the interaction is specific. In this assay, Bcl-xL binding to LBD could not be observed, potentially due to interference of the fusion protein to establish a functional recombination of the cytokine receptor.

Taken together, our data demonstrate that the K87 residue is crucial for the interaction of Bcl-xL with the IP3R, where it is involved in its binding to both the LBD and the Fragment 3.

K87 residue is critical for Bcl-xL-mediated IP3R inhibition in living cells

Next, we examined the role of the K87 residue in Bcl-xL-mediated IP3R inhibition. We used Fura-2-loaded COS-7 (Fig. 4a–c) and HeLa (Fig. 4d–g) cells with overexpressed mCherry and either Bcl-xL or Bcl-xLK87D. We used mCherry to identify transfected cells. We first studied the effect of Bcl-xL and Bcl-xLK87D overexpression in COS cells on IP3R-mediated Ca2+ signals elicited by 500 nM ATP, a relatively high concentration provoking a Ca2+ response in about 75% of the cells. Extracellular Ca2+ was chelated with EGTA, so the reported Ca2+ signals only originate from internal stores. Under these conditions, ATP-induced Ca2+ signals appeared as a single transient (Fig. 4a). While about 75% of the cells expressing the empty vector displayed a response to ATP, only 40% of the cells expressing Bcl-xL responded (Fig. 4b). Cells expressing Bcl-xLK87D displayed similar responsiveness to ATP as empty vector-expressing cells with about 75% responding cells. Quantification of the amplitude of the ATP-induced Ca2+ transient in the responding cells yielded similar trends (Fig. 4c). Overexpression of Bcl-xL provoked a decrease in the peak [Ca2+]i provoked by ATP, while overexpression of Bcl-xLK87D failed to do this.

Then, we aimed to study the effect of Bcl-xL in HeLa cells, well-known to display long-lasting Ca2+ oscillations in response to extracellular agonists [31, 32]. Here, we exposed HeLa cells to a very low [ATP] (70 nM), thereby mimicking basal, pro-survival Ca2+ oscillations and enhancing the likelihood of observing different Ca2+ signaling profiles. Single peak responses, long-lasting responses and baseline Ca2+ oscillations (Fig. 4d). Consistent with an inhibitory effect of Bcl-xL on IP3Rs, we found that long-lasting responses were clearly impaired upon overexpression of Bcl-xL (Fig. 4d, e). Interestingly, this effect was not observed upon overexpression of Bcl-xLK87D. Furthermore, the area under the curve (Fig. 4f) and the peak amplitude (Fig. 4g) were reduced upon overexpression of Bcl-xL, but not Bcl-xLK87D. This demonstrates that Bcl-xL’s inhibitory action on IP3Rs is critically dependent on the K87 residue.

Finally, we also determined that overexpressed Bcl-xL or its mutant did not alter the ER Ca2+ store content, by monitoring ER Ca2+ release in HeLa cells following sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) inhibition by 1 µM thapsigargin (Fig. S5). These data are consistent with the differences observed upon ATP stimulation not being as a result of altered ER Ca2+ levels, but instead are due to the specific effect of Bcl-xL on IP3R-mediated Ca2+ release.

Purified Bcl-xL can directly suppress IP3R single-channel opening

As all our functional data were obtained in intact cells, we also wished to provide more direct evidence for IP3R inhibition by Bcl-xL through electrophysiology. This is important because in intact cell systems Bcl-xL may have other targets besides IP3Rs that impact cytosolic Ca2+ signals. In addition, these experiments can be performed in tightly controlled conditions, including different IP3 and Bcl-xL concentrations. Therefore, we aimed to study the
impact of purified Bcl-xL proteins on IP3Rs. We generated 6xHis-tagged versions of full-length Bcl-xL, Bcl-xLΔTMD and full-length Bcl-xLK87D that enabled their purification from E. coli using NiNTA columns (Fig. S6a). Next, we tested the effect of the different recombinantly expressed and purified 6xHis-Bcl-xL variants on IP3R1 single-channel activity using the on-nucleus patch-clamp technique (Fig. 5). Channel opening in isolated nuclei obtained from DT40-3KO cells ectopically expressing IP3R1 was triggered by 1 μM IP3 (Fig. 5a). We used purified Bcl-2ΔTMD as a benchmark (Fig. 5b), which we previously validated to inhibit IP3R1 single-channel openings [24]. We subsequently first tested whether Bcl-xLΔTMD could inhibit the opening of IP3R1 channels induced by 1 μM IP3, but this protein failed to modulate (inhibit/sensitize) IP3R1 channels (Fig. 5c). Next, we assessed the effect of 1 μM full-length Bcl-xL (Fig. 5d), a concentration previously proposed to have a stimulatory effect on IP3R [26]. Consistent with the data obtained in intact cells and similarly to 1 μM Bcl-2ΔTMD (Fig. 5b), application of 1 μM full-length 6xHis-Bcl-xL resulted in a significantly decreased open probability (P0) of IP3R1 channels in the presence of 1 μM IP3 (Fig. 5d). Clearly, these results contradict previous data that reported that Bcl-xL sensitizes IP3Rs [25, 26]. In these reports, the effect of Bcl-xL on IP3Rs was shown to exhibit a bell-shaped dependence with 1 μM of Bcl-xL optimally sensitizing IP3Rs [26]. Hence, to ensure that we did not apply Bcl-xL at too high concentrations, we also examined the effects of 300 nM (Fig. 5e) and 100 nM (Fig. 5f) full-length Bcl-xL. These lower Bcl-xL concentrations also inhibited IP3R1 single-channel opening, though with lower potency compared to 1 μM full-length Bcl-xL. Next, we examined the effect of 1 μM full-length Bcl-xLK87D protein on IP3R1 single-channel openings activated by 1 μM IP3 (Fig. 5g). Consistent with our in vitro binding assays and our Ca2+ imaging studies in intact cells, Bcl-xLK87D failed to inhibit IP3R1 single-channel activity. Quantification of all conditions is shown in Fig. 4.
Fig. 5  Purified Bcl-xL, but not Bcl-xL<sup>K87D</sup>, suppresses IP<sub>3</sub>R1 single-channel opening. Representative IP<sub>3</sub>R1 single-channel recordings from DT40-3KO cells ectopically expressing IP<sub>3</sub>R1. The channel opening was evoked by 1 µM IP<sub>3</sub> (a–h), 100 nM IP<sub>3</sub> (i to m) or 10 µM IP<sub>3</sub> (n–p) at 200 nM Ca<sup>2+</sup> and 5 mM ATP in the presence of PBS (a, i, n) or in the presence of 1 µM 6xHis-Bcl-2<sup>TMD</sup> (b, j), 1 µM 6xHis-Bcl-xL<sup>TMD</sup> (c), 1 µM 6xHis-Bcl-xL (d, k, o), 0.3 µM 6xHis-Bcl-xL (e), 0.1 µM 6xHis-Bcl-xL (f), or 1 µM 6xHis-Bcl-xL<sup>K87D</sup> (g, l) purified proteins. Each vertical bar represents one single-channel current recording; C: closed; O: opened. Histogram depicting the P<sub>o</sub> ± SEM (N = 4 or 5) for the IP<sub>3</sub>R1 current recordings (h, m, p). Statistically significant differences were determined using a one-way ANOVA (*P < 0.05).
Another potential explanation could be that the conditions in which we have measured IP$_3$R1 opening favor the detection of inhibitory effects and we may have missed potential sensitizing effects. We therefore measured the impact of purified Bcl-xL proteins on IP$_3$R1 single-channel openings induced by threshold concentrations of IP$_3$ (Fig. 5i–m). In the presence of 100 nM IP$_3$, the $P_0$ was reduced to ~0.005 (Fig. 5i), compared to a $P_0$ of ~0.25 at 1 µM IP$_3$ (Fig. 5a). Such conditions, which initiate threshold IP$_3$R1 opening, should favor the detection of any potential sensitization of the channel. Nevertheless, similarly to Bcl-2, IP$_3$ might compete with Bcl-xL for the LBD of IP$_3$Rs, thereby alleviating IP$_3$R inhibition by Bcl-2 [24]. Here, we have established that 6xHis-Bcl-xL is also able to interact with the LBD of IP$_3$Rs, prompting us to test the effect of purified 6xHis-Bcl-xL on IP$_3$R1 single-channel activity triggered by high concentrations of IP$_3$. With 10 µM IP$_3$, the $P_0$ reached more than 0.65 (Fig. 5n), compared to a $P_0$ of about 0.25 at 1 µM IP$_3$ (Fig. 5a). In those conditions, 6xHis-Bcl-xL did not alter channel activity, reflecting a loss of capacity to inhibit the channel at high [IP$_3$] (Fig. 5a, p). These results suggest that, similarly to our observations made for Bcl-2, IP$_3$ might compete with Bcl-xL for the LBD of IP$_3$Rs, thereby rendering Bcl-xL less effective in inhibiting IP$_3$Rs at high IP$_3$ concentrations.

