Functional and physical interaction between Bcl-X<sub>L</sub> and a BH3-like domain in Beclin-1

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The anti-apoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> bind and inhibit Beclin-1, an essential mediator of autophagy. Here, we demonstrate that this interaction involves a BH3 domain within Beclin-1 (residues 114–123). The physical interaction between Beclin-1 and Bcl-X<sub>L</sub> is lost when the BH3 domain of Beclin-1 or the BH3 receptor domain of Bcl-X<sub>L</sub> is mutated. Mutation of the BH3 domain of Beclin-1 or of the BH3 receptor domain of Bcl-X<sub>L</sub> abolishes the Bcl-X<sub>L</sub>-mediated inhibition of autophagy triggered by Beclin-1. The pharmacological BH3 mimetic ABT737 competitively inhibits the interaction between Beclin-1 and Bcl-2/Bcl-X<sub>L</sub>, antagonizes autophagy inhibition by Bcl-2/Bcl-X<sub>L</sub> and hence stimulates autophagy. Knockout or knockdown of the BH3-only protein Bad reduces starvation-induced autophagy, whereas Bad overexpression induces autophagy in human cells. Gain-of-function mutation of the sole BH3-only protein from Caenorhabditis elegans, EGL-1, induces autophagy, while deletion of EGL-1 compromises starvation-induced autophagy. These results reveal a novel autophagy-stimulatory function of BH3-only proteins beyond their established role as apoptosis inducers. BH3-only proteins and pharmacological BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin-1 and Bcl-2 or Bcl-X<sub>L</sub>.

**Introduction**

Two self-destructive processes, apoptosis (self-killing) and autophagy (self-eating), have captured the attention of cell biologists over the last decades. While apoptosis involves the activation of catabolic enzymes leading to the demolition of cellular structures and organelles, autophagy is a slow, localized phenomenon in which parts of the cytoplasm are sequestered within double-membrane autophagic vacuoles and finally digested by lysosomal hydrolases (Gozuacik and Kimchi, 2004; Kroemer and Jaattela, 2005). The relationship between apoptosis and autophagy is complex and autophagy may either contribute to cell death (Shimizu et al., 2004; Yu et al., 2004) or constitute a cellular defense against acute stress, in particular stress induced by starvation from nutrients or obligate growth factors (Boya et al., 2005; Lum et al., 2005).

The cross-talk between autophagy and apoptosis is mediated at least in part by the functional and physical interaction between Beclin-1, an essential autophagy gene, and Bcl-2, one of the paradigmatic apoptosis-inhibitory proteins (Liang et al., 1999; Pattingre et al., 2005; Takacs-Vellai et al., 2005).

Bcl-2 is the prototype of a family of proteins containing at least one Bcl-2 homology (BH) region. The family is split into anti-apoptotic multidomain proteins (such as Bcl-2 and Bcl-X<sub>L</sub>), which contain four BH domains (BH1234), pro-apoptotic multidomain proteins (prototypes Bax and Bak), which contain three BH domains (BH123), and the pro-apoptotic BH3-only protein family (Letai et al., 2002). As a rule, BH1234 proteins (Bcl-2, Bcl-X<sub>L</sub>, Mcl-1) mainly reside in mitochondria, protecting these organelles against mitochondrial outer membrane permeabilization (MOMP), one of the rate-limiting events of apoptosis induction. Either of the two BH123 proteins (Bax and Bak) are required for MOMP, in a series of different models of apoptosis induction (Wei et al., 2001). BH3-only proteins are suggested to kill cells by interacting with the BH3 receptor, which is a hydrophobic groove formed by apposition of the BH1, BH2 and BH3 domains, hence activating BH123 proteins and/or by neutralizing BH1234 proteins. The so-called ‘BH3 mimetics’, pharmacological compounds that bind to BH3 receptors, can induce apoptosis or facilitate apoptosis induction in cancer cells (Letai et al., 2002; Oltersdorf et al., 2005).

Beclin-1 (also called ATG6) is a phylogenetically conserved protein that is essential for the initiation of autophagy, perhaps via its interaction with the class III phosphatidylinositol-3-kinase Vps34 (Zeng et al., 2006). Originally, human Beclin-1 has been identified as an interactor of Bcl-2 (Liang et al., 1999). Caenorhabditis elegans Beclin-1 (BEC-1) interacts with the Bcl-2 homolog CED-9, and inactivation of the C. elegans bec-1 gene causes apoptosis (Takacs-Vellai et al., 2005).

