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The N-terminal helix of Bcl-x_L targets mitochondria

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Abstract

Anti- and pro-apoptotic Bcl-2 family members regulate the mitochondrial phase of apoptotic cell death. The mitochondrial targeting mechanisms of Bcl-2 family proteins are tightly regulated. Known outer mitochondrial membrane targeting sequences include the C-terminal tail and central helical hairpin. Bcl-x_L also localizes to the inner mitochondrial membrane, but these targeting sequences are unknown. Here we investigate the possibility that the N-terminus of Bcl-x_L also contains mitochondrial targeting information. Amino acid residues 1-28 of Bcl-x_L fused to EGFP are sufficient to target mitochondria. Although positive charges and helical propensity are required for targeting, similar to import sequences the N-terminus is not sufficient for efficient mitochondrial import.

Keywords

Bcl-x_L; mitochondrial inner membrane; localization; BH4; Bcl-2; apoptosis

1. INTRODUCTION

Mammalian Bcl-2 family proteins are best known as regulators of cytochrome *c* release across the outer mitochondrial membrane, a key event during apoptotic cell death in mammals. Dysregulation of Bcl-2 proteins can lead to prolonged cell survival contributing to tumorigenesis (Letai, 2008). Bcl-x_L is an anti-apoptotic Bcl-2 family protein that is abundantly expressed in adult neurons and some tumors (Friess et al., 1998; Karbowski et al., 2002). Growing evidence indicates that Bcl-x_L and other family members have additional non-apoptotic functions in healthy cells, including roles in mitochondrial dynamics, cellular metabolism and neuronal activity (Berman et al., 2009; Chen et al., 2011; Fannjiang et al., 2003; Yi et al., 2011). These apoptosis-independent functions are suggested

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to require mitochondrial localization. Outer mitochondrial membrane (OMM) localization is critical for the classical apoptosis functions of both anti- and pro-death Bcl-2 family members (Leber et al., 2010). However, localization to the endoplasmic reticulum (ER) and to the inner mitochondrial membrane (IMM) may be important for both cell death and non-apoptotic functions (Alavian et al., 2011; Annis et al., 2001; Chen et al., 2011).

The primary mitochondrial targeting sequence of Bcl-2 family proteins for the OMM and ER is the C-terminal hydrophobic tail (helix α_9) (Kaufmann et al., 2003). In addition to the tail, the central helical hairpin α_5 - α_6 is suggested to insert into membranes, and also may contain OMM-specific targeting information (Garcia-Saez et al., 2004; George et al., 2010). The long-held view that Bcl-2 family proteins are present exclusively on the OMM and not the IMM was recently overturned (Hardwick et al., 2012), but how some Bcl-2 family proteins target to the IMM is not known. The Bcl-2 family member Mcl-1 has a predicted N-terminal mitochondrial import sequence and its function requires a mitochondrial membrane potential (Huang and Yang-Yen, 2010; Perciavalle et al., 2012). However, there is disagreement about whether Mcl-1 is fully imported into the mitochondrial matrix or is only partially imported while its C-terminal tail remains anchored in the OMM.

Recent evidence indicates that endogenous Bcl-x_L prominently localizes to the inner membrane of mitochondria and is required to prevent large fluctuations in membrane potential based on analysis of cultured neuron derived from conditional knockout mice (Chen et al., 2011). However, Bcl-x_L lacks a predicted N-terminal mitochondrial import sequence, and the mechanisms responsible for targeting Bcl-x_L to the IMM are unexplored. In this study we examined the Bcl-x_L N-terminus for novel mitochondrial localization signals and identified a mitochondrial targeting sequence. Although this sequence appears to engage the import machinery, it is not sufficient for efficient import into the mitochondrial matrix.

2. MATERIALS AND METHODS

2.1 Plasmids and Transfection

N-terminal segments of human Bcl-x_L were PCR amplified, fused to the N-terminus of EGFP in pSG5 (Stratagene #216201) and verified by sequencing. COS7 and HeLa cells were cultured on flamed uncoated 2 cm² glass cover slips in high glucose Dulbecco's Modified Eagle Medium (Invitrogen #12491-015) with 10% fetal bovine serum and penicillin/streptomycin. Cells were transfected with 375 ng of each plasmid using Lipofectamine 2000 for 4 h in OptiMeM (Invitrogen) and analyzed 48 h later except where indicated. Mito-RFP contains human COX8A amino acids 1-29 fused to red fluorescent protein DsRed2 as described (Berman et al., 2009).

