## Introduction

Bcl-xL is an antiapoptotic Bcl-2 family member that is required for embryonic development and can contribute to cancer cell survival (Letai, 2008; Hardwick and Youle, 2009). The traditional viewpoint is that anti- and proapoptotic Bcl-2 family proteins actively engage each other to determine cell fate after a death stimulus (Galonek and Hardwick, 2006; Youle and Strasser, 2008). The best-characterized cell survival activity of Bcl-xL is its ability to inhibit Bax-induced pores in the outer mitochondrial membrane (Billen et al., 2008). In this manner, Bcl-xL prevents release of mitochondrial cytochrome c into the cytoplasm, where cytochrome c induces apoptosis.

Mammalian Bcl-xL protein localizes to the outer mitochondrial membrane, where it inhibits apoptosis by binding Bax and inhibiting Bax-induced outer membrane permeabilization. Contrary to expectation, we found by electron microscopy and biochemical approaches that endogenous Bcl-xL also localized to inner mitochondrial cristae. Two-photon microscopy of cultured neurons revealed large fluctuations in inner mitochondrial membrane potential when Bcl-xL was genetically deleted or pharmacologically inhibited, indicating increased total ion flux into and out of mitochondria. Computational, biochemical, and genetic evidence indicated that Bcl-xL reduces futile ion flux across the inner mitochondrial membrane to prevent a wasteful drain on cellular resources, thereby preventing an energetic crisis during stress. Given that F$_1$F$_0$-ATP synthase directly affects mitochondrial membrane potential and having identified the mitochondrial ATP synthase β subunit in a screen for Bcl-xL-binding partners, we tested and found that Bcl-xL failed to protect β subunit–deficient yeast. Thus, by bolstering mitochondrial energetic capacity, Bcl-xL may contribute importantly to cell survival independently of other Bcl-2 family proteins.
is consistent with the limited reserve capacity of bcl-x-deficient neurons and their susceptibility to cell death. This function of Bcl-xL involves the mitochondrial F1F0 ATP synthase.

Results

Defective control of mitochondrial membrane potential in bcl-x-deficient neurons

To explore the function of Bcl-xL in healthy neurons, several mitochondrial parameters were analyzed by two-photon laser-scanning fluorescence microscopy, comparing control and bcl-x conditional knockout (cKO) cortical neuron cultures (Berman et al., 2009). Both unfloxed and bcl-x–floxed littermates express neuron-specific knockin NEX-Cre recombinase starting around embryonic day 12 (E12) to delete bcl-x. Staining for Cre recombinase serves as a positive marker for the survival of bcl-x–deficient (and control unfloxed) cortical neurons (Fig. 1 A; Berman et al., 2009). Mitochondrial membrane potential ($\Delta \Psi_{m}$) was assessed in immature cortical cultures with the potentiometric dye tetramethylrhodamine methyl ester (TMRM; nonquench mode), revealing higher fluorescence intensity in the mitochondria-enriched regions of bcl-x knockout cortical neurons (Fig. 1, B and C [left]). This is not a result of increased mitochondrial biomass because bcl-x–deficient neurons have lower, not higher, mitochondrial biomass in these and other cell types based on several criteria (Kowaltowski et al., 2002; Berman et al., 2009). Thus, it appears that bcl-x deficiency may result in a higher mitochondrial membrane potential.

In respiring cells, three direct mechanisms (Fig. 1 D, dashed boxes) of proton flux across the inner membrane (Fig. 1 D, blue arrows) are main determinants of $\Delta \Psi_{m}$: (1) the ETC, (2) the F1F0 ATP synthase, and (3) uncoupling proteins or other molecularly undefined leak mechanisms (protons not used for ATP synthesis; Nicholls and Ferguson, 2002). To further explore these parameters in bcl-x knockout neurons, reactive oxygen species (ROS) production by the ETC was assessed in the same mitochondrial areas where TMRM was evaluated. Unexpectedly, bcl-x knockout neurons have lower mitochondrial ROS. This suggests either a high rate of electron flow through the respiratory chain or that bcl-x knockout neurons are more dependent on glycolysis than on mitochondrial respiration for energy production (Fig. 1, A and B). The same mitochondrial areas of bcl-x knockout neurons have modestly higher mitochondrial NAD(P)H levels, which is consistent with an earlier study (Schwartz et al., 2007). Higher levels of the complex I substrate NADH indicate more than adequate supplies of NADH either because the respiratory chain is inactive or other metabolic processes are altered, such as decreased anaplerosis (Abramov et al., 2007; Cheng et al., 2011). However, no inherent defects in respiratory chain activity were detected when complexes I–IV, II–IV, and IV were assessed by measuring oxygen consumption in isolated brain mitochondria (Fig. S1, A and B). The relative contributions of glycolysis versus the mitochondrial F1F0 ATP synthase to the levels of total cellular ATP.

(Vander Heiden et al., 2001; Levine et al., 2008; Li et al., 2008). Thus, Bcl-2 proteins may have alternative biochemical functions independent of their proapoptotic Bcl-2 family binding partners, or they may participate in other machineries before engaging classical apoptosis.

One nonapoptosis role of Bcl-2 family proteins in mammals and worms is regulation of mitochondrial fission and fusion (Karbowska et al., 2006; Berman et al., 2009; Montessuit et al., 2010; Hoppins et al., 2011). This role appears to contribute importantly to Bcl-xL–induced mitochondrial localization at neuronal synapses, neuronal activity, and seizure behaviors (Fannjiang et al., 2003; Li et al., 2008). However, regulation of fission and fusion rates is not sufficient to explain the ability of endogenous and overexpressed Bcl-xL to increase mitochondrial biomass (Berman et al., 2009). Therefore, we pursued alternative functions of Bcl-xL in mitochondria. Consistent with an evolutionarily conserved function, Bcl-2 family proteins have been linked to control of mitochondrial energetics by regulating the voltage-dependent anion channel in the outer membrane or the adenine nucleotide transporter (ANT)/adenine nucleotide carrier in the inner membrane, which are the primary conduits through which ATP and ADP are exchanged between the cytosol and the mitochondrial matrix (Vander Heiden et al., 2001; Belzacaq et al., 2003; Cheng et al., 2003). The relative contributions of antiapoptotic activity versus alternative functions of Bcl-xL for overall survival are unclear.