In mammalian cells, knockdown of beclin-1 sensitizes to apoptosis induction by starvation (Boya et al., 2005; Lum et al., 2005). However, Beclin-1 downregulation can also inhibit cell death induction by conditions in which essential
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pro-apoptotic MOMP or caspase activation are blocked (Shimizu et al., 2004; Yu et al., 2004), and restoration of normal Beclin-1 levels in Beclin-1 deficient tumor cells can facilitate the induction of cell death by vitamin D analogues (Hoyer-Hansen et al., 2005). Importantly, beclin-1 is a hallmark tumor suppressor gene (Qu et al., 2003; Yue et al., 2003). Transfection-enforced overexpression of Beclin-1 stimulates autophagy, and this autophagy-stimulatory effect is enhanced by depletion of Bcl-2 and reduced by Bcl-2 overexpression (Pattingre et al., 2005).

Based on these premises and incognita, we explored the fine mechanisms which govern the interaction between Beclin-1 and Bcl-2/Bcl-XL. As shown here, Beclin-1 possesses a BH3-like domain, thus elucidating the structural basis for the functional Beclin-1-Bcl-2/Bcl-XL interaction. Disrupting this interaction by BH3-only proteins or BH3 mimetics increases the autophagic activity of Beclin-1, thus revealing a novel physiological role for BH3 domains.

Results

Identification of a BH3-like domain in Beclin-1

In multiple yeast two-hybrid screens, using complex human DNA libraries, a number of Beclin-1 fragments interacted with Bcl-2 as well as with Bcl-XL, allowing us to narrow down the precise interaction domain of Beclin-1 to amino
The BH3-mimetic compound ABT737 (Oltersdorf et al, 2005), inhibits the binding of Beclin-1 BH3 peptide to Bcl-X<sub>L</sub> in a competitive manner, with an IC<sub>50</sub> in the micromolar range, as determined by fluorescence polarization of synthetic peptide binding to purified recombinant Bcl-X<sub>L</sub> <em>in vitro</em> (Figure 2A). Pretreatment of cells with ABT737 inhibited the co-immunoprecipitation of Flag-tagged Bcl-X<sub>L</sub> and His-tagged (Figure 2B) or endogenous Beclin-1 (Figure 2C). ABT737 also reduced the interaction between Bcl-2 and Beclin-1 (Figure 2D), yet had no effect on the interaction between Mcl-1 and Beclin-1 (Figure 2E). This can be explained by the selectivity of ABT737, which binds to Bcl-2 and Bcl-X<sub>L</sub> but not to Mcl-1, and hence has a Bad-like profile (Oltersdorf et al, 2005, Van Delft et al, 2006). It is important to note that ABT737 also abolished the interaction between endogenous Bcl-2 and Beclin-1 (see below), meaning that these effects cannot be attributed to overexpression-associated artifacts. Altogether, these data indicate that the BH3-like domain of Beclin-1 binds to the BH3 receptor region of Bcl-X<sub>L</sub>/Bcl-2 and that ABT737 competitively disrupts this interaction.

**The BH3 mimetic ABT737 stimulates Beclin-1-dependent autophagy**

If the physiological function of the physical Beclin-1-Bcl-X<sub>L</sub>/Bcl-2 interaction were to control Beclin-1-initiated autophagy (Pattingre et al, 2005), then inhibition of this interaction order of magnitude similar to Bax-BH3 (145 nM) or Bak-BH3 (40 nM), although lower than Bad-BH3 (10 nM) (data not shown). In contrast Bcl-X<sub>L</sub> carrying a single mutation (G138A) in the BH3 binding groove (Ottillie et al, 1997) was unable to interact with the Beclin-derived BH3-like peptide.