2.2 Fluorescence microscopy

To quantify mitochondrial morphology, live cell images were randomly acquired (manually focused after blind selection of 10 fields per condition) on a Nikon Eclipse TE200 inverted microscope using SPOT Advanced software with a Modulation Optics 20x ELWD Flour lens. For mitochondrial staining, live cells were incubated 10 min with MitoTracker Red (Invitrogen, 200 nM) in phosphate buffered saline (PBS: 6.2 mM Na₂HPO₄ • 2 H₂O, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4), washed and incubated in growth medium 1 h at 37°C. MitoTracker-stained cells were washed with cold PBS, fixed 10 min in 4% paraformaldehyde and permeabilized 5 min with 0.2% Triton X-100. Fixed cells were blocked 30 min with 2% goat serum in PBS, incubated 1.5 h with protein disulfide isomerase (PDI) antibody 1D3 (Assay Designs #SPA-891; 1:1,000 in 2% goat serum) and secondary Alexa Fluor antibody (Invitrogen #A21125; 1:1000 in 2% goat serum) for 1 h.

Fixed coverslips were stained 10 min with DAPI, mounted onto glass slides with Prolong Gold (Invitrogen) and stored flat at 4°C. Images (2 μ m Z-stacks) were acquired on a Nikon Eclipse 90i fluorescence microscope with a Nikon Plan Apo 100x lens, and deconvoluted using Volocity software.

2.3 Immunoblot analysis

Cell lysates were prepared 24 h after transfection in buffer [50 mM Tris-HCl, 0.15 M NaCl, 1% Igepal CA-630 (Sigma-Aldrich), 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM EDTA, 2 mM MgCl₂, 1mM dithiothreitol, pH 7.4] and immunoblots were probed with rabbit polyclonal anti-GFP (1:5,000; Invitrogen), mouse anti-actin (C4, 1:10,000; MP Biomedicals), mouse anti-ATP synthase β (1:10,000; 3D5, Invitrogen), donkey anti-rabbit horseradish peroxidase-linked antibody (1:10,000; GE Healthcare), or sheep anti-mouse horseradish peroxidase-linked antibody (1:10,000; GE Healthcare).

2.4 Mitochondrial Isolation

COS7 cells were trypsin-treated 24 h posttransfection and resuspended in fresh MS buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/ml fatty acid free bovine serum albumin, 1 mM EGTA, pH 7.4 at 4°C), permeabilized with digitonin until 90-95% of cells stained positive for trypan blue, and centrifuged 3 min at 2,822 g at 4°C to generate supernatant S1. The corresponding pellet was homogenized in cold MS buffer with 10 strokes in a glass-teflon homogenizer and centrifuged 5 min at 850 g at 4°C. The resulting pellet (P1) was re-suspended in buffer (see 2.3), and the corresponding supernatant was centrifuged 10 min at 16,000 g at 4°C to generate supernatant S2 and the heavy membrane (HM) pellet (Moreadith and Fiskum, 1984). All samples were stored frozen in buffer (see 2.3) with 1x protease inhibitor cocktail (Thermo Scientific). Treatment of mitochondria with digitonin and protease digestion was performed as reported (Chen et al., 2011). Briefly, purified mitochondria were incubated with proteinase K (1 μ g/ml) at 37°C for 30 min. Mitochondria were subsequently treated with digitonin to selectively permeabilize the cholesterol-rich OMM. Selective permeabilization of the OMM was verified by digestion of the IMS proteins, e.g. EndoG but not matrix localized proteins F1 β subunit of the ATPase complex (not shown).

2.5 Immuno-gold Electron Microscopy

Immunogold electron microscopy was performed as described (Chen et al., 2004). Briefly, 80% confluent COS7 cells were fixed (4% formaldehyde, 0.1% glutaraldehyde in 100 mM phosphate buffer with 3 mM CaCl₂), scraped, cryoprotected (2.3 M sucrose with 20% polyvinylpyrrolidone, SIGMA PVP-10), mounted on aluminum stubs, frozen in liquid nitrogen, sectioned on a Reichert-Jung ultramicrotome with an RMC CR-X cryosystem, and placed on formvar-coated 200-mesh nickel grids. Grids were incubated with rabbit anti-GFP antibody (1:10; Invitrogen #A6455) in PBS plus 10% FBS and secondary goat anti-rabbit IgG coupled to 12-nm colloid gold (1:20 in PBS plus 10% FBS). Grids were subsequently fixed in 2% glutaraldehyde in PBS (postfixation in 2% osmium tetroxide) and stained in 2% uranyl acetate. Grids were imaged with a Hitachi 7600 transmission electron microscope at 120 kV.

