Bcl-x<sub>L</sub> regulates mitochondrial energetics by stabilizing the inner membrane potential

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Mammalian Bcl-x<sub>L</sub> 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-x<sub>L</sub> also localized to inner mitochondrial cristae. Two-photon microscopy of cultured neurons revealed large fluctuations in inner mitochondrial membrane potential when Bcl-x<sub>L</sub> was genetically deleted or pharmacologically inhibited, indicating increased total ion flux into and out of mitochondria. Computational, biochemical, and genetic evidence indicated that Bcl-x<sub>L</sub> 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<sub>1</sub>F<sub>0</sub>-ATP synthase directly affects mitochondrial membrane potential and having identified the mitochondrial ATP synthase β subunit in a screen for Bcl-x<sub>L</sub>-binding partners, we tested and found that Bcl-x<sub>L</sub> failed to protect β subunit–deficient yeast. Thus, by bolstering mitochondrial energetic capacity, Bcl-x<sub>L</sub> may contribute importantly to cell survival independently of other Bcl-2 family proteins.

Introduction

Bcl-x<sub>L</sub> 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 (Galunek and Youle, 2006; Youle and Strasser, 2008). The best-characterized cell survival activity of Bcl-x<sub>L</sub> is its ability to inhibit Bax-induced pores in the outer mitochondrial membrane (Billen et al., 2008). In this manner, Bcl-x<sub>L</sub> prevents release of mitochondrial cytochrome c into the cytoplasm, where cytochrome c induces apoptosome formation to trigger caspase-dependent death of mammalian cells. Attention has been focused on the functional interactions and the binding specificities between anti- and proapoptotic Bcl-2–related proteins, leading to new therapeutic strategies (Oltersdorf et al., 2005).

The evolutionary conservation of Bcl-2–like proteins cannot be uniformly linked to apoptosis regulation (for example, the Bcl-2 homologues of Drosophila melanogaster and viruses; Bellows et al., 2002; Graham et al., 2008; Galindo et al., 2009). Many other binding partners have been reported for human Bcl-x<sub>L</sub>, linking Bcl-x<sub>L</sub> to other cellular processes including mitochondrial dynamics, energetics, and autophagy.

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brane drives rotation of the central stalk against the catalytic
(oligomycin-sensitive fraction) embedded in the inner mem-
2006). In this manner, proton flux through F O is coupled to
by glycolysis, reversing the flow of protons through F O to
fusion (Karbowski et al., 2006; Berman et al., 2009; Montessuit
mals and worms is regulation of mitochondrial fission and
2008). Thus, Bcl-2 proteins may have alternative biochemi-
cal 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 mam-
mals and worms is regulation of mitochondrial fission and
fusion (Karbowski et al., 2006; Berman et al., 2009; Montessuit
et al., 2010; Hoppins et al., 2011). This role appears to con-
tribute importantly to Bcl-xL–induced mitochondrial localiza-
tion at neuronal synapses, neuronal activity, and seizure behaviors (Fannjiang et al., 2003; Li et al., 2008). However, regu-
lation of fission and fusion rates is not sufficient to ex-
plain the ability of endogenous and overexpressed Bcl-xL to
increase mitochondrial biomass (Berman et al., 2009). There-
fore, 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; Belzacq 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 phos-
phate 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 trans-
port chain (ETC; or respiratory chain) fueled by NADH. Re-
entry of protons into the mitochondrial matrix via the F0 ring
(oligomycin-sensitive fraction) embedded in the inner mem-
brane drives rotation of the central stalk against the catalytic
F1, a ring of three α and three β 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 mito-
chondrial 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 mito-
chondrial morphology changes, the details are complex (Benard
and Rossignol, 2008).

By analyzing bcl-xL–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 cur-
rent 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
is consistent with the limited reserve capacity of bcl-xL–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-xL-
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-xL conditional knockout (cKO) cortical neuron cultures (Berman et al., 2009). Both unfloxed and bcl-xL–floxed litter-
mates express neuron-specific knockin NEX-Cre recombin-
ase starting around embryonic day 12 (E12) to delete bcl-xL.
Staining for Cre recombinase serves as a positive marker for
the survival of bcl-xL–deficient (and control unfloxed) cortical
neurons (Fig. 1 A; Berman et al., 2009). Mitochondrial membrane potential (∆Ψm) 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-xL knockout cortical neurons (Fig. 1, B and C [left]). This
is not a result of increased mitochondrial biomass because
bcl-xL–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-xL 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 ∆Ψm: (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-xL knockout neu-
rons, reactive oxygen species (ROS) production by the ETC
was assessed in the same mitochondrial areas where TMRM
was evaluated. Unexpectedly, bcl-xL knockout neurons have
lower mitochondrial ROS. This suggests either a high rate
of electron flow through the respiratory chain or that bcl-xL
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-xL 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 indi-
cate 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 con-
sumption in isolated brain mitochondria (Fig. S1, A and B).