To corroborate the role of the BH3-like domain for the Beclin-1 interaction with Bcl-X<sub>L</sub>, cells were transfected with wild-type or mutant Beclin-1 and/or with Bcl-X<sub>L</sub>, followed by co-immunoprecipitation assays. The BH3-disrupting mutation L116A almost abolished the interaction of Beclin-1 with Bcl-X<sub>L</sub> (Figure 1E), as well as that of Beclin-1 with Bcl-2 and Mcl-1 (not shown). Moreover, F123A mutation reduced the Beclin-1-Bcl-X<sub>L</sub> interaction (Figure 1E). The G138A mutation within the BH3-binding cleft of Bcl-X<sub>L</sub> abrogated the binding of Beclin-1 in the cellular context (Figure 1F). These results suggest that a novel BH3-like domain in Beclin-1 is critical for the interaction of Beclin-1 with anti-apoptotic members of the Bcl-2 family. The BH3-like domain of Beclin-1 is phylogenetically conserved, because the Beclin-1 orthologs from fugu (<em>Takifugu rubripes</em>), latipes (<em>Oryzias latipes</em>) and zebrafish (<em>Danio rerio</em>) exhibit sequence homology within their BH3-like domain with human Beclin-1 (Supplementary Figure 1A). Peptides corresponding to these BH3-like domains induced apoptosis in a Bax-dependent manner when they were microinjected into human HCT116 cells (Supplementary Figure 1B and C), and the peptides from fugu and latipes (but not the ones from zebrafish) are able to displace the Bak BH3 peptide from recombinant Bcl-X<sub>L</sub> or Bcl-2 protein <em>in vitro</em> (Supplementary Figure 1D and E). The fact that the zebrafish-derived Beclin-1 peptide did not bind to human Bcl-X<sub>L</sub> yet induced Bax-dependent apoptosis, suggests that this particular peptide induces cell death by acting on a multidomain Bcl-2 family protein other than Bcl-X<sub>L</sub>.
Figure 3 Beclin-1-dependent autophagy stimulated by ABT737. (A, B) Detection of autophagic vacuoles by LC3-GFP and their modulation by ABT737 and by Beclin-1-specific siRNAs. HeLa cells were transfected with control or Beclin-1-specific siRNAs, 24 h later re-transfected with LC3-GFP, cultured in complete medium (CM) for 24 h, and finally kept 12 h either in CM or in nutrient-free (NF) conditions, in the presence or absence of 1 μM ABT737. Representative microphotographs of cells cultured in NF medium are shown in (A) and the percentage (means ± s.d., n = 3 separate experiments) of LC3-GFP-transfected cells bearing LC3-GFP aggregates in the cytoplasm (LC3-GFPvac) are quantified in (B). The insert in (B) demonstrates the efficiency of the Beclin-1-specific siRNAs, as quantified by immunoblot. (C, D) Detection of cytoplasmic vacuoles using chloromethylfluorescein diacetate (CMFDA). Cells were transfected with control or Beclin-1-specific siRNAs, cultured for 48 h in CM, washed, cultured in CM (D) or NF (C, D) for 12 h, stained with CMFDA, and either photographed (C) or subjected to the quantification of the cells that bear at least one discernible cytoplasmic vacuole (arrow head) (means ± s.d., n = 3 separate experiments). (E) Ultrastructure of autophagic vacuoles induced by ABT737. Transmission electron microphotographs are shown. (F) Detection of dead and dying cells in the cultures. Cells treated as in (A) were stained with the ΔΨm-sensitive dye DiOC6(3) and the vital dye propidium iodide (PI). The black portions of the columns refer to the DiOC6(3)low PI Newly formed population (dead) and the remaining part of the column corresponds to the DiOC6(3)low PI (dying) population. Results are means ± s.d. of three independent experiments.
would be expected to stimulate autophagy. Accordingly, ABT737 increased the frequency of cells that manifested cytoplasmic (non-nuclear) aggregation of the marker of autophagic vacuoles, LC3-GFP. This aggregation of LC3-GFP (which, in non-autophagic cells, is diffuse in the cytosol as well as in the nucleus) is an established sign of autophagy (Mizushima et al., 2004), and was induced both in complete medium as well as in conditions of nutrient depletion (Figure 3A). Knockdown of Beclin-1 by two distinct small interfering RNA (siRNA) heteroduplexes (insert in Figure 3B) inhibited the ABT737-stimulated LC3-GFP aggregation (Figure 3A and B). Similarly, knockdown of other essential ATG proteins (ATG5, 10, 12) reduced ABT737-induced LC3-GFP aggregation, confirming that ABT737 engages the classical autophagic pathway (Supplementary Figure 2S). Since LC3-GFP might interfere with the normal regulation of autophagy, cytoplasmic vacuoles (which are bona fide autophagic vacuoles) were detected by staining with CMFDA, without prior transfection with LC3-GFP. Again, ABT737 induced signs of autophagy that could be completely suppressed by the knockdown of Beclin-1 (Figure 3C and D). These results could be confirmed by transmission electron microscopy showing double-membraned autophagic vacuoles that were elicited by ABT737 and suppressed by Beclin-1-specific siRNAs (Figures 3E and 4). The ABT737-stimulated autophagy was oranelle-specific in the sense that LC3-GFP colocalized with the mitochondrial marker HSP60 (Supplementary Figure 3A and C) but not with the endoplasmic reticulum (ER) marker calreticulin (Supplementary Figure 3B and C). It is noteworthy that these results were obtained by observing viable, adherent cells (Figure 3A–E) and that the cytotoxic pro-apoptotic effects of ABT737 were minor. Thus, ABT737 failed to induce a major loss of the mitochondrial transmembrane potential (which may be associated with apoptosis or necrosis) (Figure 3F) or phosphatidylserine exposure (which is associated with apoptosis) (Supplementary Figure 4), unless Beclin-1 was depleted simultaneously. This is in accord with the notion that ABT737 as a single agent only kills a limited set of transformed cell lines (Oltersdorf et al., 2005). ABT737 can stimulate autophagy without inducing cell death.