3. RESULTS

3.1 N-terminus of Bcl-x_L is sufficient for mitochondrial localization

N-terminal Bcl-x_L segments of different lengths fused to EGFP were expressed at similar levels in transiently transfected cells (Figure 1A and B). Bcl-x_L residues 1-28 containing the N-terminal alpha helix of Bcl-x_L efficiently targeted EGFP to mitochondria based on nearly

uniform co-localization with membrane potential-dependent MitoTracker Red in both COS7 and HeLa cells (Figure 1C and D). In contrast, co-localization was not observed with PDI (Figure 1D). This suggests that Bcl-x_L(1-28) lacks ER targeting information, consistent with a previous report testing Bcl-x_L(1-28) in microsomal membranes in vitro (Garcia-Saez et al., 2004). The shorter Bcl-x_L segment of residues 1-16, which interrupts helix α 1 and the putative Bcl-2 homology motif BH4 (residues 4-24), lacked mitochondrial localization, similar to untargeted EGFP. Bcl-x_L residues 1-60 (containing α 1 and part of the long unstructured loop), and residues 1-85 (α 1 + entire loop) also exhibited mitochondrial localization, although less frequently (Figure 1C).

Biochemical fractionation was used to further examine sub-cellular localization of the 28-residue segment of Bcl-x_L that prominently colocalized with MitoTracker by microscopy. Similar to untargeted-EGFP, some Bcl-x_L(1-28)-EGFP was released from mildly permeabilized cells (Figure 2A, supernatant S1). This is consistent with the partial cytosolic fluorescence we observed by fluorescence microscopy, and is similar to endogenous Bcl-x_L in some cell lines (Jeong et al., 2004). Like the mitochondrial matrix protein F₁ beta, Bcl-x_L(1-28)-EGFP was enriched in the heavy membrane fraction containing mitochondria (Figure 2A), similar to endogenous Bcl-x_L in the brain (Chen et al., 2011). The longer construct Bcl-x_L(1-85)-EGFP was also partially localized in mitochondria-enriched fractions, although less efficiently. Therefore, to further examine its sub-mitochondrial localization, the mitochondrial fraction isolated as above was subjected to proteinase K digestion at a dose that efficiently removed OMM protein Tom20 (Figure 2B). Unlike TOM20, Bcl-x_L(1-85)-EGFP was only partially digested, suggesting that a small fraction of the protein is protected by the OMM. These results indicate that most of the Bcl-x_L(1-85)-tagged EGFP protein is located at the OMM, and that only a small fraction may be embedded or translocated across the OMM. Sub-mitochondrial fractionation of transfected cells (not shown) indicate that little or no Bcl-x_L(1-28)-EGFP is imported into mitochondria. Consistent with this, the shorter Bcl-x_L(1-28) was predominantly localized to the OMM based on immuno-gold electron microscopy, with only occasional cristae-localized gold particles (Figure 2C). Thus, it appears that the N-terminus is not sufficient to explain the prominent localization of Bcl-x_L to mitochondrial cristae as well as the OMM and ER using similar techniques (Alavian et al., 2011; Chen et al., 2011). However, Bcl-x_L(1-28)-EGFP and mito-RFP unexpectedly exhibited completely non-overlapping mitochondrial patterns (>99.9% of cells, Figure 2D). Thus, Bcl-x_L(1-28)-EGFP appears to interfere with mitochondrial localization of mito-RFP, despite co-localization of this mito-RFP construct (red fluorescent protein targeted to mitochondria via the N-terminal transit peptide residues 1-29 of COX8) with full-length Bcl-x_L (Berman et al., 2009). This is in stark contrast to full-length Bcl-x_L or the C-terminal Bcl-x_L tail linked to EGFP, which uniformly co-localize with mito-GFP/mito-mRFP containing the same COX8 presequence reported by others (Delivani et al., 2006; Ospina et al., 2011). Thus, Bcl-x_L(1-28) may directly engage the mitochondrial import machinery, as we observed only 1 or 2 cells in multiple experiments with partial co-localization of Bcl-x_L(1-28)-EGFP and mito-RFP (see Discussion).

3.2 Disruption of charge or helical propensity impairs mitochondrial targeting

Classical N-terminal presequences that constitute mitochondrial transit peptides are characterized by an amphipathic α -helix and basic charges that are necessary to translocate across the OMM (Neupert and Herrmann, 2007). Comparing N-terminal sequences from the most divergent Bcl-x_L proteins in the database revealed that the three basic residues are among the most conserved residues. Furthermore, some species have additional basic residues, fewer acidic residues, or have the preferred Arg in place of Lys20 in human Bcl-x_L (Figure 3A). To determine if these three basic residues present in the N-terminal 28 amino acids of human Bcl-x_L are required for mitochondrial localization, these residues were