Given that our findings were diametrically opposite to the previously reported findings, we sought to validate that the purified full-length 6xHis-Bcl-xL proteins used were properly folded and displayed bona fide anti-apoptotic functions. We first determined the CD spectrum of both Bcl-xL, which indicated that wild-type Bcl-xL, Bcl-xL$^{K87D}$ and Bcl-xL$^{ΔTMD}$ had a proper α-helical folding (Fig. 5b). Moreover, we performed a thermal ramping experiment. Unfolding of wild-type Bcl-xL was characterized by two apparent melting temperatures Tm1: 67 °C and Tm2: 55.47 °C, which were shifted to the left for Bcl-xL$^{K87D}$ (Tm1: 46.11 °C and Tm2: 53.2 °C), indicating some destabilizing effect of the mutation. These observations very much resemble the effect of K17D mutation in purified Bcl-2 [27]. We also measured Bcl-xL$^{ΔTMD}$, which was characterized by one melting temperature Tm1: 76 °C, indicating that Bcl-xL$^{ΔTMD}$ is much more stable than wild-type Bcl-xL (Fig. 5c). Next, we employed an in vitro Bax-liposome permeabilization assay, where purified Bax is incubated with liposomes encapsulating both a quencher (DPX) and a fluorophore (ANTS) (Fig. 6). Bax-pore formation can be triggered by cBid (Fig. 6a) or Bim (Fig. 6b) proteins, two “activator” BH3-only proteins. Full-length 6xHis-Bcl-xL potently inhibited cBid and Bim-triggered Bax-pore formation (IC$_{50}$ of about 20 nM). Of note, 6xHis-Bcl-xL$^{K87D}$ too inhibited Bax-pore formation, but was less efficacious (IC$_{50}$ of about 80 nM) than 6xHis-Bcl-xL (Fig. 6c, d). This might relate to the reduced Bax binding observed in the coIPs using cell lysates (Fig. 3c). Consistent with previous reports [33], Bcl-xL$^{ΔTMD}$ failed to inhibit Bax-pore formation.

Overall, our electrophysiological studies provide strong evidence that recombinant Bcl-xL with validated anti-apoptotic properties directly inhibits IP$_3$R1 single-channel opening with a critical role for K87 in Bcl-xL. Furthermore, the data suggest that Bcl-xL’s TMD is not only important for inhibiting Bax [33, 34] but also for inhibiting IP$_3$R opening. Yet, the significance and the role of the TMD of Bcl-xL in a cellular context for IP$_3$R modulation remains to be elucidated.

**Bcl-xL$^{K87D}$ is impaired in protecting cells against staurosporine-induced apoptosis**

Next, we wished to validate the importance of the IP$_3$R/Bcl-xL interaction for the protective effects of Bcl-xL against Ca$^{2+}$-dependent pro-apoptotic stimuli (Fig. 7, Fig. S7). We therefore used STS, which has been previously validated to provoke Ca$^{2+}$-permeabilization experiments using 500 nM of purified Bax and 20 nM cBid or Bim, in absence of Bcl-xL (Fig. 6a, b). Representative traces of liposomes permeabilized by Bax or Bim are shown in Fig. 6a and b. The percentage of ANTS/DPX release was measured in the presence of single purified proteins (20 nM Bim, 20 nM cBid, 100 nM Bax, or 500 nM Bcl-xL variants) or with liposomes only. Data represents mean ± SEM (N = 3).

![Fig. 6](Image104x143 to 500x349)

**Fig. 6** Purified Bcl-xL and Bcl-xL$^{K87D}$ proteins inhibit Bax-mediated permeabilization of liposomes. ANTS/DPX-encapsulated liposomes were incubated with 100 nM unlabeled Bax and 20 nM unlabeled cBid (a and c) or Bim (b and d), as well as a range of seven concentrations (from 500 to 8 nM) of unlabeled 6xHis-Bcl-xL, 6xHis-Bcl-xL$^{K87D}$ or 6xHis-Bcl-xL$^{ΔTMD}$ purified proteins. Upon Bax oligomerization and pore formation, fluorescence increases due to loss of fluorescent molecule-quencher proximity. a and b Representative traces of liposomes permeabilization experiments using 500 nM of purified 6xHis-Bcl-xL proteins. Liposomes permeabilization is represented as the percentage of ANTS/DPX release. c and d Summary of liposomes permeabilization experiments performed with the concentration range of 6xHis-Bcl-xL proteins mentioned before. ANTS/DPX release was normalized to the maximal permeabilization (Bax + cBid or Bim, in absence of Bcl-xL proteins). Each point represents mean ± SEM (N = 3). e ANTS/DPX release was measured in the presence of single purified proteins (20 nM Bim, 20 nM cBid, 100 nM Bax, or 500 nM Bcl-xL variants) or with liposomes only. Data represents mean ± SEM (N = 3).
driven apoptosis [35–37]. Here, we assessed whether STS provoked apoptosis through IP3-R-mediated Ca2+ elevations. First, we measured long-term Ca2+ dynamics in HeLa cells exposed to 0.5 µM STS for 1 hour (Fig. 7a). Using live single-cell Ca2+ imaging in Fura-2-AM-loaded cells, we observed that STS triggered long-lasting Ca2+ elevations in wild-type HeLa cells. Contrary to IP3-R activation with physiological agonists (Fig. 1, Fig. 4), this Ca2+ release is rather slow on onset and prolonged over a long period of time. We then used a HeLa cell model in which all three IP3Rs have been knocked out (HeLa-3KO). In these cells, the STS-induced Ca2+ release events were virtually absent (Fig. 7a, b). Having validated that STS treatment in HeLa cells provoked long-lasting IP3-R-mediated Ca2+ elevations, we determined whether IP3-Rs contributed to STS-induced cell death in HeLa cells (Fig. 7c). We therefore monitored apoptotic cell death in HeLa cells exposed to 0.5 µM STS for six hours by determining the ratio of cleaved poly(ADP-ribose) polymerase (PARP) in relation to total PARP [38]. Strikingly, in wild-type HeLa, about 90% of the total PARP was converted to the cleaved form, while only 30% of the total PARP appeared in the cleaved form in HeLa-3KO cells, 

Wild-type Bcl-xL, but not Bcl-xL K87D, protects HeLa cells against IP3R/Ca2+ -driven cell death using staurosporine. a–b Ca2+ measurements in Fura-2-loaded wild-type HeLa (black) and HeLa-3KO cells (blue). Cells were exposed to 0.5 µM staurosporine (STS), after addition of 3 mM EGTA to chelate extracellular Ca2+ (not shown). Representative traces of Ca2+ release are shown (a), along with areas under the curve (b) calculated for one hour following STS addition. For each experiment (N = 2), two wells were monitored per condition and about 20–30 cells were analyzed by well. Each trace and each point represent one cell. Statistically significant differences were determined using a t test (paired, two-tailed, *P < 0.05). 

c–d Wild-type HeLa and HeLa-3KO cells were treated with 0.5 µM STS or vehicle (DMSO) for 6 h. The samples were analyzed via western blot (IB: immunoblot). Representative western blots assessing uncleaved (top band) and cleaved PARP (lower band) as well as vinculin (c). The immunoreactive bands from independent experiments, using each time freshly prepared cell lysates, were quantified (d) and PARP cleavage was calculated as the ratio of cleaved PARP over total PARP. The data are plotted as mean ± SD (N = 6). Statistically significant differences between the "+" STS conditions were determined using a one-way ANOVA (*P < 0.05).