The relative contributions of glycolysis versus the mitochon-
drial F1F0 ATP synthase to the levels of total cellular ATP
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-x 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 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 anti-apoptotic 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...
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 immunooaffinity 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 (White et al., 2005), we investigated a role for calcium in mitochondrial membrane potential fluctuation. We found that basal cytosolic calcium levels were uniformly steady 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
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 F_{1}F_{0} 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 mitochondria 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, hippocampal neurons expressing scrambled or bcl-x–specific short hairpin RNA (shRNA) were monitored for TMRE fluorescence intensity in time (Fig. S4), and the SD of fluorescence intensities was significantly greater for the bcl-x knockdown than the control (Fig. 4 G). These data again suggest that Bcl-xL stabilizes the mitochondrial membrane potential by limiting total ion flux across the mitochondrial membrane.

Bcl-xL stabilizes the mitochondrial membrane potential to conserve energy

Figure 4. Bcl-xL–deficient mitochondria have fluctuating membrane potentials. (A) Continuous recordings (3.5-s intervals) of TMRE intensities per ROI of individual cortical neurons for at least 250 s (DIV4–5; Fig. 1). Traces are representative of multiple neurons in three independent experiments. (B) Fluorescence intensity (arbitrary units [a.u.]) of TMRE stain per pixel was determined for the marked mitochondria-rich region of a single neuron in bcl-x–deficient (cKO) and control (Cont) cultures shown in Fig. 1 C. (C) SDs were calculated for mean TMRE fluorescence intensities per pixel for 200 individual mitochondria derived from 20 different cells per genotype monitored every 2.5 s for at least 90 s. Each symbol represents one mitochondrion. An F-test for variance comparing control and bcl-x-deficient neuronal mitochondria was performed; P < 0.0001. (D) Continuous recordings of intracellular calcium levels at 4-s intervals in DIV3–4 cortical neurons. Initial intracellular calcium levels from four independent experiments are graphed. *, P = 0.039. (E) Mitochondrial membrane potential fluctuation increases with 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 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. (F and G) Data are presented as the mean ± SD.
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. 5A, 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. 5A, c), we measured the magnitude of total ion flux through the pumps (Fig. 5A, a) and the channels (Fig. 5A, 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. 5B). The additional amount of ion movement (Fig. 5A, a and b) produced by the small transient current fluctuations (Fig. 5A, 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. 5A) 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. 5A (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-xL-deficient neurons**

Our vesicle models predict that the increased membrane leakiness (productive and nonproductive ion flux) across the inner mitochondrial membrane in bcl-xL-deficient neurons will result in decreased energetic performance. To test this prediction, cultured bcl-xL 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
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 $\approx 50\%$ 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 F$_0$, to disrupt the proton path (Walker and Dickson, 2006), and a crystal structure reveals that aurovertin B inhibits the enzymatic F$_1$ subunit by binding near the ATP-binding site on F$_1$ 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 F$_1$/F$_0$ ATP synthase was an important contributor to ATP production and concomitant dissipation of membrane potential in both genotypes before treatment.
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-xL–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 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.

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 negative 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 Bac protein (Lam et al., 1998; Basañez et al., 2002). Thus, the ion-conducting activity of Bcl-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 Bcl-xL (Alavian et al., 2011). It is conceivable that the Bcl-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 (Billen et al., 2008). The capacity of a non-Bax/Bak-binding mutant of Bcl-xL (mt1) to interact with the F1β subunit and to inhibit cell death in mammalian cells argues against this possibility. Furthermore, Bcl-xL can inhibit cell death in wild-type yeast, which lack Bcl-2 family and BH3-only proteins, but Bcl-xL cannot protect yeast lacking F1β, which shares 89% amino acid sequence homology with humans. The inner membrane function and the antideath function of Bcl-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, Bcl-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 closing 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 Bcl-xL–overexpressing cells (Alavian et al., 2011).