changed to glycine (RKK/GGG) to disrupt both the charge and helical structure. These mutations nearly abolish mitochondrial localization of Bcl-x_L(1-28)-EGFP (Figure 3B and C). When these same residues were changed to alanine (RKK/AAA) to disrupt charge while maintaining α -helical structure, some activity was retained though significantly reduced. Thus, both basic residues and alpha helical structure of α 1/BH4 appear to be critical for mitochondrial targeting. Potential phosphorylation sites are concentrated on one side of helix α 1 opposite the basic residues. Mutation of three serines to acidic residues (Glu) that favor alpha helical structure but have unfavorable negative charge potentially mimicking phosphorylation, also greatly impaired mitochondrial localization. In contrast, changing these same residues to Ala (SSS/AAA) had no significant effect in either cell type (Figure 3B and C). Thus, negative charges suppress mitochondrial localization, leaving open the possibility that post-translational modification may impact mitochondrial localization of Bcl-x_L. Although these mutant Bcl-x_L-EGFP fusion proteins were expressed at somewhat different levels in both COS7 and HeLa cells, these differences did not correlate with mitochondrial localization (Figure 3D). The evidence presented suggests that both basic charges and amphipathic features of helix α 1 contribute importantly to mitochondrial targeting of Bcl-x_L, yet additional elements of Bcl-x_L are required for localization to the IMM.

4. DISCUSSION

We examined the N-terminus of human Bcl-x_L and identified a novel mitochondrial targeting sequence within residues 1-28. Despite having several features of mitochondrial transit peptides, the Bcl-x_L helix α 1 alone appears insufficient to explain the IMM localization of endogenous Bcl-x_L (Chen et al., 2011). In this regard, it is worth noting, that many dual targeted mitochondrial proteins are characterized by lower mitochondrial targeting sequence parameters and a lower total net positive charge, similar to the situation described here for the N-terminus of Bcl-x_L (Dinur-Mills et al., 2008). Bcl-x_L, which localizes to several intracellular compartments including mitochondria and ER, could belong to this recently defined subgroup of mitochondrial proteins that in yeast comprise a quarter of the mitochondrial proteome (Dinur-Mills et al., 2008). Such weak mitochondrial presequences, which often contain lysines instead of arginines, can direct selective/cell-specific mitochondrial import/localization depending on the magnitude of the mitochondrial membrane potential ($\Delta\Phi$) (Martin et al., 1991; Matthews et al., 2010). Nevertheless, this sequence may be one component of the IMM import signal of Bcl-x_L, potentially analogous to PINK1, which is suggested to have several regions involved in targeting to the OMM and IMM (Becker et al., 2012). Bcl-x_L(1-28) may engage the import machinery as it prevented mitochondrial localization/import by the COX8 transit peptide. If Bcl-x_L-EGFP can plug import pores randomly distributed along the surface of mitochondria, then Bcl-x_L-EGFP and mito-RFP might have co-localized to the same mitochondrion. Instead, they unexpectedly segregated to separate organelles, possibly because they can distinguish distinct mitochondrial subsets, though both subsets are polarized (MitoTracker).

Previously, Bcl-x_L has been shown to localize to the OMM via a C-terminal transmembrane domain, which is flanked by positive charges that further specify mitochondria over ER membranes (Kaufmann et al., 2003). However, Bcl-2 family proteins Bfl1/A1 and Bid lack a C-terminal transmembrane domain and still localize to mitochondria (Aouacheria et al., 2012; Brien et al., 2009; Kim et al., 2004). Although the C-terminal tail of Bcl-x_L is not essential for membrane insertion, dimerization or anti-death activity, its tail contributes importantly to all of these functions (Basañez et al., 2001; Bhat et al., 2012; Jeong et al., 2004; Thuduppathy et al., 2006). The C-terminal tail may cooperate with the α 5- α 6 hairpin to carry out membrane functions of Bcl-x_L.

In contrast to the C-terminus, the N-terminal helix $\alpha 1$ is required for anti-death activity of Bcl-x_L, and is sufficient to inhibit cell death in some models (Clem et al., 1998; Martorana et al., 2012; Monaco et al., 2012; Sugioka et al., 2003). Furthermore, removal of the Bcl-x_L N-terminus by engineered deletion or by caspase cleavage results in a highly active/toxic protein in lipid vesicles, in mitochondrial membranes, in cell lines and in the brain as knockin caspase-resistant Bcl-x_L mice are protected from ischemic injury (Basañez et al., 2001; Clem et al., 1998; Jonas et al., 2004; Ofengeim et al., 2012). The Bcl-x_L helix $\alpha 1$ was previously suggested to interact with membranes based on studies with micelles, (Losonczi et al., 2000), but another study did not detect lipid membrane insertion of Bcl-x_L helix $\alpha 1$, in contrast to helix $\alpha 1$ of Bax (Garcia-Saez et al., 2004). Thus, it is possible that Bcl-x_L helix $\alpha 1$ targets mitochondria via protein-protein interactions with factors involved in importing other COX8-RFP or with other Bcl-2 family proteins (Ding et al., 2010).