STS-induced Ca2+ release events were virtually absent (Fig. 7a, b). Having validated that STS treatment in HeLa cells provoked long-lasting IP3-R-mediated Ca2+ elevations, we determined whether IP3-Rs contributed to STS-induced cell death in HeLa cells (Fig. 7c). We therefore monitored apoptotic cell death in HeLa cells exposed to 0.5 µM STS for six hours by determining the ratio of cleaved poly(ADP-ribose) polymerase (PARP) in relation to total PARP [38]. Strikingly, in wild-type HeLa, about 90% of the total PARP was converted to the cleaved form, while only 30% of the total PARP appeared in the cleaved form in HeLa-3KO cells,
indicating that IP$_3$Rs are crucial for STS-induced cell death in HeLa cells (Fig. 7c, d). In a more general way, this is the first time that, to our knowledge, that IP$_3$Rs were directly implicated in STS-evoked pro-apoptotic Ca$^{2+}$ flux and directly linked to cell death. Next, using live, single-cell Ca$^{2+}$ imaging, we studied the impact of overexpressing Bcl-xL-P2A-mCherry and Bcl-xL$^{K87D}$ P2A-mCherry on STS-induced Ca$^{2+}$ elevations in Fura-2-AM-loaded wild-type HeLa cells. Ca$^{2+}$ signals were measured in mCherry-positive cells. Strikingly, Bcl-xL overexpression strongly suppressed prolonged Ca$^{2+}$ elevations induced by 0.5 µM STS compared to empty vector-expressing cells, while Bcl-xL$^{K87D}$ overexpression was much less effective (Fig. 7e, f, Fig. 5a, b). We then examined whether IP$_3$R modulation by Bcl-xL contributed to the anti-apoptotic action of Bcl-xL (Fig. 7g). We first confirmed that the transfection of the cells with the 3xFLAG plasmids did not provoke cell death by itself (Fig. 7g, “STS” conditions). Bcl-xL overexpression strongly suppressed PARP cleavage in wild-type HeLa cells exposed to 0.5 µM STS for 6 h compared to empty vector-expressing cells (Fig. 7g, “+ STS” conditions). In contrast, Bcl-xL$^{K87D}$ overexpression was much less effective than wild-type Bcl-xL in suppressing PARP cleavage in wild-type HeLa cells (Fig. 7g, h). This suggests that Bcl-xL protects against STS through inhibition of IP$_3$Rs, since Bcl-xL$^{K87D}$ is much less effective in doing so.

We then focused on IP$_3$R-independent cell death mechanisms. We have shown that Bcl-xL$^{K87D}$ binding to Bax appeared to be somewhat impaired compared to wild-type (Fig. 3c). Furthermore, the ability of Bcl-xL$^{K87D}$ to neutralize Bax pore formation also appeared attenuated (Fig. 6). Since STS partially acts independently of IP$_3$Rs and since PARP cleavage also occurs in HeLa-3KO cells, though to a lesser extent (Fig. 7d), we wanted to discriminate Bcl-xL anti-apoptotic effect between IP$_3$R inhibition versus IP$_3$R-independent processes, such as Bax inhibition. Hence, we examined the effect of Bcl-xL and Bcl-xL$^{K87D}$ overexpression on STS-induced cell death in HeLa-3KO cells (Fig. 7i, j). Consistent with the ability of Bcl-xL to bind and neutralize Bax, we found that Bcl-xL could suppress STS-induced PARP cleavage in HeLa-3KO cells. Of particular interest and in contrast to the results obtained in wild-type HeLa cells, Bcl-xL$^{K87D}$ was equally effective as wild-type Bcl-xL in dampening STS-induced PARP cleavage in HeLa cells lacking IP$_3$Rs. This implies that although Bax binding/inhibition is somewhat affected by the K87D mutation in Bcl-xL, there is sufficient residual Bax-binding and inhibition capacity of Bcl-xL$^{K87D}$ to prevent cell death in cells.

We wished to further validate that the K87D mutation does not affect the protection afforded by Bcl-xL towards IP$_3$R-independent cell death triggers. Hence, we chose the BH3 mimetic venetoclax/ABT-199, a selective Bcl-2 inhibitor [16] previously established to neither interfere with the ability of Bcl-2 to inhibit IP$_3$Rs nor to alter Ca$^{2+}$ signaling [39, 40]. Venetoclax (25 µM; 24 h) triggered ~80% PARP cleavage in HeLa cells (Fig. 7k, l). The level of PARP cleavage was similar between wild-type HeLa and HeLa-3KO, thereby validating that venetoclax indeed acted in an IP$_3$R-independent manner. Bcl-xL and Bcl-xL$^{K87D}$ were equally effective in counteracting venetoclax-induced PARP cleavage by about 40–50% (Fig. 7k, l). Moreover, the anti-apoptotic effect of Bcl-xL and Bcl-xL$^{K87D}$ was also comparable between wild-type HeLa and HeLa-3KO. These data strongly indicate that K87D mutation impairs Bcl-xL’s protective effect against IP$_3$R-dependent cell death but does not significantly affects its canonical anti-apoptotic function, thereby antagonizing Bax/Bak.

**Bcl-xL protects breast cancer cells from IP$_3$R-mediated cell death**

Finally, by knocking down Bcl-xL in a Bcl-xL-dependent cell model, we examined whether also endogenous Bcl-xL could inhibit IP$_3$Rs. We used a breast cancer model, the mammary gland adenocarcinoma cell line MDA-MB-231, in which Bcl-xL is important for survival [41] and migration [42]. We transfected MDA-MB-231 cells with a siRNA targeting Bcl-xL, thereby lowering its protein levels by about 50% (Fig. 8a, b). Interestingly, Bcl-xL knock-down in MDA-MB-231 cells did not induce apoptosis by itself (Fig. 8a, c). This was important to exclude that any potential changes in Ca$^{2+}$ signaling in cells with decreased Bcl-xL levels were a consequence of ongoing cell death rather than due to a decrease in Bcl-xL protein levels. By using thrombomodulin, we next validated in MDA-MB-231 cells that the ER Ca$^{2+}$-store content is not altered following Bcl-xL depletion (Fig. 8d, e, f). Therefore, changes in agonist-induced Ca$^{2+}$ signaling in Bcl-xL-depleted cells are not an indirect consequence of changes in ER Ca$^{2+}$ loading. We then measured IP$_3$R-mediated Ca$^{2+}$ release elicited by ATP (0.5 µM) in individual MDA-MB-231 cells pre-treated with extracellular Ca$^{2+}$ chelator EGTA, thereby ensuring that Ca$^{2+}$ signals only arise from internal stores. Compared to the cells transfected with a non-target siRNA, the cells treated with a siRNA targeting Bcl-xL displayed a strikingly higher ATP-induced Ca$^{2+}$ response (Fig. 8g, h). We calculated a significant increase in the number of responding cells (Fig. 8i), the area under the curve (Fig. 8j) and in the maximal peak amplitude (Fig. 8k) in MDA-MB-231 cells in which Bcl-xL-protein levels were lowered. To be certain that this effect was not due to a potential downregulation of the Bcl-xL-related Bcl-2 protein, which is a prominent inhibitor of IP$_3$Rs, we analyzed the Bcl-2 protein levels via western blotting (Fig. S8). However, Bcl-2 protein levels were not decreased. Instead, Bcl-2 protein levels increased, potentially as a compensatory mechanism that could help sustain the survival of the cells in which Bcl-xL was downregulated. Nevertheless, the overall Bcl-2-protein levels remained extremely low in these MDA-MB-231 cells, when benchmarked against the Bcl-2-protein levels present in OCSV-LY1 cells, a Bcl-2 dependent diffuse large B-cell lymphoma cell line. In any case, these data indicate that endogenous Bcl-xL suppresses IP$_3$R activity in breast cancer cells, independently of Bcl-2 levels. To determine whether Bcl-xL could also counteract IP$_3$R-mediated apoptotic Ca$^{2+}$ release in those cells, we exposed the MDA-MB-231 cells to STS (0.5 µM) (Fig. 8i). In MDA-MB-231 cells transfected with a non-target siRNA, STS only provoked limited PARP cleavage, indicating that these cells are rather resistant to STS. However, cells treated with the siRNA against Bcl-xL displayed a prominent increase in STS-induced PARP cleavage resulting in about 50% PARP cleavage. This indicates that lowering endogenous Bcl-xL-protein levels rendered MDA-MB-231 cells very sensitive to STS-induced cell death (Fig. 8i). Altogether, these results reveal that endogenous Bcl-xL suppresses IP$_3$R-mediated Ca$^{2+}$ release and confers cell death protection against Ca$^{2+}$-dependent cell death stimuli.