**BH3 dependency of the functional interaction between Beclin-1 and Bcl-XL/Bcl-2**

Overexpression of Beclin-1 stimulates autophagy (Liang et al., 1999; Hoyer-Hansen et al., 2005; Pattingre et al., 2005), and this effect was increased by ABT737 (Figure 5A and B). The Beclin-1 mutants L116A and F123A were more efficient than wild-type Beclin-1 in stimulating autophagy, in line with the fact that these mutations disrupt the interaction with the Beclin-1 inhibitors Bcl-2/Bcl-XL. This result was obtained when using two different read-outs, namely LC3-GFP aggregation (Figure 5A and B) and CMFDA-quantifiable vacuolization (Figure 5C and D). In the presence of ABT737, the differential capacity of wild-type Beclin-1 and of its mutants L116A and F123A to induce autophagy was matched, suggesting that it was indeed the interaction between the BH3-like domain of Beclin-1 and a BH3 receptor (inhibitable by ABT737) that regulated the pro-autophagy activity of Beclin-1. These data also suggests that endogenous Mcl-1 (which is not inhibitable by ABT737) (van Delft et al, 2006) does not play a crucial role in controlling autophagy induced by over-expressed Beclin-1. Moreover, Bcl-XL (but not Bcl-XL G138A), Bcl-2 and Mcl-1 all inhibited the induction of autophagy by Beclin-1 (Figure 5E). The autophagy-inhibitory effect of Bcl-XL and Bcl-2 was abrogated by ABT737. However, ABT737 did not affect the suppression of autophagy by Mcl-1 (Figure 5E), in accord with its incapacity to block the Beclin-1–Mcl-1 interaction (Figure 2E). Altogether, these data suggest that ABT737 stimulates (or de-inhibits) autophagy. 

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**Figure 3** Continued.
A siRNA emerin or siRNA emerin + ABT737
B siRNA beclin 110 or siRNA beclin 110 + ABT737

Figure 4 Quantification of autophagic vacuoles induced by ABT737. HeLa cells transfected with the indicated siRNAs (specific for Emerin or for Beclin-1 at 0 h) were re-transfected with LC3-GFP and cultured in nutrient-free (NF) conditions (60–72 h), in the presence or absence 1 μM ABT737 and then subjected to electron microscopic detection of immature (AV1) or mature (AV2) autophagic vacuoles. Representative pictures are shown in (A). The number of AV1 and AV2 was determined for a minimum of 50 cells (means ± s.e.m.) (B).

Spatially restricted, regulated interactions between Bcl-2 and Beclin-1 are inhibited by ABT737
ABT737 as well as nutrient depletion reduced the amount of endogenous Beclin-1 that immunoprecipitated with endogenous Bcl-2, both in HeLa and in MV4.11 cells (Figure 6A), confirming that physiological levels of these proteins can interact in a fashion that is inhibited by nutrient depletion or ABT737. Bcl-2 associates both with ER and mitochondrial membranes (Germain and Shore, 2003). Wild-type and ER-targeted Bcl-2 (Bcl-2-Cb5) but not mitochondrion-targeted Bcl-2 (Bcl-2-Acta) inhibits starvation-induced autophagy (Pattingre et al., 2005), suggesting that the autophagy-regulatory pool of Bcl-2 is localized on ER. To investigate this, we determined the inhibitory effect of ABT737 and nutrient depletion on the Bcl-2–Beclin-1 interaction in cell lines that stably express wild-type Bcl-2, Bcl-2-Cb5, or Bcl-2-Acta. The amount of Beclin-1 that co-immunoprecipitated with wild type and Bcl-2-Cb5 diminished after treatment with ABT737 or starvation, whereas the amount of Beclin-1 that co-immunoprecipitated with Bcl-2-Acta remained constant (Figure 6B). These results could be further substantiated by subcellular fractionation. The interaction between wild-type Bcl-2 and Beclin-1 measurable in microsomes (ER) was reduced by ABT737 or starvation, but remained constant within the heavy membrane fraction (mitochondria) (Figure 6C and D). Hence, only the ER-targeted pool of Bcl-2 is relevant to the inhibition of autophagy.