Implications for the F1F0 ATP synthase
Because Bcl-xL is not found in the 3D structures of F1F0 ATP synthase, we considered other potential functions for partnering. Given a structural resemblance between Bcl-xL and Diphtheria toxin, a polypeptide translocator, we considered that Bcl-xL could facilitate entry of the β subunit into mitochondria. However, we found that bcl-x–deficient mitochondria appear to have normal levels of β subunit. Bcl-xL could interact with fully assembled ATP synthase or participate in the assembly process, which requires many factors not present in active complexes (Rak et al., 2011). Alternatively, the effects of Bcl-xL on membrane curvature could influence the ATP synthase (Basañez et al., 2002; Paumard et al., 2002).

Our biochemical and pharmacological analyses are consistent with a Bcl-xL–regulated leak mechanism involving the F1F0 ATP synthase. However, the differential effects of two F1F0 inhibitors are somewhat puzzling. Both aurovertin B (acting on F1) and oligomycin (acting on F0) block ATPase/synthase activity and proton movement through F1, because of the coupling between F1 and F0. Yet, only aurovertin B inhibited mitochondrial depolarization in bcl–x–deficient cells, possibly by triggering closure of the leak channel by binding the β subunit (van Raaij et al., 1996). In contrast, the Bcl-xL–regulated leak channel appears to be oligomycin resistant, though it is not clear whether this is the long-sought-after leak channel that explains continued mitochondrial respiration with oligomycin treatment (Nicholls and Ferguson, 2002). We speculate that the F1F0 ATP synthase is involved in leaking ions and that the regulation of this function is defective in bcl–x–deficient neurons. These studies further extend the long-standing link between Bcl-2 family proteins and the ATP synthase (Matsuyama et al., 1998; Vander Heiden et al., 2001; Belzacq et al., 2003).

Materials and methods

Primary cortical neuron cultures
Conditional bcl-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′-GCCACCTCATACGTGGG-3′ and 5′-TCAGAAGCCGCAATCCC-3′. The NEX-CRE locus was identified with primers 5′-CTTTTCATGCTGCTGGG-3′ and 5′-GCCGATAACC-AGTGAACACG-3′, and the wild-type allele was identified with 5′-CAAGTGTCCCTGGAAGAGC-3′ and 5′-GATACAGACAG-AGGGAGGG-3′. All experiments were performed on density-matched cultures. All animal procedures were approved by the Animal Care and Use Committee. For immunofluorescence microscopy, cortical neuron cultures were quickly washed with Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 1.0 mM MgCl2, 5 mM Heps, and 10 mM glucose, pH 7.4), fixed for 15 min in 4% PFA, permeabilized for 5 min with 0.2% Triton X-100, blocked for 30 min at RT with 5% normal goat serum, and incubated with primary antibodies at 4°C overnight followed by 1 h at RT with secondary antibodies.

Two-photon laser-scanning microscopy
Potentiometric dye TMRM, which accumulates in the matrix according to its Nernst potential, was used at 100 nM [the lowest workable concentration; nongenoch mod verified with carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone] to monitor mitochondrial membrane potential ΔΨm. Accumulation of ROS was monitored simultaneously with 2 μM CM-H2DCFDA (5′,6′-chloromethyl-2′,7′-dichlorofluorescein diacetate). Fluorescence probes were loaded into cortical neuron cultures (3–5 days in vitro [DIV3–5]) for at least 20 min, and images were recorded using a two-photon laser-scanning microscope (MRC-1024MP; Bio-Rad Laboratories) with an excitation at 740 nm (Tsunami Ti:Sa laser; Spectra-Physics) to measure fluorescence intensity of TMRM (605 ± 25 nm), CM-DCF (525 ± 25 nm), and intrinsic autofluorescence of endogenous NADH/NADPH (<490 nm; Aon et al., 2003). For single recordings, three to five fields per culture dish were imaged in immediate succession. For time-lapse recordings, images of the same field were captured every 3.5 s for up to 5 min using 50% laser intensity to limit photo damage to live samples. Region of interests (ROIs) were drawn and analyzed using ImageJ (National Institutes of Health) for all neurons per image. Mean fluorescence intensity per pixel in each ROI at the three emission wavelengths was calculated as arbitrary fluorescence units. Background from cell-free areas was subtracted for each wavelength. Photodamage-induced fluctuations specifically in knockout cells is unlikely, as fluctuations are evident at the earliest time points and with single-photon microscopes (Fig. 4), and treatment with antioxidant N-acetyl cysteine does not inhibit depolarization.