The mitochondrial F1F0 ATP synthase synthesizes ATP in the mitochondrial matrix using cytosolic ADP and phosphate as substrates (Hong and Pedersen, 2004). This process requires a potential across the inner mitochondrial membrane that is generated by pumping out protons via the electron transport chain (ETC; or respiratory chain) fueled by NADH. Reentry of protons into the mitochondrial matrix via the F0 ring (oligomycin-sensitive fraction) embedded in the inner membrane drives rotation of the central stalk against the catalytic F1, a ring of three $\alpha$ and three $\beta$ subunits (Walker and Dickson, 2006). In this manner, proton flux through F0 is coupled to ATP synthesis. Because mitochondrial membrane potential is required for essential functions other than ATP synthesis, there are alternative strategies for building a potential. Reversal of the F1F0 ATP synthase hydrolyzes cytoplasmic ATP produced by glycolysis, reversing the flow of protons through F0 to stabilize a potential (Nicholls and Ferguson, 2002; Abramov et al., 2007). A membrane potential is also required for mitochondrial fusion, and depolarization of the potential leads to Parkin-dependent mitophagy (Narendra et al., 2008; Twig et al., 2008). Although mitochondrial energetics are linked to mitochondrial morphology changes, the details are complex (Benard and Rossignol, 2008).

By analyzing bcl-x–deficient neurons, we uncovered a new function of Bcl-xL. We found that Bcl-xL can localize to the inner mitochondrial membrane/matrix, which is contrary to current opinion. Importantly, Bcl-xL is required to stabilize the membrane potential across the inner mitochondrial membrane. By decreasing excess ion flux across the inner mitochondrial membrane, Bcl-xL increases overall energetic efficiency, which

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were also similar between bcl-x knockout and control cultures. ATP levels (relative to total protein) were slightly reduced in the bcl-x knockout cortical cultures, though this was a result in part of 15% lower cell viability compared with controls (Fig. S1, C and D). In sum, no defects were detected to explain the altered mitochondrial parameters of bcl-x knockout neurons.

Localization of endogenous Bcl-xL includes mitochondrial cristae

To pursue the role of Bcl-xL in regulating mitochondrial parameters, we determined the subcellular localization of endogenous Bcl-xL protein in neurons of the brain. Endogenous Bcl-xL in HeLa cells resides predominantly in the cytosol as a homodimer and translocates to mitochondria via its C-terminal transmembrane domain after a death stimulus (Jeong et al., 2004). However, crude fractionation of mouse cortex suggests that a significant proportion of endogenous Bcl-xL localizes to mitochondria in the brain (Fig. 2 A), which is consistent with an earlier finding (Soane et al., 2008). Deletion of bcl-x (except in interneurons and glial cells where NEX-Cre is not expressed; Berman et al., 2009) did not significantly alter other mitochondrial markers (Fig. 2 A).

Immunogold EM was used to more precisely determine the subcellular localization of endogenous Bcl-xL in tissue slices of mouse brain hippocampus. Approximately 90% of gold-labeled anti-Bcl-xL (BioCarta) is associated with membranes, and at least half of these membranes (54%) can be clearly identified as mitochondria (Fig. 2, B and C). Surprisingly, most of the mitochondrial staining was inside mitochondria, where the colabeled ATP synthase $\beta$ subunit was also found (Figs. 2 [B and C] and S2). The frequency of labeled mitochondria with inner membrane/matrix Bcl-xL gold label (58%) argues strongly against the possibility of contamination from the outer membrane as a result of edge skimming, folding of the slice preparation, or random background. Gold particles on mitochondria were also detected in polar clusters (Fig. 2 C, arrowheads) and on membranes adjacent to mitochondria, possibly marking mitochondrial fission/fusion sites or where the outer mitochondrial membrane may be tethered to the ER (Fig. 2 C, line arrows), though patchy staining can reflect the uneven epitope accessibility in ultrathin cryosections.
Biochemical purification of Bcl-xL with inner membrane components

The possibility that Bcl-xL regulates mitochondrial membrane potential by acting at the inner mitochondrial membrane led us to revisit our earlier yeast two-hybrid screen (Chau et al., 2000). Seeking to identify prosurvival functions distinct from antiapoptotic functions of Bcl-xL in an unbiased screen, the BH1 domain mutant of Bcl-xL (mt1; F131V/D133A), which inhibits cell death without binding prodeath family members Bax or Bak (Cheng et al., 1996), was used to screen a human B cell library (Chau et al., 2000). Among the six hits, we identified an unexpected Bcl-xL–binding partner, the β subunit of the mitochondrial F1FO ATP synthase. This interaction was confirmed in a secondary yeast two-hybrid screen in which the β subunit interacted with wild-type Bcl-xL and Bcl-2 but did not interact with mutants lacking antideath activity (Bcl-xL mt7 and mt8) and did not interact with Bax or Bak (Fig. 3 A). Because Bcl-xL mt1 could potentially inhibit mammalian cell death by binding BH3-only proteins (Billen et al., 2008), we assayed the function of mt1 and mt8 in yeast, which lack Bcl-2 and BH3-only proteins. Bcl-xL mt1 but not mt8 protected yeast from dose-dependent cell death (Fig. 3 B).