Starvation-induced induction of autophagy via the BH3-only protein Bad
The results above imply that, to the very least in certain instances, induction of Beclin-1-mediated autophagy should correlate with its release from inhibitory complexes. Upon starvation, the amount of endogenous Beclin-1 that co-immunoprecipitated with Bcl-XL declined within the first hour of serum and nutrient withdrawal, whereas the amount of the BH3 protein Bad (whose activation is known to be triggered by serum withdrawal) (Danial and Korsmeyer, 2004) that co-immunoprecipitated with Bcl-XL increased (Figure 7A). In contrast, addition of rapamycin (which induces autophagy by inhibition of mTOR) (Sarbassovods et al., 2005) or other autophagy inducers that affect the level of phosphatidylinositol-3-phosphate (Sarkar et al., 2005) had less marked effects on the interactions between Beclin-1, Bcl-XL, and Bad (Figure 7B). Upon starvation (but not upon ABT737 addition), the amount of endogenous Bad that immunoprecipitated with endogenous Bcl-2 also increased (Figure 7C), at the same time as the Bcl-2–Beclin-1 interaction was reduced (Figure 6A). The siRNA-mediated depletion of Bad (Figure 7D) reduced the starvation-induced activation of autophagy, yet had no or little effect on rapamycin-induced autophagy, whereas depletion of Vps34 inhibited both starvation and rapamycin-induced autophagy (Figure 7E). Bad−/− mouse embryonic fibroblasts (MEF) (Ranger et al., 2003) also exhibited a decreased starvation-induced autophagy as compared with WT MEF, although this difference disappeared in the presence of ABT737 (Figure 7F). The absence of Bad
compromised the disruption of the Beclin-1/Bcl-2 interaction induced by starvation, yet had no effect on the disruption of this interaction by ABT737 (Figure 7G). The effect of Bad depletion/deletion on starvation-induced autophagy (Figure 7D and F) and on the changing Beclin-1/Bcl-2 interaction (Figure 7G) was partial, suggesting that Bad is not the sole BH3 protein linking starvation to autophagy induction or that BH3-independent mechanisms may be involved. Transfection-enforced overexpression of Bad was sufficient to induce autophagy both in normal conditions and conditions of caspase inhibition (Figure 7H). These results indicate that BH3-only proteins including Bad may partake in the activation of autophagy by starvation.

**Regulation of autophagy by the BH3-only protein EGL-1 in C. elegans**

EGL-1 is the sole pro-apoptotic BH3-only protein in *C. elegans* and is required for developmental cell death in this nematode (Conradt and Horvitz, 1998, 1999). Hence, this model organism is uniquely suitable to determine the phylogenetically conserved regulation of autophagy by BH3-only proteins. In *C. elegans*, lgg-1 (the nematode orthologue of Atg8/LC3) is ubiquitously expressed throughout development and induced in conditions of autophagy (Melendez et al., 2003). Autophagy was monitored using an LGG-1::DsRED reporter in embryos that carry a wild-type, gain-of-function or deletion allele of *egli-1*, in normal conditions or after starvation. Starvation strongly induced autophagy, and this induction was blunted in *egli-1*-deficient nematode embryos. In contrast, the gain-of-function mutation of *egli-1* caused an increase in constitutive autophagy that was not enhanced further by starvation (Figure 8). These results underscore the phylogenetic conservation of autophagy control by BH3-only proteins.

**Discussion**

As shown here, Beclin-1 possesses a BH3 domain that dictates its interaction with the BH3 receptor domain of anti-apoptotic proteins of the Bcl-2 family including Bcl-2, Bcl-XL, and Mcl-1. A recent report, which was published when this paper was under review, revealed the crystallographic structure of human Bcl-XL interacting with a peptide (aa 107–135) derived from human Beclin-1 and corroborated the hypothesis that Beclin-1 possesses a BH3-like amphipathic α-helix that can bind to the conserved hydrophobic groove of Bcl-XL (Oberstein et al., 2007). As shown here, this interaction is physiologically important because its inhibition by the BH3-only protein Bad and by a Bad-like BH3 mimetic can stimulate autophagy.

Beclin-1 possesses a BH3 domain that, in the form of a synthetic peptide excised from the context of the whole protein, induces Bax-dependent apoptosis. Upon overexpression, however, it promotes Bcl-XL-inhibitable autophagy rather than apoptosis. Beclin appears therefore as a new type of BH3 protein, in which this domain plays a regulatory, rather than an effector function.