We considered the possibility that this N-terminal region of Bcl-x_L, when removed from its native protein, fortuitously targets EGFP to mitochondria and does not reflect a physiological function of $\alpha 1$. In support of this potential caveat, removing the C-terminal transmembrane domain of Bcl-x_L largely abolished its mitochondrial localization in transfected cells (not shown) (Jeong et al., 2004; Kaufmann et al., 2003). However, overexpression of the cDNA for mitochondrial matrix protein F₁ beta also results in little mitochondrial import despite having a bona fide N-terminal transit peptide (Margeot et al., 2002). Related oddities occur with membrane targeting sequences of other Bcl-2 family proteins. Curiously, the C-terminal transmembrane domain of Bax (residues 172-192) fused to the C-terminus of GFP is not sufficient to target GFP to mitochondria (Nechushtan et al., 1999). Additionally, there are mixed reports about whether the N-terminus of Bax contains mitochondrial targeting information (Cartron et al., 2003; George et al., 2010) or interacts with Tom22, a mitochondrial import receptor (Bellot et al., 2007; Ross et al., 2009). However, each of these studies analyzes slightly different segments of the Bax N-terminus using different approaches. Thus, it is challenging to segregate the functional elements of Bcl-2 family proteins. Furthermore, how the N-terminus of Bcl-2 family proteins and a number of other mitochondrial proteins are imported into mitochondria remains unknown (Schmidt et al., 2010).

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Abbreviations

Bcl-2	B-cell lymphoma/leukemia protein 2
BH4	Bcl-2 homology motif 4
ER	endoplasmic reticulum
IMM	inner mitochondrial membrane
OMM	outer mitochondrial membrane
PDI	protein disulfide isomerase

REFERENCES

Alavian KN, Li H, Collis L, Bonanni L, Zeng L, Sacchetti S, Lazrove E, Nabili P, Flaherty B, Graham M, Chen Y, Messerli SM, Mariggio MA, Rahner C, McNay E, Shore GC, Smith PJ, Hardwick JM,