**DISCUSSION**

The main finding of this study is that the anti-apoptotic Bcl-xL protein functions as an inhibitor of IP$_3$R channels both in intact living cells and at the single-channel level. These data challenge the presumed role of Bcl-xL as an IP$_3$R-sensitizing protein [25, 26, 43]. This supposition is strongly supported by several independent lines of evidence. Molecular studies reveal that Bcl-xL targets the same regions in IP$_3$Rs as Bcl-2 (e.g., LBD and Fragment 3), which are responsible for inhibition of IP$_3$Rs. Further, we demonstrate that Bcl-xL, in a similar fashion to Bcl-2, possesses a lysine residue that is critical for IP$_3$R binding and inhibition. The critical lysine identified in Bcl-xL (K87) spatially resembles and substitutes for the previously identified critical lysine in Bcl-2 (K17) [27]. Mutation of K87 abrogates the ability of Bcl-xL to bind and inhibit IP$_3$Rs. The findings are further underpinned by single-channel recordings of IP$_3$R1 channels, whose open probability is reduced upon exposure to purified Bcl-xL, but not Bcl-xL$^{K87D}$. Moreover, by inhibiting IP$_3$Rs, K87 in Bcl-xL is important for Bcl-xL’s ability to protect cells against STS, a stimulus that triggers...
apoptosis in an IP$_3$R/Ca$^{2+}$-dependent manner. Finally, we demonstrate that also in MDA-MB-231, a Bcl-xL-dependent breast cancer cell model, endogenous Bcl-xL inhibits IP$_3$R function.

Over the past two decades, several, mainly anti-apoptotic, members of the Bcl-2-protein family, have emerged as critical modulators of Ca$^{2+}$ homeostasis and dynamics [21]. The two most studied proteins are Bcl-2 and Bcl-xL, which are consistently reported to be localized in the ER and to control the flux through ER-resident Ca$^{2+}$-release channels [20]. While reports suggest that Bcl-2 may also lower ER Ca$^{2+}$-store content and thus the likelihood
for pro-apoptotic Ca\textsuperscript{2+} release to mitochondria \[19, 44, 45\], other evidence has emerged that anti-apoptotic Bcl-2 is a direct inhibitor of Ca\textsuperscript{2+} flux through IP\textsubscript{3}Rs without markedly affecting the ER Ca\textsuperscript{2+}-store content \[46\]. This results from Bcl-2 binding to IP\textsubscript{3}Rs \[8\]. Subsequent work revealed the interaction domains in both IP\textsubscript{3}Rs and Bcl-2 that are responsible for the complex formation. In Bcl-2, we identified the BH4 domain \[23\] and the C-terminal TMD \[11\] as critical for IP\textsubscript{3}R inhibition in intact cells. For IP\textsubscript{3}Rs, we found that the LBD \[24\], a stretch of 20 a.a. in the central, modulatory domain \[8\] and the C-terminus of IP\textsubscript{3}Rs \[11\] participate in Bcl-2 binding. Important, the hydrophobic cleft of Bcl-2 is not necessary for IP\textsubscript{3}R modulation \[11, 39\] and, as a result, BH3 mimetic drugs do not impact IP\textsubscript{3}R modulation by Bcl-2. In various cancer cell models, disrupting the complex between IP\textsubscript{3}Rs, and Bcl-2 was even sufficient to provoke cell death through Ca\textsuperscript{2+} overload \[47–49\]. Our current model is that Bcl-2 acting via its BH4 domain inhibits IP\textsubscript{3}Rs by targeting the LBD and the central, modulatory domain. The occurrence of inhibition is aided by the “effective concentration” of Bcl-2 in the close proximity of IP\textsubscript{3}Rs as a result of the interaction between the C-terminal regions of both proteins.

Our new data demonstrate that, similarly to Bcl-2, Bcl-xL inhibits IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release by targeting precisely the same regions as the BH4 domain of Bcl-2, namely the ligand-binding domain and the central, modulatory domain. This challenges the currently accepted concept that Bcl-xL sensitizes IP\textsubscript{3}Rs \[25, 26, 43\]. We have previously proposed that the distinct modulation of IP\textsubscript{3}R by Bcl-2 and Bcl-xL could be due to differences in their BH4 domain \[27\]. Yet, \textit{in silico} superposition of Bcl-2 and Bcl-xL indicated that the K17 residue critical for Bcl-2\’s BH4 domain spatially resembled K87 of Bcl-xL \[28\]. Our functional results prompted us to revisit the modulation of IP\textsubscript{3}Rs by Bcl-xL, revealing that Bcl-xL, inhibited IP\textsubscript{3}Rs through an interaction mediated by K87, an evolutionary conserved residue located in the BH3 domain of Bcl-xL. Moreover, similarly to Bcl-2, inhibition of IP\textsubscript{3}Rs by Bcl-xL was dependent on the agonist/IP\textsubscript{3}C concentration, whereby high agonist/IP\textsubscript{3}C concentrations abrogated the inhibitory effect of Bcl-xL on IP\textsubscript{3}Rs. This is consistent with our molecular studies showing that Bcl-xL can target the LBD, the region where IP\textsubscript{3} binds. Thus, similarly to Bcl-2 \[24\], Bcl-xL binding to LBD might be antagonized by IP\textsubscript{3}.

This interaction also accounts for Bcl-xL\’s protective effect against IP\textsubscript{3}R-mediated, Ca\textsuperscript{2+}-driven apoptosis by using STS. Exposing wild-type versus IP\textsubscript{3}R-knockout HeLa cells, we demonstrated that STS triggered long-lasting Ca\textsuperscript{2+} rises that depended on IP\textsubscript{3}Rs and that STS-induced cell death was for a large part driven by IP\textsubscript{3}Rs, though not exclusively. Bcl-xL overexpression suppressed STS-induced Ca\textsuperscript{2+} rises and cell death. We also observed that mutating K87 into aspartic acid in Bcl-xL also mildly impacted the ability of Bcl-xL to bind Bax and the potency of Bcl-xL to prevent Bax-pore formation. Therefore, we wanted to exclude that the impaired protection against STS-induced cell death could be due to the slightly weakened Bax-binding properties of Bcl-xL\textsuperscript{K87D}. We therefore used the HeLa-3KO cells to exclude any IP\textsubscript{3}R-independent mechanism, revealing that wild-type Bcl-xL and Bcl-xL\textsuperscript{K87D} were equally potent in protecting HeLa cells that lacked IP\textsubscript{3}Rs against STS. In addition, we found that Bcl-xL and Bcl-xL\textsuperscript{K87D} were equally effective in protecting cells against IP\textsubscript{3}R-independent cell death stimuli, such as venetoclax \[11, 39, 50\]. Hence, this demonstrates that reduced anti-apoptotic properties of Bcl-xL\textsuperscript{K87D} are related to reduced inhibition of IP\textsubscript{3}Rs rather than non-IP\textsubscript{3}R-related targets such as Bax or BAK.