The prevalence of the autophagic activity of BH3-containing Beclin-1 and its failure to induce apoptosis in an in vivo context raises important mechanistic questions. Regulatory sequences in the Beclin-1 full-length protein, or the structure of the Beclin-1 BH3 domain itself, may normally prevent the BH3 motif in Beclin-1 from activating apoptosis, perhaps because of different affinities between BH3-only proteins for anti-apoptotic Bcl-2 family members in a cellular context. Indeed, in the recent report of the structure of Beclin-1 BH3 peptide binding to Bcl-XL, interaction features suggest a distinct binding specificity (Oberstein et al., 2007).
**Figure 7** Impact of the BH3-only protein Bad on autophagy. (A, B) Interactions between Bcl-XL, Beclin-1 and Bad in conditions of autophagy induction. Cells were either treated by nutrient depletion (A) or addition of 1 μM rapamycin, 1 mM lithium chloride, 100 μM L-690,330 or 50 μM carbamazepine (B), followed by immunoprecipitation of Bcl-XL [as in Figure 2C] and revealing the immunoblots by antibodies specific for Bcl-LC3-GFP, and then subjected to nutrient depletion (NF) and/or treatment with ABT737 (18h; means ± s.d., n ≥ 3). The asterisks denotes a significant (P<0.05) effect of Bad deficiency. (C) Interaction between endogenous Bad and Bcl-2. HeLa cells were treated with ABT737 (1 μM) or nutrient-depleted, followed by immunoprecipitation of Bcl-2 and immunodetection of Bad. (D, E) Impact of Bad depletion on autophagy. Cells were transfected with LC3-GFP together with siRNAs specific for Bad, siRNAs specific for emerin or the Beclin-1-associated PI3 kinase Vps34. Forty-eight hours later, when the siRNAs had down-regulated the proteins of interest (D), cells were subjected by nutrient depletion (NF) or addition of rapamycin (E) and the frequency of cells exhibiting LC3-GFP aggregation as a marker of autophagy was assessed after 18h (means ± s.d., n = 3 separate experiments). (F) Effect of the Bad knock-out on autophagy. Wild Type (WT) or Bad−/− MEF were transfected with LC3-GFP and then subjected to nutrient depletion (NF) and/or treatment with ABT737 (18h; means ± s.d., n = 3). The asterisks denotes a significant (P<0.05) effect of Bad deficiency. (G) Effect of the Bad knockout on the Beclin-1/Bcl-2 interaction. WT or Bad−/− MEF were transfected with LC3-GFP alone (Control), together with vector-only or with a vector encoding Bad, and subjected 48 h later to nutrient depletion (NF). Cells were cultured in the continuous presence or absence of the pan-caspase inhibitor Z-VAD-fmk (50 μM) for 16h, followed by assessment of autophagy as in (E) (means ± s.d., n = 3 separate experiments, *P<0.01). Immunoblots are representative of at least three independent experiments.
The BH3 domain protein EGL-1 modulates starvation-induced autophagy in *C. elegans*. L4 Larvae expressing an LGG-1::DsRED reporter (whose expression level provides an indication of autophagy) as well as a gain-of-function mutation of *egl-1* (n487) or a deletion allele of *egl-1* (ok1418) were maintained in rich medium or starved overnight. (A) Representative images of wt and *egl-1* mutant embryos, bearing the p*lgg*-DsRED::LGG-1 reporter transgene are shown under conditions of normal growth and under starvation. Scale bars denote approximately 50 μm. (B) Quantification of LGG-1::DsRED fluorescence as determined in (A). Each point represents the measurement for one individual embryo. Horizontal bars denote means and gray areas denote standard errors.

Alternatively, there could be different pools of anti-apoptotic multidomain Bcl-2 proteins, some sequestering pro-apoptotic Bcl-2 family members, others Beclin-1, and that these may be selectively displaced by other BH3 proteins, perhaps Bcl-2 family members, others Beclin-1, and that these may be selectively displaced by other BH3 proteins, again perhaps reflecting their different affinities and/or subcellular compartmentalization.

In favour of this latter possibility, we found that ABT737 as well as nutrient withdrawal only affected the interaction between Beclin-1 and ER-localized Bcl-2. The mechanisms through which ABT737 may effect the interaction between ER-localized Bcl-2-Beclin-1 complexes (but not those found on mitochondria) are elusive. On the one hand, the affinity of ABT737 for Bcl-2 may be influenced by post-transcriptional alterations (such as Bcl-2 phosphorylation; Konopleva et al., 2006) that may correlate with its subcellular localization (Ruvolo et al., 1998). On the other hand, the nature of the physicochemical interaction between Bcl-2 and Beclin-1 might be conditioned by the presence of additional organelle-specific proteins, such as IP₃R (1,4,5-inositol trisphosphate receptor) in ER (Chen et al., 2004; Criollo et al., 2007) and VDAC (voltage-dependent Anion Channel) in mitochondria (Shimizu et al., 1999). While mitochondrial Bcl-2 and Bcl-Xₐ could be the preferential target for the pro-apoptotic function of BH3-only proteins, ER-resident Bcl-2-Beclin-1 complexes could be selectively disrupted by competing BH3 domains (such as that of Bad) or BH3 mimetics for the induction of autophagy, which is agreement with the findings of Pattingre et al. (2005) showing that only ER-targeted Bcl-2 inhibits autophagy.