An independent biochemical purification scheme also identified the β subunit as the prominent binding partner of Bcl-xL.
endogenous Bcl-xL. WEHI 7.1 membrane preparations were solubilized with CHAPS to avoid detergent-induced dimerization with Bax during extract preparation (Hsu and Youle, 1997). Bcl-xL-containing complexes were purified by sequential ion exchange and immunoaffinity chromatography followed by preparative SDS-PAGE (Fig. 3 D). The only major Coomassie-stained species copurifying with Bcl-xL was ~54 kD. Direct sequencing of two tryptic peptides derived from this extracted band yielded exact matches with the human/mouse F1F0 ATP synthase β-subunit residues 244–253 (NDLYHEMIES) and 389–404 (IAELGIYPAVDPLDST).

A screen of 80 detergents yielded a strategy for purifying enzymatically active F1F0 ATP synthase from mitoplasts isolated from rat liver mitochondria for the purpose of 3D structure determination (Ko et al., 2003). Immunoblot analyses of these preparations revealed monomeric Bcl-xL, which decreased in abundance with purification as a band approximately the size of Bcl-xL dimers was enriched with purification (Fig. 3 C). Detergents likely induced SDS-stable dimers of Bcl-xL, which are frequently encountered with purified Bcl-xL (O’Neill et al., 2006). On parallel blots, both Bcl-xL bands were eliminated when the antibody was preadsorbed with recombinant Bcl-xL protein, indicating that Bcl-xL is enriched in highly purified preparations of the ATP synthase from liver. To determine whether Bcl-xL is monomeric or present in larger complexes inside cells, CHAPS-solubilized lysates were separated by column chromatography, revealing that all of the Bcl-xL was in complexes >70 kD that overlap fractions containing the β subunit (Fig. 3 E). An association of Bcl-2 with the F1F0 ATP synthase has also been observed by the laboratories of J. Downward (London Research Institute, London, England, UK), Y. Tsujimoto (Osaka University, Osaka, Japan), and S. Korsmeyer and G. Linette (Washington University in St. Louis, St. Louis, MO; personal communication).

Membrane potential fluctuations in bcl-x-deficient mitochondria

Because the mitochondrial F1F0 ATP synthase is an important control point for proton flux across the inner mitochondrial membrane, mitochondrial membrane potential was further evaluated by time-lapse imaging (3.5-s intervals). TMRM intensity in mitochondria-enriched regions fluctuates modestly in control neurons, which is consistent with an earlier study (Vergun et al., 2005). Membrane potential fluctuations in cultured bcl-x knockout and control cortical neurons (Fig. 4 D). Although compiled data indicate a small but significant calcium elevation in bcl-x knockout neurons, fluctuations in potential appear not to be controlled by paired fluctuations in cytosolic calcium.

To investigate the possibility that Bcl-xL has a direct role in stabilizing the mitochondrial membrane potential, tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity was...
maintained by expending energy (Nicolis and Prigogine, 1977). Moreover, the additional time-dependent flux of ions across the inner membrane that drives these fluctuations in potential can result in an overall ion flux (both inward and outward directions) that is greater than what is required simply to maintain a nonfluctuating membrane potential at a steady negative value. Thus, the fluctuations in mitochondrial membrane potential in bcl-x knockout implies that more energy is required to maintain ion gradients across the inner membrane. To illustrate this concept, we constructed a simple numerical model to investigate the effect of fluctuations on the dissipation of ion gradients across the mitochondrial membrane. A vesicle (1 µm in diameter) was used to represent a mitochondrion (Fig. 5 A). This vesicle was equipped with active ion pumps (Fig. 5 A, b) capable of pumping out protons/ions (approximating the respiratory chain) to build a negative potential (−180 mV) and with ion channels (Fig. 5 A, a) that can partially dissipate this potential by allowing ions to reenter the vesicle (approximating the F1FO ATP synthase and nonproductive leaks). We first modeled steady-state conditions in which the inward flux and monitored in cultured hippocampal neurons treated only briefly with ABT-737, a specific inhibitor of Bcl-xL designed to fit the binding pocket on Bcl-xL and block its antiapoptotic function (Oltersdorf et al., 2005). Individual mitochondrion exhibited greater fluctuations in TMRE fluorescence intensity after only 10 min of ABT-737 (in 0.1% DMSO) compared with DMSO alone (Fig. 4, E and F). To confirm the specificity of ABT-737, fluorescence intensities were measured in small puncta (estimated to be one mitochondrion) near the soma in cultured rat hippocampal neurons (DIV14–16) stained with 5 nM TMRE. Relative fluorescence intensities (collected at 1/s) for the same puncta/mitochondria treated with 0.1% DMSO before and after addition of 1 µM ABT-737 (in 0.1% DMSO) for 10 min are shown. (F) SDs of TMRE intensity measurements as in E; data are for 30 measurements for each of 12 puncta in six cells in two independent experiments and are similar to three additional experiments with protocol variations. Paired t test was used; *, P = 0.02. (G) SD of TMRE as in F, except 4 d after transfection with shRNA vector with scrambled (n = 10) or bcl-x-specific shRNAs (n = 17). Fluorescent images were taken every 3 s for 8 min. Paired t test was used; **, P = 0.00027. Data are presented as the mean ± SD.