- Jonas EA. Bcl-xL regulates metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP synthase. *Nat Cell Biol.* 2011; 13:1224–1233. [PubMed: 21926988]
- Annis MG, Zamzami N, Zhu W, Penn LZ, Kroemer G, Leber B, Andrews DW. Endoplasmic reticulum localized Bcl-2 prevents apoptosis when redistribution of cytochrome c is a late event. *Oncogene.* 2001; 20:1939–1952. [PubMed: 11360178]
- Aouacheria A, Laval VR, Combet C, Hardwick JM. Evolution of Bcl-2 homology motifs: homology versus homoplasy. *Trends Cell Biol.* 2012
- Basañez G, Zhang J, Chau BN, Maksaev GI, Frolov VA, Brandt TA, Burch J, Hardwick JM, Zimmerberg J. Pro-apoptotic cleavage products of Bcl-xL form cytochrome c-conducting pores in pure lipid membranes. *J Biol Chem.* 2001; 276:31083–31091. [PubMed: 11399768]
- Bellot G, Cartron PF, Er E, Oliver L, Juin P, Armstrong LC, Bornstein P, Mihara K, Manon S, Vallette FM. TOM22, a core component of the mitochondria outer membrane protein translocation pore, is a mitochondrial receptor for the proapoptotic protein Bax. *Cell Death Differ.* 2007; 14:785–794. [PubMed: 17096026]
- Berman SB, Chen YB, Qi B, McCaffery JM, Rucker EB 3rd, Goebbels S, Nave KA, Arnold BA, Jonas EA, Pineda FJ, Hardwick JM. Bcl-x L increases mitochondrial fission, fusion, and biomass in neurons. *J Cell Biol.* 2009; 184:707–719. [PubMed: 19255249]
- Bhat V, McDonald CB, Mikles DC, Deegan BJ, Seldeen KL, Bates ML, Farooq A. Ligand binding and membrane insertion compete with oligomerization of the BclXL apoptotic repressor. *J Mol Biol.* 2012; 416:57–77. [PubMed: 22197371]
- Brien G, Debaud AL, Robert X, Oliver L, Trescol-Biemont MC, Cauquil N, Geneste O, Aghajari N, Vallette FM, Haser R, Bonnefoy-Berard N. C-terminal residues regulate localization and function of the antiapoptotic protein Bfl-1. *J Biol Chem.* 2009; 284:30257–30263. [PubMed: 19759007]
- Cartron PF, Priault M, Oliver L, Meflah K, Manon S, Vallette FM. The N-terminal end of Bax contains a mitochondrial-targeting signal. *J Biol Chem.* 2003; 278:11633–11641. [PubMed: 12529375]
- Chen JQ, Delannoy M, Cooke C, Yager JD. Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. *Am J Physiol Endocrinol Metab.* 2004; 286:E1011–1022. [PubMed: 14736707]
- Chen YB, Aon MA, Hsu YT, Soane L, Teng X, McCaffery JM, Cheng WC, Qi B, Li H, Alavian KN, Dayhoff-Brannigan M, Zou S, Pineda FJ, O'Rourke B, Ko YH, Pedersen PL, Kaczmarek LK, Jonas EA, Hardwick JM. Bcl-xL regulates mitochondrial energetics by stabilizing the inner membrane potential. *J Cell Biol.* 2011; 195:263–276. [PubMed: 21987637]
- Clem RJ, Cheng EH, Karp CL, Kirsch DG, Ueno K, Takahashi A, Kastan MB, Griffin DE, Earnshaw WC, Veluona MA, Hardwick JM. Modulation of cell death by Bcl-XL through caspase interaction. *Proc Natl Acad Sci U S A.* 1998; 95:554–559. [PubMed: 9435230]
- Delivani P, Adrain C, Taylor RC, Duriez PJ, Martin SJ. Role for CED-9 and Egl-1 as regulators of mitochondrial fission and fusion dynamics. *Mol Cell.* 2006; 21:761–773. [PubMed: 16543146]
- Ding J, Zhang Z, Roberts GJ, Falcone M, Miao Y, Shao Y, Zhang XC, Andrews DW, Lin J. Bcl-2 and Bax interact via the BH1-3 groove-BH3 motif interface and a novel interface involving the BH4 motif. *J Biol Chem.* 2010; 285:28749–28763. [PubMed: 20584903]
- Dinur-Mills M, Tal M, Pines O. Dual targeted mitochondrial proteins are characterized by lower MTS parameters and total net charge. *PLoS ONE.* 2008; 3:e2161. [PubMed: 18478128]
- Fannjiang Y, Kim CH, Haganir RL, Zou S, Lindsten T, Thompson CB, Mito T, Traystman RJ, Larsen T, Griffin DE, Mandir AS, Dawson TM, Dike S, Sappington AL, Kerr DA, Jonas EA, Kaczmarek LK, Hardwick JM. BAK alters neuronal excitability and can switch from anti- to pro-death function during postnatal development. *Dev Cell.* 2003; 4:575–585. [PubMed: 12689595]
- Friess H, Lu Z, Andren-Sandberg A, Berberat P, Zimmermann A, Adler G, Schmid R, Buchler MW. Moderate activation of the apoptosis inhibitor bcl-xL worsens the prognosis in pancreatic cancer. *Ann Surg.* 1998; 228:780–787. [PubMed: 9860477]
- Garcia-Saez AJ, Mingarro I, Perez-Paya E, Salgado J. Membrane-insertion fragments of Bcl-xL, Bax, and Bid. *Biochemistry.* 2004; 43:10930–10943. [PubMed: 15323553]