It is noteworthy that the regulation of autophagy by BH3-only proteins is phylogenetically old. This does not only apply to the conservation of the BH3-like domain of Beclin-1 in non-mammalian species but extends to the observation that EGL-1, the sole BH3-only protein of *C. elegans*, suffices to trigger autophagy and is a mediator of starvation-induced autophagy. It should be noted that loss-of-function mutants of *egl-1* (in *C. elegans*) and deletion/deletion of Bad (in human and mouse cells) did not completely abolish starvation-induced autophagy, in line with the presence of redundant regulatory pathways (Shintani and Klionsky, 2004) in which BH3-only proteins play a prominent role but not an exclusive role.

By virtue of its BH3-mimetic action, ABT737 exerts two distinct cellular effects. On the one hand, it inhibits the anti-apoptotic action of Bcl-2 or Bcl-Xₐ, as established previously (Ottersdorf et al., 2005), thus triggering apoptosis in cells in which neutralization of Bcl-2 or Bcl-Xₐ is sufficient to inhibit the mitochondrial cell death pathway. On the other hand, ABT737 stimulates autophagy, which can be viewed as a cytoprotective mechanism (Boya et al., 2005; Lum et al., 2005). Although at present no clinically applicable autophagy inhibitors are available, these results point to the possibility of using autophagy inhibitors for sensitizing tumor cells to BH3 mimetics or indirect activators of BH3-dependent apoptotic pathways.

In conclusion, our data support a novel function of the BH3-binding site of Bcl-2 homolog, namely regulation of autophagy. Beyond their established function as cell death inducers, BH3-only proteins or BH3 mimetics can induce autophagy by competitively disrupting the interaction of Beclin-1 with Bcl-2/Bcl-Xₐ. Our findings indicate a hitherto unexpected crosstalk between self-killing and self-eating. Both phenomena are controlled by BH3-only proteins.

**Materials and methods**

**Cell lines and culture conditions**

HeLa cells were cultured in DMEM containing 10% fetal calf serum (FCS), 1 mM pyruvate and 10 mM Hepes at 37°C under 5% CO₂, MV4.11 cells were cultured in RPMI 1640 medium containing 10% FCS, 1 mM pyruvate, 10 mM Hepes, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 5 μg/ml Gramacyte/Macrophage Colony-Stimulating Factor (GM-CSF) at 37°C under 5% CO₂, wild type and Bad⁻/⁻* MEF (Ranger et al., 2003), as well as cells expressing Bcl-2 Acta or Bcl-2 Cb5 (Zhu et al., 1996) were maintained in DMEM supplemented with 10% FCS, 1 mM pyruvate, 10 mM Hepes and 1% non-essential amino acids (NEAA, 5GMA) at 37°C under 5% CO₂. All media and supplements were purchased from Gibco-Invitrogen (Carlsbad, USA). For serum and amino-acid starvation, cells were cultured in serum-free Earle’s Balanced Salt Solution.
Solution medium (Sigma) (Vahsen et al., 2004), optionally supplemented with ABT737 (1 μM; synthesized by Servier).

Plasmids, transfection and RNA interference
Cells were cultured in six-well plates and transfected at 80% confluence with Oligofectamine (Invitrogen), in the presence of lipofectamine DNA complexes of siRNAs specific for human Beclin-1 and other autophagy genes (Boya et al., 2005; Gonzalez-Polo et al., 2005). siRNA1 from Jin (2005 and siRNA2 from Byfield et al., 2005), a scrambled siRNA or an siRNA targeting the unrelated protein emerin (Harborth et al., 2000), siRNA effects were controlled by immunoblot analysis with suitable antibodies specific for Beclin-1 (SantaCruz), Bad (SantaCruz), Vps (Zymed) and Beclin L116A, Beclin-1 F123A, Bcl-XL wild type, Bcl-XL G138A, Bcl-2 wild type, or Mcl-1.