Bcl-xL stabilizes the mitochondrial membrane potential to conserve energy

It is known that when any chemical system is not at thermodynamic equilibrium, as is the case for respiring mitochondria, the occurrence of persistent fluctuations or oscillations can only be maintained by expending energy (Nicolis and Prigogine, 1977). Moreover, the additional time-dependent flux of ions across the inner membrane that drives these fluctuations in potential can result in an overall ion flux (both inward and outward directions) that is greater than what is required simply to maintain a nonfluctuating membrane potential at a steady negative value. Thus, the fluctuations in mitochondrial membrane potential in bcl-x knockouts imply that more energy is required to maintain ion gradients across the inner membrane. To illustrate this concept, we constructed a simple numerical model to investigate the effect of fluctuations on the dissipation of ion gradients across the mitochondrial membrane. A vesicle (1 µm in diameter) was used to represent a mitochondrion (Fig. 5 A). This vesicle was equipped with active ion pumps (Fig. 5 A, b) capable of pumping out protons/ions (approximating the respiratory chain) to build a negative potential (−180 mV) and with ion channels (Fig. 5 A, a) that can partially dissipate this potential by allowing ions to reenter the vesicle (approximating the F1FO ATP synthase and nonproductive leaks). We first modeled steady-state conditions in which the inward flux and
outward flux of ions are exactly matched in time, and the membrane potential does not fluctuate in amplitude. These conditions approximate the steady-state conditions of mitochondria in wild-type cells. Next, we modeled fluctuations in membrane potential by introducing small currents across the vesicle membrane (Fig. 5 A, c). These small currents (set arbitrarily at 5 ms with a fixed amplitude between 0 and 10 pA) were allowed to occur randomly (averaging 1/s) to drive fluctuations in the potential across the vesicle membrane. To assess the effects of these external current amplitudes (Fig. 5 A, c), we measured the magnitude of total ion flux through the pumps (Fig. 5 A, a) and the channels (Fig. 5 A, b). In all cases, the total amount of ion flux (measured in picocoulombs) was increased when current fluctuations were introduced and was further increased with increasing external current amplitude (Fig. 5 B). The additional amount of ion movement (Fig. 5 A, a and b) produced by the small transient current fluctuations (Fig. 5 A, c) represents a futile dissipation of the ion gradient that has to be balanced by pump activity to restore the mean membrane potential. Thus, Bcl-xL could improve mitochondrial energetics simply by preventing futile ion flux.

Collectively, our results suggest the possibility that Bcl-xL regulates an inner mitochondrial membrane ion-conducting channel and that this channel has an increased probability of opening in the absence of Bcl-xL. This increased channel opening (analogous to point c in Fig. 5 A) could result in the increased membrane potential fluctuations observed in the bcl-x knockout. To investigate the effects of such a Bcl-xL-regulated channel on mitochondrial membrane potential fluctuations, we made a second computational model that more closely represents known properties of the inner mitochondrial membrane. This enabled us to test explicitly the effect of very brief transient openings of a nonselective inner membrane channel on the mitochondrial membrane potential measured over time. A vesicle (1.5 µm in diameter) was equipped with a proton pump and a proton leak pathway as in Fig. 5 A (a and b). The steady-state proton concentration of the mitochondrion was further regulated by a fixed proton buffer and a proton–cation exchange pathway (Garlid and Paucek, 2003). Finally, we introduced a nonselective cation channel representing the one regulated by Bcl-xL, which is permeable to both protons and to other cations and has a reversal potential of 0 mV (Lam et al., 1998; Vander Heiden et al., 2001; Alavian et al., 2011). Opening of the nonselective cation channel (mean open time of 0.33 ms) was allowed to occur stochastically with different opening probabilities of 0–0.1. We found that opening of the nonselective channel produced fluctuations in the membrane potential that increased with increased probability of channel opening (Fig. 5, C and D). The simulation further reveals that the very brief increases in internal proton concentration produced by influx through the channel resulted in proton pump activation, resulting in an overall hyperpolarization of the membrane as the frequency of channel openings increased (Fig. 5, C and D). This is consistent with transient hyperpolarization of mitochondria in bcl-x knockout cells as a result of overshooting by the respiratory chain after the channel opens.

Draining resources in bcl-x-deficient neurons

Our vesicle models predict that the increased membrane leakiness (productive and nonproductive ion flux) across the inner mitochondrial membrane in bcl-x-deficient neurons will result in decreased energetic performance. To test this prediction, cultured bcl-x cKO and control cortical neurons were energetically stressed by the addition of mitochondrial ATP synthase inhibitors and analyzed for ATP levels and for mitochondrial parameters by two-photon microscopy. Extensive genetic and

Figure 5. Bcl-xL stabilizes the mitochondrial membrane potential to conserve energy. (A) A simplified model of ion flux across the inner mitochondrial membrane. Ions may enter or leave the matrix through leakage channels at point a [e.g., the F1F0 ATP synthase through which protons enter the mitochondrial matrix], and overall stability and membrane potential are maintained by active pumps at point b (e.g., the ETC). The double arrow represents the source of fluctuation in potential. (B) Numerical simulations of the additional ion flux that occurs as a result of fluctuations in membrane potential using the 1-µm membrane vesicle model in A. An external perturbation with a fixed amplitude between 0 and 10 pA (5-ms duration) was allowed to occur randomly with a mean interval of 1 s, and the total ion flux was integrated over 20-s periods. The graph plots the increase in total integrated flux that results from fluctuations of increasing amplitude. (C) Numerical simulations of changes in mitochondrial inner membrane potential produced by stochastic opening of a nonselective cation channel. No openings occur when probability of channel opening (Po) equals 0, and the membrane is maintained at a steady level (−180 mV; dashed lines). Indicated opening rates of the channel produce fluctuations in membrane potential accompanied by net hyperpolarization (more negative potentials). (D) Mean ± SD of membrane potentials for traces in C.
In contrast to controls, bcl-x–deficient cortical neurons consistently underwent delayed mitochondrial depolarization 30–45 min after the addition of oligomycin (Fig. 6, C and E). Consistent with an energy-wasting crisis unique to bcl-x–deficient neurons, oligomycin also causes mitochondrial NAD(P)H levels to decline to <50% of pretreatment levels in <1 h, whereas NAD(P)H levels rebound and stabilize after oligomycin treatment in controls (Fig. 6 F). These results suggest that bcl-x–deficient mitochondria continue to deplete the substrate of complex I, as would be expected for a leaky mitochondrial membrane that allows the respiratory chain to continue running.