Mitochondrial respiration
Mouse forebrain mitochondria were isolated from littermates of control and cKO mice (postnatal day 2–7 [P2–P7]) by modification of a standard protocol (Rosenthal et al., 1987). Mitochondria [primarily nonsynaptosomal] were

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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. S1 A), 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 cortexes 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 collected by centrifugation (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 Na2VO4 (sodium orthovanadate and phosphate inhibitors), and 10 mM HEPES. Total homogenates were solubilized with 0.5% Triton X-100, and 10% SDS-PAGE and blotting were verified with antibody against ATP synthase [Subunit (BD) and/or anti–Bcl-xL/S antibody, each at 1:2,000; Santa Cruz Biotechnology, Inc.), and anti–ATP synthase subunit (clone 7H8.2C12, 1:1,000; Thermo Fisher Scientific), anti–Tom20 (sc-11415, 1:2,000; Santa Cruz Biotechnology, Inc.), and anti–ATP synthase subunit (1:1,000). The ATP synthase fractions used were as previously described (Ko et al., 2003) and were solubilized in lithium dodecyl sulfate for separation by SDS-PAGE.

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 isonicotinic acid (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 isonicotinic 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 immunopurified with 1 ml anti–murine Bcl-xL antibody, 7D9 (1:10,000; Hsu et al., 2003) bound to beads (2 mg antibody/ml Sepharose 4B, 20 mg/ml BSA, 0.1% Triton X-100, 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 hippocampal neurons were prepared from E18 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 mOsM) 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 63x 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 Fluo-2 acetoxymethyl ester at 37°C 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 MetaFlour 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 Ca2+ by using a video imaging system (Intracellular Imaging Inc.) and commercial reference standards (Invitrogen) by the formula

\[
\frac{[Ca^{2+}]}{R_{\text{calc}} - R_{\text{min}}} = \frac{R_{\text{max}} - R_{\text{calc}}}{R_{\text{max}} - R_{\text{min}}} = \frac{F_{\text{max}}}{F_{\text{min}}} - \frac{F_{\text{calc}}}{F_{\text{calc}} - F_{\text{min}}}
\]

where R equals the ratio of 510-nm emission intensities excited at 340 nm relative to 380 nm, Rcalc equals the ratio at zero free Ca2+, Rmin equals the ratio at saturating Ca2+ (39 µM), 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+.

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Numerical simulations
To estimate the flux of ions in the model vesicle (Fig. 5 A), we integrated the equation $\frac{dV}{dt} = I_b + I_{\text{out}}$, where $V$ is the membrane potential across the vesicle, $I_b$ represents the capacitance of the vesicle, $I_{\text{out}}$ is the ionic current flowing through the channel in the membrane, and $I_{\text{in}}$ is an additional fluctuating current that is applied across the membrane. $I_{\text{out}}$ was defined by the equation $I_{\text{out}} = g_i(V_{\text{m}} - V)$, where $g_i$ is the conductance of the membrane and, under steady-state conditions, provides a membrane potential of $-180$ mV ($V_{\text{m}} = -180$ mV). $I_{\text{in}}$ was either fixed at 0 or was allowed to fluctuate from 0 to a value $i_{\text{max}}$ for 5 ms periods. These fluctuations occurred randomly with a mean period of 1 s. In different simulations, the value of $i_{\text{max}}$ was increased from 0.01 to 10 pA. Equations were integrated for a time span of 20 s, and the ion 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 ($V_{\text{m}} = -180$ 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 cation channel. The open probability, $P_0$, of the channel was determined by the rate constants for channel activity, $P_k$, and $P_{\text{out}}$, respectively. The values for $P_k$ were set at 0, 0.0005, 0.0010, and 0.0335 ms$^{-1}$, providing mean open probabilities of 0, 0.0017, 0.0233, and 0.1040.