Yeast two-hybrid system
Bcl-XL and Bcl-2 without their C-terminal transmembrane domains (Bcl-XL 1–624 and Bcl-2 1–630) were PCR amplified (Pfu, Stratagene) and cloned C-terminal to the lexA DNA binding domain in the pB24 vector. DNA libraries constructed in pP6 vector (Fromont-Racine et al., 2002) were transformed into the Y187 yeast strain. Ten million independent yeast colonies were collected, pooled and stored at −80°C as equivalent aliquots fractions of the same library. The mating protocol has been described (Fromont-Racine et al., 2002). Each screen was performed to ensure a minimum of 50 million interactions. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5’ and 3’ junctions on a PE3700 Sequencer. The resulting sequences were used to identify the corresponding gene in the GenBank database (NCBI).

Peptides and recombinant protein
Recombinant GST fused to Bcl-XL wild type or Bcl-XL G138A (pGEX 5X vector, Amersham) deleted of the C-terminal 21 aa were produced in Escherichia Coli (BL21) and purified on glutathione-Sepharose resin (Amersham). HPLC-purified Beclin wild-type (GTIENLSRRLKVTGDLFDMSGQTDV), Beclin L116A (GTIENLSRRLKVTGDLADIMSGQTDV) and Bak BH3 (DLADIMSGQTDV) and Bak BH3 (GQAQRQLAIGGDDINRR) peptides were obtained from NeoSystem.

Fluorescence polarization assays
Fluorescence polarization (FP) assays (measured with a Fusion FLuorescence polarization assays) were obtained from Neosystem. DLADIMSGQTDV) and Bak BH3 (GQAQRQLAIGGDDINRR) peptides were affinity purified with formaldehyde (4% w/v) for LC3-GFP and immunofluorescence assays (Obed et al., 2007). Cells presenting a diffuse distribution of LC3-GFP in the cytoplasm and nucleus were classified as autophagic. Each LC3-GFP staining was read by two independent investigators (MC Mauri and A Criollo or E Tasdemir). Transmission electron microscopy was performed as described (Gonzalez-Polo et al., 2005).

Nematode experiments
To generate the P_egl-1::DsRED::LGG-1 reporter construct, we fused DsRED to the N-terminus of C. elegans LGG-1, a 750 bp fragment containing the lgg-1 gene was amplified using the following oligos: 5’-GGATTCAGAATGGCAAGCAGACAAGGAGGA-3’ and 5’-GCAATTCGTTCTTCTTGGATCATCG-3’. Two independent investigators (MC Mauri and A Criollo or E Tasdemir). Transmission electron microscopy was performed as described (Gonzalez-Polo et al., 2005).

Western blot analysis
Cells were washed with cold PBS at 4°C and lysed. Forty μg of protein were loaded on a 10% SDS–PAGE and transferred to nitrocellulose. The membrane was incubated for 1 h in PBS-Tween 20 (0.05%) containing 5% nonfat milk. Primary antibody anti-Beclin1 (SantaCruz), Bad (SantaCruz), iVps (Zymed) were incubated for 15 h at 4°C and then washed peroxidase-labeled secondary antibodies (Southern Biotechnologies Associates) plus the SuperSignal West Pico chemiluminescent substrate (Pierce). Anti-GAPDH (Chemicon), anti-Hsp60 (Sigma) or anti-calreticulin (Stressgen) antibody was used to ensure equal loading.

Flow cytometry
The following fluorochromes were employed to determine apoptosis-associated changes by cytofluorometry: 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3) (40 nM) for quantification of the mitochondrial transmembrane potential (ΔΨm) and propidium iodide (PI; 1 μg/ml) for determination of cell viability (Molecular probes) (Gonzalez-Polo et al., 2005). Trypsinized cells were labeled with the fluorochromes 30°C, followed by cytofluorometry analysis with a fluorescence-activated cell sorter (FACS) (Becton Dickinson).

Light microscopy, immunofluorescence, and electron microscopy
Cells cultured on coverslips were stained with Cell Tracker Green 5-chloromethylfluorescein diacetate (CMFDA (1 μM); Molecular Probes) and Hoechst 33342 (2 μM; Sigma). Alternatively, cells were fixed with paraformaldehyde (4% w/v) for LC3-GFP and immunofluorescence assays (Obed et al., 2007). Cells presenting a mostly diffuse distribution of LC3-GFP in the cytoplasm and nucleus were considered as non-autophagic, whereas cells presenting several intense punctate LC3-GFP aggregates with no nuclear LC3-GFP were classified as autophagic. Each LC3-GFP staining was read by two independent investigators (MC Mauri and A Criollo or E Tasdemir). Transmission electron microscopy was performed as described (Gonzalez-Polo et al., 2005).
Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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