biochemical evidence indicates that oligomycin inhibits mitochondrial ATP synthesis by acting on F0, to disrupt the proton path (Walker and Dickson, 2006), and a crystal structure reveals that aurovertin B inhibits the enzymatic F1 subunit by binding near the ATP-binding site on β subunit (van Raaij et al., 1996). Treatment with oligomycin or with aurovertin B caused cellular ATP levels to decline similarly in control and knockout neurons (Fig. 6, A and B). Therefore, the F1F0 ATP synthase was an important contributor to ATP production and concomitant dissipation of membrane potential in both genotypes before treatment.
Bcl-xL requires the β subunit for antideath activity in yeast

To test whether Bcl-xL increases cell survival through a functional interaction with F₁F₀ ATP synthase and independently of any other Bcl-2 family members, we tested the ability of human Bcl-xL to inhibit cell death of yeast lacking β subunit (Δatp2) of the F₁F₀ ATP synthase. Using a novel heat ramp cell death assay (Teng et al., 2011), we found that Bcl-xL failed to protect yeast in which the ATP2 gene was deleted. In contrast, Bcl-xL protected yeast with mutations in both the mitochondrial fission protein FIS1 and WHI2, which lack mitochondrial fission and have respiratory function defects (Δfis1; Figs. 7 C and S5; Fannjiang et al., 2004; Cheng et al., 2008). Yeast have no recognizable Bcl-2 family members or BH3-only proteins yet have a highly conserved F₁F₀ ATP synthase. Our results indicate that Bcl-xL promotes cell survival through an interaction with the ATP synthase.

Discussion

Our evidence indicates that endogenous Bcl-xL prevents a futile ion flux across the mitochondrial inner membrane, thereby preventing pronounced irregular fluctuations in mitochondrial membrane potential observed in bcl-x knockout cells. The additional energy required for fueling excessive ion flux across the mitochondrial membrane would place bcl-x-deficient cells at a distinct disadvantage during cell stress. Unable to sustain a potential across a more leaky inner membrane, bcl-x-deficient mitochondria depolarize and subsequently die. Prominent localization of endogenous Bcl-xL with the inner mitochondrial membrane is consistent with a close link between Bcl-xL and the membrane leak channels. Copurification of Bcl-xL with the F₁F₀ ATP synthase raises the possibility that a novel leak channel could be within the ATP synthase itself or a functionally interacting component. This function of Bcl-xL can be expected to alter many other aspects of mitochondrial and cellular physiology, though, like many mitochondrial proteins, the mechanism by which Bcl-xL enters mitochondria is not known.

Consistent with this conclusion, rates of oxygen uptake by cells decrease with overexpression of Bcl-xL and increase with shRNA knockdown of Bcl-xL (Alavian et al., 2011). NAD(P)H depletion and membrane depolarization were not simply a result of inhibition of mitochondrial ATP synthesis because NAD(P)H levels and membrane potential were sustained for at least 1 h after aurovertin B treatment, although at lower steady-state levels relative to controls (see Discussion; Fig. 6 G). To verify that depletion of NAD(P)H and mitochondrial depolarization is not simply a marker of cell death, oligomycin was washed away from depolarized bcl-x–deficient neurons in a flow chamber. Upon washout, we observed simultaneous increases in NAD(P)H levels and TMRM intensity, indicating cell recovery (Fig. 7 A). The evidence presented suggests that Bcl-xL increases the efficiency of mitochondrial energetics by decreasing inner membrane leakiness, thereby preventing membrane potential fluctuations and the resulting energy deficits (Fig. 7 B).

Bcl-xL inhibits mitochondrial membrane leakiness

These findings are consistent with a conceptually simple mechanism in which Bcl-xL acts at the inner mitochondrial membrane to close a molecularly undefined leak, thereby preventing large swings in membrane potential. This could be achieved if Bcl-xL directly closes the leak channel. Our computational models predict that opening of this Bcl-xL–inhibited leak channel results in membrane potential fluctuations, which is consistent with fluctuations observed in bcl-x–deficient cells. The models further predict that the large fluctuations are a result of transient overcompensations by the respiratory chain, which is consistent with the observed transient hyperpolarizations in bcl-x–deficient cells. Our numerical simulations also suggest that the greater total flux of ions across the mitochondrial inner membrane in bcl-x–deficient mitochondria requires more energy to maintain ionic homeostasis, analogous to other fluctuating or oscillating biochemical systems (Kaczmarek, 1976). Even if the additional ion flux in bcl-x–deficient mitochondria was coupled to ATP synthesis by F₁F₀, additional energy would be required to move ions out of the matrix when the potential is fluctuating compared with a steady state with little or no fluctuations. Our simulations are consistent with the notion that the stabilizing effect of Bcl-xL on inner mitochondrial membrane potential...
contributes importantly to the efficiency of energy production. An expected consequence of excess ion flux is that a sudden deficit in nutrients or a sudden increase in energy demand cannot be readily satisfied by an inefficient system. 