Our understanding of the role of Bcl-xL in Ca\textsuperscript{2+} signaling has been shaped by previous studies from the Foskett lab \[25, 26, 43\]. Therefore, the previous model is that anti-apoptotic Bcl-xL proteins enhance IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release by sensitizing the channels to IP\textsubscript{3}. In support of these ideas, Bcl-xL promoted IP\textsubscript{3}R-driven Ca\textsuperscript{2+} oscillations to drive mitochondrial bio-energetics and ATP production \[25, 43\]. In contrast to Bcl-2, Bcl-xL was proposed to bind IP\textsubscript{3}Rs via its hydrophobic cleft responsible for scaffolding pro-apoptotic Bcl-2-family members \[26\]. Thus, BH3-mimetic Bcl-xL inhibitors interfere with the ability of Bcl-xL to modulate IP\textsubscript{3}Rs. Moreover, the effect of Bcl-xL on IP\textsubscript{3}Rs was concentration-dependent with an optimal IP\textsubscript{3}R-sensitizing effect observed at 1 µM Bcl-xL protein. In contrast, our present data demonstrate that Bcl-xL inhibits, rather than sensitizes, IP\textsubscript{3}R channels. Furthermore, our live single-cell measurements show that Bcl-xL suppressed Ca\textsuperscript{2+} signals, even when induced by very low concentrations of agonist. By doing so, Bcl-xL seems to shift the profile of Ca\textsuperscript{2+} signals from long-lasting responses towards transient peaks and spontaneous oscillations (Fig. 4e). Thus, while Bcl-xL indeed increases the number of cells displaying Ca\textsuperscript{2+} oscillations, we argue that this is due to IP\textsubscript{3}R inhibition.

The reason for the discrepancy with the earlier studies is not clear, but we can exclude a number of obvious factors. First, we exclude that differences might be attributed to different cell models (here: HEK-293 and HeLa cells; \[25\]: DT40 cells). In the present study, we also used permeabilized DT40 cells in our electrophysiology experiments and the results we obtained were consistent with the experiments we performed in intact HEK-293 and HeLa cells. Furthermore, by sensitizing IP\textsubscript{3}Rs, Bcl-xL was also reported to lower ER Ca\textsuperscript{2+} levels in DT40 cells \[51\]. However, overexpressing Bcl-xL in wild-type DT40 cells did not lower the thapsigargin-induced Ca\textsuperscript{2+} release, indicating that in our hands Bcl-xL did not affect ER Ca\textsuperscript{2+}-stores in these cell models (Fig. S9). We thus assume that the discrepancy with earlier studies is not
related to different cell models. Second, we also controlled some other factors and validated that they can also be ruled out. i. It could be argued that in the present study, very high Bcl-xL levels were used, or that experimental conditions were not favorable to observe sensitization of Ca\(^{2+}\) release. However, in our single-channel recordings, we applied 1 μM Bcl-xL, a concentration previously reported to maximally cause IP\(_3\)R sensitization [26]. Nevertheless, we also used even lower Bcl-xL concentrations (100–300 mM), which also inhibited IP\(_3\)R1 single-channel openings. ii. In intact cell experiments and in IP\(_3\)R1 single-channel recordings, we also used low concentrations of extracellular agonist and IP\(_3\), which should prime the system to observe IP\(_3\)R sensitization. iii. We validated that both overexpressed Bcl-xL in living cells and purified Bcl-xL proteins used in this study are bona fide anti-apoptotic proteins and can exert anti-apoptotic functions. iv. We mainly focused on IP\(_3\)R1 in this study as it was the IP\(_3\)R isoform that was analyzed in depth in the original reports [25, 43]. We have not formally ruled out IP\(_3\)R isoform-dependence in the inhibitory effect of Bcl-xL. Nevertheless, previous work indicated that all three IP\(_3\)R isoforms could bind and were similarly sensitized by Bcl-xL in a similar fashion [25, 43]. Future work will determine whether Bcl-xL differentially impacts IP\(_3\)R1, IP\(_3\)R2 and IP\(_3\)R3 channels.

At the cellular level, Bcl-xL has been shown, beyond its canonical anti-apoptotic activity, to favor cell survival by enhancing mitochondrial morphological change. For instance, Bcl-xL can interact with and promote activity of the F-type ATPase [52]. Yet, in breast cancer cells, Bcl-xL improves the metabolic capacities by more efficiently coupling the mitochondrial proton motive force with ATP production [53]. Although the involvement of Ca\(^{2+}\) signaling in these processes is still unknown, sensitization of IP\(_3\)R by Bcl-xL has been shown to optimize mitochondrial bioenergetics, which may relate to Bcl-xL’s ability to promote Ca\(^{2+}\) flux to mitochondria [25, 54]. But if Bcl-xL does not sensitize IP\(_3\)Rs, then how does Bcl-xL promote mitochondrial bioenergetics? In our work, we demonstrate that Bcl-xL inhibits Ca\(^{2+}\) release from the ER to the cytosol in various cell systems, including in breast cancer cells, thereby protecting the cells from IP\(_3\)R-mediated apoptosis. Furthermore, our group has previously established that Bcl-xL inhibits the voltage-dependent anion channel 1 (VDAC1) [55]. Recently, Bcl-xL has been reported to dampen VDAC1-mediated mitochondrial Ca\(^{2+}\) uptake in breast cancer cells [56]. This mechanism has been proposed to alter mitochondrial ATP generation and increase ROS production, thereby promoting breast cancer cell migration [42]. Since Ca\(^{2+}\) transfers between the ER and the mitochondria are tightly connected, we speculate that Bcl-xL could inhibit both VDAC1 and IP\(_3\)Rs in breast cancer cells to promote cancer malignant features.

Finally, since the interaction profile of Bcl-2 and Bcl-xL for IP\(_3\)R binding is very similar, it is possible that peptides similar to those disrupting IP\(_3\)R/Bcl-2 complexes, such as the Bcl-2/IP\(_3\)R disruptor 2 (BIRD-2) [57], can affect IP\(_3\)R/Bcl-xL complexes. Disrupting such IP\(_3\)R/Bcl-xL complexes could therefore result in Ca\(^{2+}\)-driven cell death, as observed in several Bcl-2-dependent cancer in which Bcl-2 was displaced from IP\(_3\)Rs [22, 58] or antagonize breast cancer cell migration, a process controlled by Bcl-xL at the level of the IP\(_3\)R [42].

Overall, this work reassesses the model and mechanism of anti-apoptotic action of Ca\(^{2+}\)-signaling events modulated by Bcl-xL. In contrast to the previous model, we argue that Bcl-xL, in a similar manner to Bcl-2, inhibits IP\(_3\)Rs and thereby can protect cells against apoptosis. Bcl-xL not only photorecipes Bcl-2 at the functional level, but also at the molecular level. This in-depth understanding of the similarities and differences in the mechanism of interaction and action of distinct anti-apoptotic Bcl-2 family members may ultimately be exploited for the design of novel therapeutic modulating apoptosis.