- George NM, Targy N, Evans JJ, Zhang L, Luo X. Bax contains two functional mitochondrial targeting sequences and translocates to mitochondria in a conformational change- and homo-oligomerization-driven process. *J Biol Chem.* 2010; 285:1384–1392. [PubMed: 19880508]
- Hardwick JM, Chen Y.-b. Jonas EA. Multipolar functions of BCL-2 proteins link energetics to apoptosis. *Trends Cell Biol.* 2012 (in press), available online May 4.
- Hsu YT, Youle RJ. Nonionic detergents induce dimerization among members of the Bcl-2 family. *J Biol Chem.* 1997; 272:13829–13834. [PubMed: 9153240]
- Huang CR, Yang-Yen HF. The fast-mobility isoform of mouse Mcl-1 is a mitochondrial matrix-localized protein with attenuated anti-apoptotic activity. *FEBS Lett.* 2010; 584:3323–3330. [PubMed: 20627101]
- Jeong SY, Gaume B, Lee YJ, Hsu YT, Ryu SW, Yoon SH, Youle RJ. Bcl-x(L) sequesters its C-terminal membrane anchor in soluble, cytosolic homodimers. *EMBO J.* 2004; 23:2146–2155. [PubMed: 15131699]
- Jonas EA, Hickman JA, Chachar M, Polster BM, Brandt TA, Fannjiang Y, Ivanovska I, Basanez G, Kinnally KW, Zimmerberg J, Hardwick JM, Kaczmarek LK. Proapoptotic N-truncated BCL-xL protein activates endogenous mitochondrial channels in living synaptic terminals. *Proc Natl Acad Sci U S A.* 2004; 101:13590–13595. [PubMed: 15342906]
- Karbowski M, Lee YJ, Gaume B, Jeong SY, Frank S, Nechushtan A, Santel A, Fuller M, Smith CL, Youle RJ. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol.* 2002; 159:931–938. [PubMed: 12499352]
- Kaufmann T, Schlupf S, Sanz J, Neubert K, Stein R, Borner C. Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. *J Cell Biol.* 2003; 160:53–64. [PubMed: 12515824]
- Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH. Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell.* 2009; 36:487–499. [PubMed: 19917256]
- Kim TH, Zhao Y, Ding WX, Shin JN, He X, Seo YW, Chen J, Rabinowich H, Amoscato AA, Yin XM. Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. *Mol Biol Cell.* 2004; 15:3061–3072. [PubMed: 15107464]
- Leber B, Lin J, Andrews DW. Still embedded together binding to membranes regulates Bcl-2 protein interactions. *Oncogene.* 2010; 29:5221–5230. [PubMed: 20639903]
- Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer.* 2008; 8:121–132. [PubMed: 18202696]
- Losonczi JA, Olejniczak ET, Betz SF, Harlan JE, Mack J, Fesik SW. NMR studies of the anti-apoptotic protein Bcl-xL in micelles. *Biochemistry.* 2000; 39:11024–11033. [PubMed: 10998239]
- Margeot A, Blugeon C, Sylvestre J, Vialette S, Jacq C, Corral-Debrinski M. In *Saccharomyces cerevisiae*, ATP2 mRNA sorting to the vicinity of mitochondria is essential for respiratory function. *Embo J.* 2002; 21:6893–6904. [PubMed: 12486010]
- Martin J, Mahlke K, Pfanner N. Role of an energized inner membrane in mitochondrial protein import. Delta psi drives the movement of presequences. *J Biol Chem.* 1991; 266:18051–18057. [PubMed: 1833391]
- Martorana F, Brambilla L, Valori CF, Bergamaschi C, Roncoroni C, Aronica E, Volterra A, Bezzi P, Rossi D. The BH4 domain of Bcl-XL rescues astrocyte degeneration in amyotrophic lateral sclerosis by modulating intracellular calcium signals. *Hum Mol Genet.* 2012; 21:826–840. [PubMed: 22072391]
- Matthews GD, Gur N, Koopman WJ, Pines O, Vardimon L. Weak mitochondrial targeting sequence determines tissue-specific subcellular localization of glutamine synthetase in liver and brain cells. *J Cell Sci.* 2010; 123:351–359. [PubMed: 20053634]
- Monaco G, Decrock E, Akl H, Ponsaerts R, Vervliet T, Luyten T, De Maeyer M, Missiaen L, Distelhorst CW, De Smedt H, Parys JB, Leybaert L, Bultynck G. Selective regulation of IP3-receptor-mediated Ca²⁺ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-XL. *Cell Death Differ.* 2012; 19:295–309. [PubMed: 21818117]

- Moreadith RW, Fiskum G. Isolation of mitochondria from ascites tumor cells permeabilized with digitonin. *Anal Biochem.* 1984; 137:360–367. [PubMed: 6731817]
- Nechushtan A, Smith CL, Hsu YT, Youle RJ. Conformation of the Bax C-terminus regulates subcellular location and cell death. *Embo J.* 1999; 18:2330–2341. [PubMed: 10228148]
- Neupert W, Herrmann JM. Translocation of proteins into mitochondria. *Annu Rev Biochem.* 2007; 76:723–749. [PubMed: 17263664]
- Ofengeim D, Chen YB, Miyawaki T, Li H, Sacchetti S, Flannery RJ, Alavian KN, Pontarelli F, Roelofs BA, Hickman JA, Hardwick* JM, Zukin* RS, Jonas* EA. N-terminally cleaved Bcl-x(L) mediates ischemia-induced neuronal death. **Nat Neurosci.* 2012; 15:574–580. [*co-corresponding; knockin mouse generated in our lab] See. [PubMed: 22366758] News & Views, *Nat Neurosci.* 515:505–506.
- Ospina A, Lagunas-Martinez A, Pardo J, Carrodeguas JA. Protein oligomerization mediated by the transmembrane carboxyl terminal domain of Bcl-XL. *FEBS Lett.* 2011; 585:2935–2942. [PubMed: 21856303]
- Perciavalle RM, Stewart DP, Koss B, Lynch J, Milasta S, Bathina M, Temirov J, Cleland MM, Pelletier S, Schuetz JD, Youle RJ, Green DR, Opferman JT. Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol.* 2012
- Ross K, Rudel T, Kozjak-Pavlovic V. TOM-independent complex formation of Bax and Bak in mammalian mitochondria during TNFalpha-induced apoptosis. *Cell Death Differ.* 2009; 16:697–707. [PubMed: 19165229]
- Schmidt O, Pfanner N, Meisinger C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat Rev Mol Cell Biol.* 2010; 11:655–667. [PubMed: 20729931]
- Sugioka R, Shimizu S, Funatsu T, Tamagawa H, Sawa Y, Kawakami T, Tsujimoto Y. BH4-domain peptide from Bcl-xL exerts anti-apoptotic activity in vivo. *Oncogene.* 2003; 22:8432–8440. [PubMed: 14627984]
- Thuduppathy GR, Terrones O, Craig JW, Basanez G, Hill RB. The N-terminal domain of Bcl-xL reversibly binds membranes in a pH-dependent manner. *Biochemistry.* 2006; 45:14533–14542. [PubMed: 17128992]
- Yi CH, Pan H, Seebacher J, Jang IH, Hyberts SG, Heffron GJ, Vander Heiden MG, Yang R, Li F, Locasale JW, Sharfi H, Zhai B, Rodriguez-Mias R, Luithardt H, Cantley LC, Daley GQ, Asara JM, Gygi SP, Wagner G, Liu CF, Yuan J. Metabolic regulation of protein N-alpha-acetylation by Bcl-xL promotes cell survival. *Cell.* 2011; 146:607–620. [PubMed: 21854985]