Seemingly contrary to these findings, recombinant Bcl-xL can induce ion channel activity in outer mitochondrial membranes and synthetic bilayers, although these channels are smaller than those formed by the related proapoptotic Bax protein (Lam et al., 1998; Basañez et al., 2002). Thus, the ion-conducting activity of Bel-xL may not be related to the Bcl-xL functions under study here. However, more complex scenarios remain possible where Bcl-xL channels open to correct other ion-conducting activities in the inner membrane, thereby preventing large swings in potential. Though the detailed molecular events remain unclear, our conclusions are supported by patch clamp recordings of mitochondrial inner membrane vesicles with Bel-xL (Alavian et al., 2011). It is conceivable that the Bel-xL–inhibited mitochondrial leak channel is related to the Bax pores that trigger apoptosis, except Bax pores are not known to occur in inner membranes (Bilien et al., 2008). The capacity of a non–Bax/Bak-binding mutant of Bel-xL (mut1) to interact with the F$_{1}$β subunit and to inhibit cell death in mammalian cells argues against this possibility. Furthermore, Bel-xL can inhibit cell death in wild-type yeast, which lack Bcl-2 family and BH3-only proteins, but Bel-xL cannot protect yeast lacking F$_{1}$β, which shares 89% amino acid sequence homology with humans. The inner membrane function and the antideath function of Bel-xL appear to be separable biochemical events (e.g., Fig. 7 A). However, yet unknown nonapoptotic activities of Bax/Bak could be involved, potentially those that regulate neuronal activity or inhibit neuronal cell death in vivo (Lewis et al., 1999; Fannjiang et al., 2003). Furthermore, Bel-xL was recently reported to regulate acetyl-CoA levels in a Bax/Bak-independent manner (Yi et al., 2011).

Given the unexpected finding that mitochondrial ROS levels in bcl-x–deficient cells are lower than controls, our data are not consistent with Bcl-xL–mediated leak closure serving to reduce ROS levels (Jastroch et al., 2010). To the contrary, the leakier/fluctuating membrane potential could be expected to increase oxygen consumption in bcl-x–deficient cells, which is consistent with reduced oxygen consumption in Bel-xL–overexpressing cells (Alavian et al., 2011).

Implications for the F$_{1}$F$_{0}$ ATP synthase

Because Bcl-xL is not found in the 3D structures of F$_{1}$F$_{0}$ ATP synthase, we consider other potential functions for partnering. Given a structural resemblance between Bel-xL and Diphtheria toxin, a polypeptide translocator, we considered that Bel-xL could interact with the F$_{1}$β subunit and to inhibit cell death in mammalian cells argues against this possibility. Furthermore, Bel-xL can inhibit cell death in wild-type yeast, which lack Bcl-2 family and BH3-only proteins, but Bel-xL cannot protect yeast lacking F$_{1}$β, which shares 89% amino acid sequence homology with humans. The inner membrane function and the antideath function of Bel-xL appear to be separable biochemical events (e.g., Fig. 7 A). However, yet unknown nonapoptotic activities of Bax/Bak could be involved, potentially those that regulate neuronal activity or inhibit neuronal cell death in vivo (Lewis et al., 1999; Fannjiang et al., 2003). Furthermore, Bel-xL was recently reported to regulate acetyl-CoA levels in a Bax/Bak-independent manner (Yi et al., 2011).

Primary cortical neuron cultures

Conditional bel-x knockout cortical neuron cultures were prepared separately from individual E16.5 mouse embryos as previously described (Berman et al., 2009). Wild-type and floxed bcl-x mice/embryos were distinguished using PCR primers 5′-GCACTCTCATGCTGGGG-3′ and 5′-TCAGAAGGCCGCAAATCCC-3′. The CRE-luciferase fusion gene was identified with primers 5′-TCTTTTTCATGTGCTCTTGG-3′ and 5′-CCGATACGCGTCTGCTGAGG-3′. All experiments were performed on density-matched cultures. All animal procedures were approved by the Animal Care and Use Committee.

Two-photon laser-scanning microscopy

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prepared as previously described for subcellular fractionation and were further washed with mannitol sucrose (MS) buffer (without EGTA). Rates of oxygen consumption by purified mitochondria (0.5 mg/ml) were measured with a Clark-type oxygen electrode (Hansatech Instruments Limited) in KCL buffer (125 mM KCl, 20 mM Trizma base, 2 mM potassium phosphate, and 1 mM MgCl₂, pH 7.2) plus substrates (Fig. S1A), 1 mM MgCl₂, and 0.25 mM EGTA and were calculated in nanomoles of O₂ per mg protein per minute based on a KCl medium oxygen content of 195 nmol/mM O₂ at 30°C.

Crude subcellular fractionation
Cerebral cortices from P3 mice were rapidly dissected, minced on ice in 2 ml MS-EGTA buffer (225 mM mannitol, 75 mM sucrose, 250 µM EGTA, 1 mg/ml fatty acid–free BSA, and 5 mM Hepes, pH 7.4), and homogenized with 15 strokes in a 2 ml Dounce. The cortical suspension was clarified at (15,000 g for 8 min), gently resuspended in MS-EGTA, and recentrifuged (for 10 min). The pellet was lysed in 100 µl MS buffer plus 1% NP-40, and the supernatant was centrifuged at 100,000 g for 30 min to clarify the cytosolic fraction.

Immunoblot analyses
Mouse cerebral cortex was dissected on ice and passed 15 times through a 25-gauge needle in 3 vol of radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, 1% NP-40, 150 mM NaCl, and 1 mM EDTA, pH 7.4) plus 1 mM NaF, 1 mM Na₃VO₄, (sodium orthovanadate and phosphatase inhibitor), and protease inhibitors (PMSF, leupeptin, pepstatin, and aprotinin). Cultured cortical neurons were washed quickly with Locke’s buffer and lysed in 150 µl of RIPA buffer. 500 µg lysates were separated by SDS-PAGE and blotted with specific antibodies (see figure legends).