**MATERIALS AND METHODS**

**Cell culture**

Wild-type human cervix carcinoma cells (HeLa cells), wild-type human embryonic kidney (HEK) 293 and HEK-293 cells deficient for all three endogenous IP\(_3\)R isoforms (3KO) stably expressing rat IP\(_3\)R1 (HEK-IP\(_3\)R1) were cultured as previously described [59]. HeLa-3KO were cultured as described before [60]. Wild-type chicken lymphoblasts DT40 were cultured as previously described [61]. COS-7 and DT40 cells lacking all three IP\(_3\)R isoforms (DT40-3KO) with ectopically expressing IP\(_3\)R1 were cultured as described before [24]. OCI-LY-1 diffuse large B-cell lymphoma cells were cultured as previously described [39]. MDA-MB-231 breast adenocarcinoma cells were obtained from Professor P. Vangheluwe (Laboratory of Cellular Transport Systems, KU Leuven) and were bought from ATCC. MDA-MB-231 cells were cultured at 37 °C in 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich/Merck, Overijse, Belgium) supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% non-essential amino acids ( Gibco/Thermo Fisher Scientific, Merelbeke, Belgium), 4 mM L-glutamine, (Gibco) 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). All cell lines were authenticated using short tandem repeats (STR) profiling (InSource [Arizona Genetics Core, Tucson, Arizona]). They were cultured in mycoplasma-free conditions and were monthly checked for myco-

**Plasmids, constructs, and protein purification**

The pCMV24-3xFLAG-Bcl-xL, the pCMV24-3xFLAG-Bcl-2, the pCMV24-P2A-mCherry and the pCMV24-3xFLAG-Bcl-2-P2A-mCherry plasmids were obtained as described before [27]. The pCMV24-3xFLAG-Bcl-xL [60, 61], the pCMV24-3xFLAG-Bcl-xL-P2A-mCherry and the pCMV24-3xFLAG-Bcl-xL-P2A-mCherry plasmids were obtained as previously described [28]. The pGEX-6p2 plasmid coding for GST-mIPI-R1/Fragment 3 and GST-mIPI-R1/Fragmen 5b were obtained as previously described [8]. The plasmid coding for GST-mIPI-R1-LBD construct was obtained as described before [26].

For 6xHis plasmids purification, cDNAs sequence-coding for Bcl-2 and Bcl-xL were cloned in pT45b plasmids. Full length or truncated sequences were inserted in the 6xHis-encoding reading frame. The Bcl-xL\(^{ATM}\) is deleted of amino acids R209 to K233 and the Bcl-2\(^{ATM}\) is deleted of amino acids L217 to K239. Following cloning of the pT45b-Bcl-xL, the K87D mutant was obtained by PCR site-directed mutagenesis as previously described [28]. All constructs were verified by sequencing (LGC Genomics, Berlin, Germany). Proteins were then purified from BL21 Escherichia coli as described [24].

For GST fusion proteins purification, BL21 Escherichia coli were transformed and amplified as described before [62], and proteins were purified as previously described [63].

Concentration of the purified proteins was determined using BCA Protein Assay Reagent (Thermo Fisher Scientific, Merelbeke, Belgium). The purity was examined by SDS-PAGE and Coomassie blue staining of the gels with the Imperial Protein Stain reagent (ThermoFisher Scientific). Quality and integrity of the proteins were confirmed by immunoblotting with anti-GST (Cell Signaling Technology, Leiden, Netherlands; #2622) and anti-Bcl-xL (Cell Signalling Technology; #2764) antibodies. Western blots were performed as previously described [59].

Bax, Bim and cBid proteins were purified from BL21 or DH5\(_a\) Escherichia coli as extensively described before [64].

**GST-pull down assays**

Two million COS-7 cells were plated in 75 cm\(^2\) plates. 24 h after seeding, cells were transfected with 10 μg of pCMV24-3xFLAG-Bcl-xL or pCMV24-3xFLAG-Bcl-xL\(^{K87D}\) plasmids. X-tremeGene HP DNA (Roche Basel, Switzerland) was used as a transfection reagent according to the manufacturer’s instructions. 48 h after transfection, COS cells were harvested and lysed as previously described [24]. In RIPA buffer consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1.5 mM MgCl\(_2\), 0.5 mM DTT, 1% Triton X-100 and protease inhibitor cocktail tablets (Roche). Cell lysates (250 μg) were pre-cleared through 1 h of incubation at 4 °C with 20 μL glutathione-Sepharose 4B beads (GE Healthcare, Diegem, Belgium). Pre-cleared lysates and GST fusion proteins were used to perform GST-pull down assays as previously described [12]. Briefly, equimolar amounts of parental GST or GST-fused fragments of IP\(_3\)R1 (250 pmol) were incubated at 4 °C with COS lysates in 500 μL RIPA buffer. After 3 h, the GST-proteins used as bait, were immobilized on glutathione-Sepharose 4B beads (20 μL) for 2 h at 4 °C. The beads were then washed five times with RIPA buffer. The GST-complexes were eluted in 40 μL 2x LDS (Invitrogen/Thermo
Gene knockdown

300,000 MDA-MB-231 cells were plated in six-well plates. 24 h after seeding, cells were transiently transfected with 5 μg of empty pCMV24 vector or pCMV24-3xFLAG-Bcl-xL or pCMV24-3xFLAG-Bcl-xLK87D constructs. X-tremeGene HP DNA (Roche) was used as a transfection reagent according to the manufacturer’s instructions. 48 h after transfection, HeLa cells were harvested and lysed as previously described [11]. Anti-DYKDDDDK affinity gel (BioLegend, Amsterdam, Netherlands) (30 μl) and HeLa lysates (500 μg) were used to perform immunoprecipitation as described before [11]. Samples were analyzed via western blotting using anti-Bax antibody (Cell Signaling Technology; #2772) or horseradish peroxidase-anti-FLAG antibody (Sigma-Aldrich; Bcl-xL or Bcl-xLK87D constructs). X-tremeGene HP DNA (Roche) was used as a transfection reagent according to the manufacturer’s instructions. At 48 h post-transfection, the cells were used for experiments.

Apoptosis induction

300,000 HeLa cells were plated in six-well plates. 24 h after seeding, cells were transiently transfected with 1 μg of empty pCMV24 vector, pCMV24-3xFLAG-Bcl-xL or pCMV24-3xFLAG-Bcl-xLK87D constructs. X-tremGene HP DNA (Roche) was used as a transfection reagent according to the manufacturer’s instructions. 48 h after post-transfection, the cells were used for experiments. Effective gene knockdown was confirmed via western blotting using anti-Bcl-xL (Cell Signaling Technology; #2764) and anti-β-actin (Sigma-Aldrich; AS5441) antibodies. An HRP-coupled anti-β-L antibody (Santa Cruz Biotechnology, Heidelberg, Germany; sc-7382 HRP) was used to assess changes in Bcl-2-protein levels after knockdown of Bcl-xL.

Electrophysiology

Isolated nuclei from DT40-3KO cells stably transfected with rat IP3R1 were prepared by homogenization as previously described [67]. Patch-clamp experiments were performed as described before [24]. Purified recombinant proteins 6xHis-Bcl-xL, 6xHis-Bcl-xL†MD, 6xHis-Bcl-xL‡MD and 6xHis-Bcl-2†MD were used during electrophysiology experiments.

Surface plasmon resonance

The following peptides (purity >80%) were obtained from LifeTein (South Plainfield, NJ, USA) and dissolved in dimethyl sulfoxide to prepare 10 mM stock solutions.

- Biotin-BH4-Bcl-2: biotin-RTGYDNRERIKMYHHXIIQLSRGQGYEW;
- Biotin-BH4-Bcl-2-scramble: biotin-WYEKQRSLHVDFLSYKLSQRGYSW;
- Biotin-BH4-Bcl-xl: biotin-MSQSNLRLWDFLSYKLSQRGYSW;
- Biotin-BH4-Bcl-xl-scramble: biotin-WYKQKSLGSLHVDFLSYKLSQRGYSW;
- Biotinylated peptides (200 ng), immobilized on streptavidin-sensor chips, and purified GST-LBD proteins, applied as analyte, were used to perform SPR assays as described before [27].