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 µM, 5 µM, 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 yfg4-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 (30–40°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 FIS1* knockout strains are more sensitive to cell death than wild type, in which Bcl-xL also protects (Fonnjung 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 F1 β subunit. Fig. S3 shows protease digestion of mitochondria detected with Bcl-xL antibody. Fig. S4 shows an example of TMRE traces and Bcl-xL blot for shRNA knockdowns in Fig. 4 G. Fig. S5 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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References


Figure S1.  **No defects in bcl-x-deficient mitochondria under normal conditions.** (A) 1 mM ADP-stimulated rates of oxygen consumption were determined for mitochondria isolated from the cerebral cortex of P5–7 control and cKO of mouse pups (5 mM malate, 5 mM glutamate, 2.5 mM succinate, 0.5 mM tetramethylphenylenediamine [TMPD], and 2 mM ascorbate) with the indicated inhibitors for the relevant upstream complex (complex I, 2 mM rotenone; complex III, 1 mM antimycin). (B) Immunoblots of mitochondria isolated from dissected P5–7 cerebral cortex (where interneurons and glia still express Bcl-xL). Cortices from four bcl-x cKO and two control (Cont) mice were pooled, and equal protein was loaded based on the BCA assay and analyzed using antibodies to ANT (sc-9299 [1:1,000; Santa Cruz Biotechnology, Inc.]), ATP synthase β subunit (1:1,000; Invitrogen), and other proteins as described in Fig. 2 A. A representative of three independent experiments is shown. VDAC, voltage-dependent anion channel. Molecular mass is indicated in kilodaltons. (C) Relative contributions of mitochondria and glycolysis to whole-cell ATP levels. Cortical neurons cultures (DIV4) were transferred to medium containing 25 mM glucose, glucose-free medium supplemented with 10 mM pyruvate to fuel mitochondria, or medium containing 25 mM glucose and 5 mg/ml of ATP synthase inhibitor oligomycin for 1 h and were then harvested for total ATP determination for each culture dish using a bioluminescence assay (Invitrogen). The absolute value of ATP was calculated from a standard curve generated for each individual experiment using fresh ATP standards (0 nM, 5 nM, 50 nM, 500 nM, 5 µM, and 25 µM) and was normalized by the protein content of each individual sample by BCA assay. Samples were analyzed immediately, or all time points were frozen instantly and analyzed together. Data are presented for three independent experiments, each with two to four different cultures/samples per genotype (control, n = 9; cKO, n = 10). A t test was used; all comparisons were not significantly different (p > 0.13). (D) Total ATP levels in cortical neuron cultures (DIV3–6). The mean value for cKO cultures was determined proportional to control and the results from multiple samples (control, n = 18; cKO, n = 19) in seven independent experiments (left). Relative ATP values for the same cortical cultures were further normalized by the mean difference in cell viability per experiment (mean ratio of cell viability for control/cKO = 1.085), determined by nuclear morphology using Hoechst (right). Data are presented as the mean ± SEM. The p value (*, P < 0.0002) was determined using a t test.
Figure S2. **Cryo-EM of bcl-x<sup>−/−</sup> mouse brain confirms mitochondrial matrix localization of endogenous Bcl-x<sub>L</sub> protein.** An example of a mitochondrion co-labeled by immunogold for the β subunit of the ATP synthase (large gold beads) and Bcl-x<sub>L</sub> (small gold; arrows) is shown. Note that the size of the antibody–bead complex will not permit colabeling of the same protein complex. Bar, 0.1 mm.

Figure S3. **Protease digestion of mitochondria detected with Bcl-x<sub>L</sub> antibody.** Bcl-x<sub>L</sub> is protected from protease digestion after permeabilization of the outer mitochondrial membrane of isolated rat brain mitochondria with digitonin. The blotting was performed as described for Fig. 2 E, except with anti–Bcl-x<sub>L</sub> antibody. Molecular mass is indicated in kilodaltons.
Figure S4. **Example of TMRE traces and Bcl-xL blots for shRNA knockdowns.** (A) Fluorescence traces for three mitochondria per condition used to calculate SDs shown in Fig. 4 G. (B) Immunoblot analysis verifying shRNA knockdown of Bcl-xL in hippocampal neurons results in Fig. 4 G. Lentivirus plasmids (pGIPZ vector) express the following shRNAs against rat BCL-xL: 5′-CGGGCTCACTCTTCAGTCGGAATAGTGAAGCCACAGATGTATTCCGACTGAAGT-GAGCCCA-3′ or scrambled (Scr.) control (cat# RHS4346; Thermo Fisher Scientific). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure S5. **Immunoblots of human Bcl-xL in yeast cell lysates.** Three independently transformed strains for each plasmid in each of the indicated yeast knockout strains are shown. Molecular mass is indicated in kilodaltons.