Immu-EM
Immuno-gold labeling of ultrathin cryosections was performed as previously described (Macleffery and Farquhar, 1995). Tissues from both genotypes were fixed in 4% PFA diluted in PBS, pH 7.4, for 15 min followed by 8% PFA for 1 h at RT. Samples were cryoprotected in 2.3 M sucrose plus 20% polyethylene glycol [400 cps] and then frozen in liquid N₂. 80-nm ultrathin cryosections cut on a microtome (Ultracut T; Reichert) were immunostained with 1:2,000 anti-pan-Bcl-xL antibody (Li et al., 2008). Mature (DIV14–16) cultures were incubated at 37°C with 1% CHAPS and clarified by centrifugation (at 31,000 g for 15 min). The supernatant (0.4 ml) was loaded onto a 38-ml Superdex 200 gel filtration column (GE Healthcare) previously equilibrated with 150 mM NaCl, 20 mM Tris (pH 7.4), and 1 mM NaF for 30 min. The column was eluted with 1 ml of radioimmunoassay (RIA) lysis buffer (125 mM KCl, 20 mM Tris, 2 mM potassium phosphate, and 1 mM MgCl₂, pH 7.2) plus substrates (Fig. S1A) and 1 mM MgCl₂, and 0.25 mM EGTA and were calculated in nanomoles of O₂ per mg protein per minute based on a KCl medium oxygen content of 195 nmol/mM O₂ at 30°C.

Biochemical purification of Bcl-xL-binding partners
WEHI 7.1 cells (~20 ml packed pellet) were lysed in 200 ml of hypotonic buffer (37.5 mM NaCl and 10 mM Hepes, pH 7.4) plus 25 µg/ml PMSF using a Dounce homogenizer. The membrane pellet (at 23,000 g for 30 min) was solubilized in 300 ml of isonicotin buffer (150 mM NaCl and 10 mM Hepes, pH 7.4) plus 1% CHAPS and was clarified by centrifugation (for 15 min at 15,000 g). The supernatant was loaded onto a 10-ml trimethylaminoethyl anion exchange column and washed with 10 column volumes of isonicotin buffer with 0.5% CHAPS, and bound proteins were eluted with a salt gradient (Bcl-xL eluted at 0.35 M NaCl). Bcl-xL-containing fractions were immunoadfinity purified with 1 ml anti–murine Bcl-xL antibody (7D9 [Hsu et al., 2003] bound to beads (2 mg antibody/ml Sepharose 4B beads) for 3 h at 4°C). Beads were washed with isonicotin buffer with 0.5% CHAPS, and Bcl-xL–containing complexes were eluted with 3 ml of 0.1 M acetic acid + 0.3% CHAPS. The sample was neutralized with 0.4 ml of 1 M Tris (pH 8.0), concentrated (Centricon-30), and separated by preparative SDS-PAGE.

Gel filtration of membrane-associated Bcl-xL
10⁵ Hela cells stably expressing Bcl-xL (Hsu et al., 2003) were lysed by Dounce homogenization in hypotonic buffer. The membrane pellet (at 31,000 g for 30 min) was solubilized in 3 ml of isonicotin buffer plus 1% CHAPS and clarified by centrifugation (at 31,000 g for 15 min). The supernatant (0.4 ml) was loaded onto a 38-ml Superdex 200 gel filtration column (GE Healthcare) previously equilibrated with 150 mM NaCl, 20 mM Tris (pH 7.4), and 1 mM NaF, and 24 kD chymotrypsinogen. 0.4-ml column fractions containing Bcl-xL were identified by immunoblot analysis with monoclonal 2H12 (Hsu and Youle, 1997).

TMRE fluctuation in hippocampal neurons
Dissected rat hippocampal neurons were prepared from 18 embryos and plated on polylysine–coated dishes in Neurobasal medium with B27 (Invitrogen; Li et al., 2008). Mature (DIV14–16) cultures were incubated at 37°C in recording buffer (5 mM KCl, 110 mM NaCl, 2 mM MgCl₂, 10 mM glucose, 10 mM Hepes, 2 mM CaCl₂, pH 7.4, and 310 mOsmin) containing TMRE (5 mM final). Individual puncta containing mitochondria at the base of a dendrite near the soma were outlined and measured by averaging 4 x 4 pixels as previously described (Li et al., 2008). Fluorescent images were collected (1/s for 30 s) with fixed exposure times (300 ms) using an inverted microscope (Axiovert 200; Carl Zeiss) with a 63× oil objective. Background fluorescence was subtracted for each image, and data were analyzed using AxioVision software (version 4.3; Carl Zeiss). For analysis of SDs, a straight baseline was subtracted from each graphed line using Origin (version 8.0 software to eliminate any artifacts due to slight argon movement during imaging.

Calcium measurement
Mouse cortical neurons (DIV3–5) grown on 15-mm coverslips were loaded with 2 µM cell-permeable Fura-2 acetoxymethyl ester (Invitrogen) for 30 min, washed with culture medium, and incubated at 37°C for 20–30 min to allow complete hydrolysis of acetoxymethyl ester. Coverslips were mounted on a AutoFluor system (Carl Zeiss) and continuously infused with Locke’s buffer. Cells were sequentially excited at 340 nm/380 nm, and fluorescence intensities (510 nm) were determined for individual neurons from images captured at ~4-s intervals. The 340:380 ratios were converted to nanomoles of Ca²⁺ using a video imaging system (Intracellular Imaging Inc.) and commercial reference standards (Invitrogen) by the formula [Ca²⁺] = K_d ([Rmin] – [R]) / [Rmax] – [R]) ([Fmax/Fmin], where R equals the ratio of 510-nm emission intensities excited at 340 nm relative to 380 nm, Rmin equals the ratio at zero free Ca²⁺, Rmax equals the ratio at saturating Ca²⁺ (39 pM), Fmax equals the fluorescence intensity excited at 380 nm for zero free Ca²⁺, and Fmin equals the fluorescence intensity excited at 380 nm in saturating Ca²⁺.
Numerical simulations
To estimate the flux of ions in the model vesicle (Fig. 5 A), we integrated the equation $dH/dt = \lambda_f - \lambda_i$, where $V$ is the membrane potential across the vesicle, $C$ represents the capacitance of the vesicle, $\lambda_f$ is the ionic current flowing through the channel in the membrane, and $\lambda_i$ is an additional fluctuating current that is applied across the membrane. $\lambda_f$ was defined by the equation $\lambda_f = g_i (V - E_i)$, where $g_i$ is the conductance of the membrane and, under steady-state conditions, provides a membrane potential of $-180$ mV ($V_i = -180$ mV). $\lambda_i$ was either fixed at 0 or was allowed to fluctuate from 0 to a value $i_{max}$ for 5 mS periods. These fluctuations occurred randomly with a mean period of 1 s. In different simulations, the value of $i_{max}$ was increased from 0.01 to 10 pA. Equations were integrated for a time span of 20 s, and the ionic flux for the entire period was calculated in picocoulombs. Activity of pumps was not simulated explicitly in these models but was incorporated implicitly because the reversal potential for ion flux ($V_i = -180$ mV) was held fixed during the simulations. Parameters for the simulations were $C = 0.314$ picofarads and $g_i = 3.14$ picosiemens.