Highlights

- We present the first evidence that the N-terminus of Bcl-x_L is sufficient for mitochondrial targeting.
- Basic residues and alpha helicity of the N-terminal 28 residues of Bcl-x_L are required for mitochondrial targeting.
- The N-terminal 28 amino acids of Bcl-x_L appear to engage mitochondrial import machinery, but do not mediate efficient mitochondrial import.

Figure 1. Amino acids 1-28 of Bcl-x_L target EGFP to mitochondria

(A) Diagram of Bcl-x_L-EGFP fusion proteins. (B) Immunoblot of transiently transfected COS7 and HeLa cell lysates expressing constructs in panel A. (C) Percent of transiently transfected cells with prominent mitochondrial morphology of Bcl-x_L-EGFP was determined by scoring randomly acquired images of live unstained cells. Data presented are mean $\pm$ SEM for 200 cells per sample in each of three independent experiments, *** p <0.0001, * p <0.01 by Fisher's exact test (p <0.0125 is significant with Bonferroni correction). (D) Representative fluorescence microscopy images (deconvoluted Z stacks, except EGFP alone) of fixed COS7 and HeLa cells 48 h post transfection co-stained with MitoTracker Red (polarized mitochondria) or anti-PDI (ER marker, red).

Figure 2. Amino acid residues 1-28 of Bcl-x_L inhibit localization of mito-RFP

(A) Immunoblot analyses of fractionated lysates prepared from COS7 cells transfected with Bcl-x_L(1-28)-EGFP or untargeted EGFP (see text and Methods). Mildly permeabilized cells yielded the cytosolic supernatant (S1). The homogenized pellet was separated into a crude pellet (P1) and a supernatant that was further separated at high-speed into supernatant (S2) and mitochondria-enriched heavy membrane pellet (HM). Samples were blotted with anti-GFP and anti-F₁ ATP synthase beta (mitochondrial matrix). A representative of 3 independent experiments is shown. (B) Sub-mitochondrial localization of Bcl-x_L(1-85)-EGFP in COS7 cells at 24 and 96 h post transfection. (C) Immuno-gold electron microscopy of COS7 cells transfected with Bcl-x_L(1-28)-EGFP using anti-GFP antibody. (D) Immunofluorescence microscopy of COS7 cells co-expressing Bcl-x_L(1-28)-EGFP and COX8A(1-29)-dsRED as described for Figure 1; representative of >3 independent experiments.

Figure 3. Mitochondrial targeting by Bcl-x_L(1-28) requires features of N-terminal transit peptides

(A) Alignment of the N-terminal 28 residues from the most divergent Bcl-x_L protein sequences available, showing conservation of basic residues (*) among absolutely conserved amino acids (blue boxes), and additional basic residues present in some species (orange/red boxes). (B) Diagram of mutant Bcl-x_L-EGFP fusion constructs with approximate structure comparison based on several Bcl-x_L structures available at <http://www.rcsb.org/pdb/home/home.do>. (C) Percent of mutant compared to wild type Bcl-x_L(1-28)-EGFP-transfected cells with prominent mitochondrial localization was determined as in Figure 1C. Data are shown as mean $\pm$ SEM for 200 cells per sample in each of three independent experiments, *** $p < 0.0001$ by Fisher's exact test. (D) Immunoblot of COS7 and HeLa cell lysates expressing the constructs from panels B and C.