Mammalian protein–protein interaction trap

MAPIT experiments were performed as previously described [28]. Briefly, the transcription 3′ of miP.R1 was cloned in a pSEL-2 bait vector, downstream of a chimeric cytokine receptor (Fragment 3 bait), consisting of the extracellular domain of the erythropoietin receptor fused to the transmembrane and cytosolic part of the leptin receptor. Bcl-xL or Bcl-xLK87D was cloned in a the pM61-GW plasmid, downstream of a part of the glycoprotein 130 receptor (Bcl-xL or Bcl-xL‡MD prey). The interaction between Fragment 3 and Bcl-xL or Bcl-xL‡MD is detected by a luciferase reporter assay driven by a STAT-responsive promoter, since the functional complementation of the chimeric cytokine receptor results in ligand-dependent downstream STAT signaling. We also used the SV40 large antigen T as an irrelevant prey to monitor the signal representing the non-specific binding to Fragment 3. As an extra negative control, binding of the chimeric cytokine receptor without the Fragment 3 fragment (no bait) to the two Bcl-xL preys was also assessed.

Liposome permeabilization assay

Liposomes encapsulating the fluorophore 8-aminophthalene-1,3,6-trisulfonic acid (ANTS) and the colloidal quencher p-xylene-bis-pyridinium bromide (DPX) were prepared as previously described [64]. Purified Bcl-xL, CBid (20 nM), Biom (20 nM) or Bax (100 nM) proteins were then incubated with the liposomes (0.04 mg/ml) and ANTS/DPX release was measured as described before [34].

CD spectrum and thermal ramping

The experiments were performed as described in [68]. CD spectra were recorded using a J-1500 spectropolarimeter (Jasco, Easton, MD, USA)
equipped with a Peltier element for temperature control and a six-position cuvette holder. Proteins were dialed in 5 mM MOPS pH 7.5; 5 mM NaCl for 15 h, at 4 °C; 3x changes; constant stirring. Aggregated material was removed by centrifugation (20,000 g; 15 min; 4 °C) before protein concentration was determined on a Nanodrop instrument (280 nm; 2000 series; Thermo). The molecular extinction coefficient and molecular weight for each protein was estimated using the ExPasy server (http://web.expasy.org/protparam/). Wavelength scan measurements (190–260 nm) were performed with 15 µM protein, at 20 °C; in 5 mM MOPS pH 7.5; 5 mM NaCl, using 1 mm quartz cuvettes (Hellma, Müllheim, Germany); data pitch: 0.5 nm; bandwidth: 1 nm; scanning speed: 50 nm·min⁻¹; DIT: 0.5 s; accumulation: 3. Variable temperature measurements (15–90 °C) were performed with 15 µM protein; in 5 mM MOPS pH 7.5; 5 mM NaCl, using 1 mm quartz cuvettes (Hellma); interval 0.5 °C gradient 1 °C·min⁻¹; DIT: 0.5 s; bandwidth: 1 nm. Data were analyzed using the spectra analysis v.2 software (Jasco); Tmupp were derived by acquiring the first derivatives of the melting curves, using the calculus function of the Origin 7 software (GE Healthcare).

Microscale thermophoresis
Both purified 6×His-Bcl-xL protein and 6×His-Bcl-xL K87D were fluorescently labeled using the Monolith His-Tag Kit transient-TR-TAG and Generation (Nano Temper Technologies, Munich, Germany) and binding affinities were evaluated using microscale thermophoresis. Concentration of 6×His-Bcl-xL and 6×His-Bcl-xL K87D was kept constant at 50 nM, whereas the GST-LBD, GST-Fragment 3, GST-Fragment 5b and GST-control proteins were titrated down from 15 µM to 5 nM. Measurements were performed in steady-state conditions using premium capillaries and subsequently recorded on a Monolith NT automated instrument (Nano Temper Technologies) with a pico-red laser channel at 5% excitation power and bandwidth: 1 nm. Data were analyzed using the Prism software (GraphPad) was used to plot the data points, fit a nonlinear regression curve and calculate the dissociation constant (KD) for each condition.

Confocal microscopy
100,000 HeLa cells were plated on 18 mm diameter coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich) in 12-well plates and cultured as described above. Twenty-four hours later, the cells were co-transfected with 1 µg pCMV24-3×FLAG-Bcl-xL or pCMV24-3×FLAG-Bcl-xL K87D along with 1 µg of plasmid coding for an ER-targeted RFP or a mitochondria-targeted RFP (mito-RFP). The RR-RFP and mito-RFP were a gift from Cell Death & Differentiation (2022) 29:788 – 805

Statistical analysis
The Prism software (GraphPad) was used for statistical analysis. Data are expressed as mean ± SEM or SD. Two-tailed Student’s t tests were used to compare two conditions and repeated-measure ANOVA with Bonferroni post-tests were performed when comparing three or more conditions. For small sample sizes, non-parametric tests were performed. Statistically significant differences were considered at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***)

REFERENCES
1. Foskett JK, White C, Cheung KH, Mak DO. Inositol triphosphate receptor Ca2⁺ release channels. Physiol Rev. 2007;87:593–658.
2. Hamada K, Mikioshiba K, IP3 receptor plasticity underlying diverse functions. Annu Rev Physiol. 2020;82:151–76.
3. Berridge MJ. The inositol triphosphate/calcium signaling pathway in health and disease. Physiol Rev. 2016;96:1261–96.
4. Bootman MD, Bultynck G. Fundamentals of cellular calcium signaling: a primer. In: Bultynck G, Bootman MD, Berridge MJ, Stutzmann GE, editors. Calcium signaling, Second Edition. New York: Cold Spring Harbor Laboratory Press; 2019. 1–16.
5. Prole DL, Taylor CW. Inositol 1,4,5-trisphosphate receptors and their protein partners as signalling hubs. J Physiol. 2016;594:2849–66.
6. Parys JB, Vervliet T. New Insights in the IP3 receptor and its regulation. Adv Exp Med Biol. 2020;1131:243–70.
7. Yoshikawa F, Iwasaki H, Michikawa T, Furuchi T, Mikoshiba K. Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. Structural and functional coupling of cleaved ligand binding and channel domains. J Biol Chem. 1999;274:316–27.
8. Rong YP, Aranoralan AS, Bultynck G, Zhong F, Li X, McColl K, et al. Targeting Bcl-2/ IP3 receptor interaction to reverse Bcl-2’s inhibition of apoptotic calcium signals. Mol Cell. 2008;31:255–65.
9. Lee B, Vermassen E, Yoon SY, Vanderheyden V, Ito J, Alfandari D, et al. Phosphorylation of IP3R1 and the regulation of [Ca2⁺]i responses at fertilization: a role for the MAP kinase pathway. Development. 2006;133:4355–65.
10. Harr MW, Rong Y, Bootman MD, Rodenkl HW, Distelhorst CW. Glucocorticoid-mediated inhibition of Lck modulates the pattern of T cell receptor-induced calcium signals by down-regulating inositol 1,4,5-trisphosphate receptors. J Biol Chem. 2009;284:31860–71.
11. Ivanova H, Ritaine A, Wagner L, Luyten T, Shapovalov G, Welkenhuysen K, et al. The trans-membrane domain of Bcl-Zalpha, but not its hydrophobic cleft, is a critical determinant for efficient IP3 receptor inhibition. Oncotarget. 2016;7:55704–20.
12. Monaco G, Beckers M, Ivanova H, Missiaen LA, Parys JB, De Smedt H, et al. Profiling of the Bcl-2/Bcl-xL-interacting sites on type I IP3 receptor. Biochim Biophys Res Commun. 2012;428:31–5.
13. Aouchacheria A, Baghdiguian S, Lamb HM, Huska JD, Pineda FJ, Hardwick JM. Connecting mitochondrial dynamics and life-or-death events via Bcl-2 family proteins. Neurochem Int. 2017;108:141–61.
14. Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of Bcl-2 family proteins. Nat Rev Mol Cell Biol. 2019;20:175–93.
15. Adams JM, Cory S. The Bcl-2 arbiters of apoptosis and their growing role as cancer targets. Cell Death Differ. 2018;25:27–36.
16. Montero J, Letai A. Why do Bcl-2 inhibitors work and where should we use them in the clinic? Cell Death Differ. 2018;25:55–64.
17. Koe J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the death and disease. Oncogene. 2016;35:5079–92.
18. Kalkavan H, Green DR. MOMP, cell suicide as a BCL-2 family business. Cell Death Differ. 2018;25:46–55.
19. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, et al. BAX and BAK regulation of endoplasmic reticulum Ca2⁺: a control point for apoptosis. Science. 2003;300:134–5.
20. Ivanova H, Vervliet T, Monaco G, Terry LE, Rosa N, Baker MR, et al. Bcl-2 protein family as modulators of IP3 receptors and other organellar Ca2⁺ channels. Cold Spring Harb Perspect Biol. 2020;12:a035089. pii
21. Kale J, Osterlund EJ, Andrews DW. BCL-2 family as modulators of IP3 receptors and other organellar Ca2⁺ channels. Cell Death Differ. 2018;25:46–55.
25. White C, Li C, Yang J, Petenko NB, Madesh M, Thompson CB, et al. The endoplasmic reticulum gateway to apoptosis by Bcl-XL, modulation of the InsP3R. Nat Cell Biol. 2005;7:1021–8.