For the second model, we tested the effects of a nonselective cation channel on the membrane potential across the inner mitochondrial membrane. For simplicity, we included a fixed proton buffer and an electroneutral proton–cation (K+) exchange pathway (Garlid and Paucek, 2003) such that, in the absence of any other channel activity, this model has a steady-state internal proton concentration of 20 nM (pH 7.7) and a membrane potential of $-180$ mV, as is typical for many mitochondria.

We integrated the following pair of coupled stochastic differential equations:

$$\frac{dH}{dt} = \lambda_f (g_i + \lambda (1g_{CHAN}) - gH) \frac{H_{out} - V}{-k_i}{\lambda_i}(200 + V)} + k_i (H_{out} - H_i)$$

$$\frac{dV}{dt} = \frac{1}{C} \left( -\lambda_f g_{CHAN} + gH \frac{H_{out} - V}{-k_i}{\lambda_i}(200 + V)} + k_i (H_{out} - H_i) \right)$$

$H$, the total bound and unbound concentration of protons in the vesicle, and $V$ is the voltage across the membrane. $H_{out}$ is the H$^+$ concentration outside the vesicle and was fixed at 40 nM, whereas $H_i$, the concentration of free protons in the vesicle, was related to the value of $H$ and to $B$, the concentration of H$^+$ buffer in the vesicle, by the following quadratic equation:

$$H_i = -K_{eq} - B - H_{out} + \frac{k_i (B - H_{out})^2 + 4K_{eq}H_{out}}{2}$$

Here, $B = 5 \times 10^{-3}$ mM, and $K_{eq} = 2.3 \times 10^{-5}$ mM.

The constant $\lambda_f$ was equal to $[1.036 \times 10^{-5}]/W$, where $W$ is the volume of the vesicle $[1.767 \text{ mm}^3]$. The capacitance of the vesicle C was $5 \times 10^{-9}$ nanofarads. The values for $g_i$ (the basal proton leak), $gH$ (the basal leak of cations), and $g_{CHAN}$ (the conductance of the nonselective cation channel) were 5, 5.55 10$^{-6}$, and 5 $\times 10^{-6}$ picosiemens, respectively. The values for $k_i$ (the rate constant for H$^+$ pumping out of the vesicle) and $k_f$ (the rate of electroneutral cation/H$^+$ exchange) were 2.5 and 5 $\times 10^{-1}$, respectively.

[If it is a stochastic function that takes on the value of 1 or 0 depending on whether the nonselective cation channel is open or closed. The open probability, $P_o$, of the channel was determined by the rate constants for channel opening ($k_f$) and closing ($k_b$) and was given by the following relation:

$$P_o = \frac{k_f}{k_i + k_b}$$

The value of $k_f$ was fixed at 0.3 ms$^{-1}$, which provided a mean open time of 3.33 ms. In the simulations of Fig. 5 C and D, the values of $k_f$ were set at 0, 0.0005, 0.00101, and 0.0033 $\times 10^{-5}$, providing mean open probabilities of 0, 0.0017, 0.323, and 0.1004.

ATP measurements
Cortical cultures were harvested as for immunoblot analysis plus a phosphatase inhibitor; mouse cortex lysates were supplemented with 50 mM atracyloside. Samples were analyzed immediately, or time points were frozen instantly and analyzed together. Protein concentration/sample (BCA assay) and fresh ATP standards [0, 25 nM, 5 nM, 500 nM, 50 nM, and 5 nM] were used to calibrate every experiment.

Yeast cell death assay
Overnight cultures of yeast strains [MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, and yfg1Δ, KanMX4, Invitrogen] transformed with modified pRS-PGK vector without/with human Bcl-xL were diluted and grown to midlog phase (synthetic complete–uracil medium) and plated before and after a heat ramp treatment to trigger cell death (30°C–4°C in 2 min, 40–51°C in 10 min, and held at 51°C for 5 min; Teng et al., 2011). Both the ATP2 and FIS1Δ knockout strains are more sensitive to cell death than wild type, in which Bcl-xL also protects (Fannjiang et al., 2004). For immunoblot analyses lysates were prepared from overnight cultures in lysis buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% NP-40, and 0.1 M PMSF) with glass beads and blotted with anti–Bcl-xL (1:5,000 rabbit monoclonal) and anti–rabbit IgG (1:20,000; GE Healthcare).

Online supplemental material
Fig. S1 shows that no respiratory defects were detected in bcl-x-deficient mitochondria. Fig. S2 shows coimmunogold EM for Bcl-xL and the F1 subunit. Fig. S5 shows protease digestion of mitochondria detected with Bcl-xL antibody. Fig. S4 shows an example of TMRE traces and Bcl-xL blots for shRNA knockdowns in Fig. 4 G. Fig. S3 shows expression levels of Bcl-xL protein in yeast. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201108059/DC1.

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