26. Yang J, Vais H, Gu W, Foskett JK. Biphasic regulation of InsP3 receptor gating by dual Ca2+ release channel BHC-like domains mediates Bcl-XL control of cell viability. Proc Natl Acad Sci USA. 2016;113:E1953–62.

27. Monaco G, Decroo E, Akh, Ponsaerts R, Vervliet T, Luypen T, et al. Selective regulation of IP3-receptor-mediated Ca2+ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-X. Cell Death Differ. 2012;19:295–309.

28. Vervliet T, Lemmens I, Vandermarliere E, Decroo E, Ivanova H, Monaco G, et al. Ryanodine receptors are targeted by anti-apoptotic Bcl-XL involving its BH4 domain and Lys87 from its BHD domain. Sci Rep. 2015;5:9641.

29. Alavian KN, Li H, Collis L, Bonanni L, Zeng L, Sacchetti S, et al. Bcl-XL regulates metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP synthase. J Biol Chem. 2012;288:19870–81.

30. Luyten T, Salviucci F, Dussmann H, Lindner AU, Lambrechts D, Prehn JHM. BCL-XL and BCL2 increase the metabolic fitness of breast cancer cells: a single-cell imaging study. Cell Death Differ. 2020;28:1512–21.

31. Williams A, Hayashi T, Wolozyn D, Yin B, Yu T, Cetbenjak M, et al. The non-apoptotic action of Bcl-XL: regulating Ca2+ signaling and bioenergetics at the ER-mitochondrion interface. J Bioenerg Biomembr. 2016;48:211–25.

32. Monaco G, Decroo E, Arbel N, van Vliet AR, La Rovere RM, De, Smedt H, et al. The BH4 domain of anti-apoptotic Bcl-XL, but not that of the related Bcl-2, limits the voltage-dependent anion channel 1 (VDAC1)-mediated transfer of pro-apoptotic Ca2+ signals to mitochondria. J Biol Chem. 2015;290:9150–61.

33. Huang H, Hu X, Eno CO, Zhao G, Li C, White C. An interaction between Bcl-XL and the voltage-dependent anion channel (VDAC) promotes mitochondrial Ca2+ uptake. J Biol Chem. 2015;290:18970–81.

34. Kale J, Chi X, Leber B, Andrews D. Examining the molecular mechanism of bcl-2 and bcl-xl inhibitors as anti-cancer therapeutics: the impact of and on calcium signaling. Biochim Biophys Acta. 2017;1864:947–56.

35. Ando H, Hirose M, Mikoshiba K. Aberrant IP3 receptor activities revealed by comprehensive analysis of pathological mutations causing spinocerebellar ataxia type 29. Proc Natl Acad Sci USA. 2018;115:12259–64.

36. Kerkhofs M, Bultynck G, Vervliet T, Monaco G. Recent advances in understanding the mechanisms contributing to BIRD-2-induced cell death in B-cell cancer cells. Cell Death Dis. 2019;10:42.

37. Luynen T, Welkenhuysen K, Roest G, Kania E, Wang L, Bittremieux M, et al. Resveratrol-induced autophagy is dependent on Ip3Rs and on cytosolic Ca2+. Biochim Biophys Acta Mol Cell Res. 2017;1864:947–56.

38. Bultynck G, Khvotko S, Henke N, Ivanova H, Schneider L, Rybalchenko V, et al. The C terminus of Bax inhibitor-1 forms a Ca2+-permeable channel pore. J Biol Chem. 2012;287:2545–57.

39. Bultynck G, Szulc K, Kaszi NN, Assessa Z, Callewaert G, Missiaen L, et al. Thimerosal stimulates Ca2+ flux through inositol 1,4,5-trisphosphate receptor type 1, but not type 3, via modulation of an isoform-specific Ca2+-dependent intramembrane interaction. Biochem J. 2004;381:851–6.

40. Sienaert I, Missiaen L, De Smedt H, Bultynck G, Casteels R. Molecular and functional evidence for multiple Ca2+-permeable channel pore. J Biol Chem. 2012;287:2545–57.

41. Kale J, Chi X, Leber B, Andrews D. Examining the molecular mechanism of bcl-2 family proteins at membranes by fluorescence spectroscopy. Methods Enzymol. 2014;544:1–23.

42. Parys JB, de Smedt H, Missiaen L, Bootman MD, Sienaert I, Casteels R. Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection. Cell Calcium. 1995;17:239–49.

43. Decqurye JP, Welkenhuysen K, Luypen T, Ponsaerts R, Deawale M, Molgo J, et al. InsP1/4,5,3P2 receptor-mediated Ca2+ signaling and autophagy induction are interrelated. Autophagy. 2017;11:1472–89.

44. Wagner LE 2nd, Yule DI. Differential regulation of the InosP3 receptor type 1 and -2 single channel properties by InsP3s. Ca2+ and ATP. J Physiol. 2012;590:3245–59.

45. Monaco G, La Rovere R, Karamanou S, Welkenhuysen K, Ivanova H, Vandermarliere E, et al. A double point mutation at residues ile14 and val15 of Bcl-2 uncovers a role for the BH4 domain in both protein stability and function. FEBS J. 2018;285:127–45.

46. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc. 2006;224:213–32.

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AUTHOR CONTRIBUTIONS
GB conceived, coordinated and supervised the project. NR, HI, JK, NL, SK, VS, IL, EV, AE, JT, DWA, JBP, DIY, and GB designed research. NR, HI, LEW, JK, RLR, KW, NL, SK, and VS performed experiments. NR, HI, LEW, JK, NL, SK, and VS analyzed and interpreted data. NR, HI, AE, JT, DWA, JBP, DIY, and GB discussed and interpreted results. KH, HA, and KM provided critical reagents. FR and JS provided critical equipment. DIY and GB acquired funding for the project. NR, HI, and GB drafted the article. NR and HI made the figures. NR, HI, JK, NL, SK, VS, KH, HA, FR, JS, KM, AE, JT, DWA, JBP, DIY, and GB revised the manuscript. NR and GB finalized the article. All authors approved the manuscript.

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ETHICS STATEMENT
Work with human cell lines has been approved by the Ethics Committee Research UZ/KU Leuven: protocol S63808.

COMPETING INTERESTS
The authors declare no competing interests.

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