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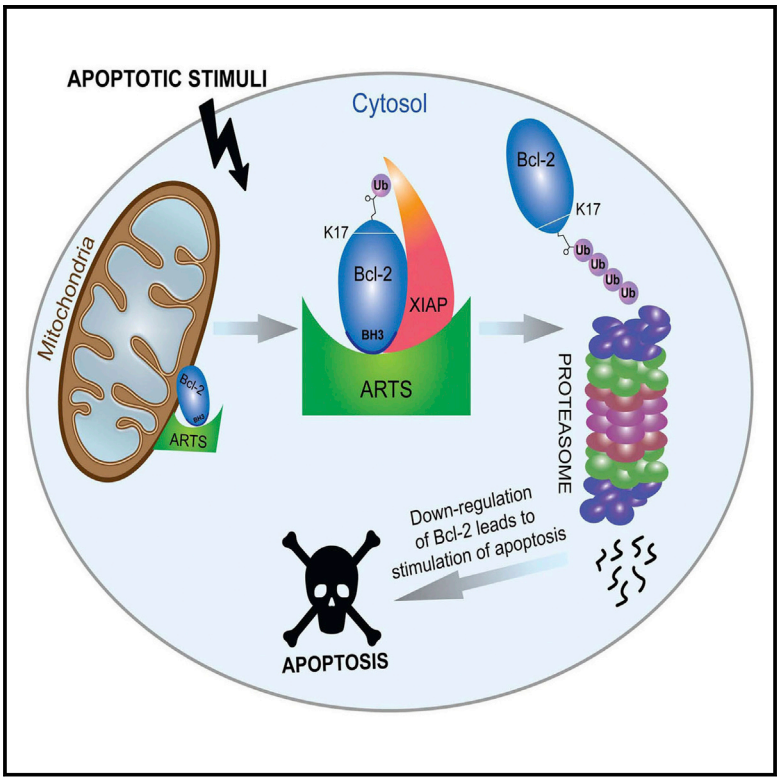
Degradation of Bcl-2 by XIAP and ARTS Promotes Apoptosis

Graphical Abstract



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In Brief

Many cancers avoid cell death (apoptosis) by expressing high levels of apoptosis inhibitors, such as Bcl-2. Thus, Bcl-2 is a major target for cancer therapy. Edison et al. describe a mechanism by which the ARTS protein promotes proteasome-mediated degradation of Bcl-2 and thereby stimulates cell death.

Highlights

- ARTS binds directly to both XIAP and Bcl-2, bringing them into a ternary complex
- ARTS bridges between XIAP and Bcl-2, allowing XIAP to serve as an E3-ligase for Bcl-2
- ARTS interacts with the BH3 domain of Bcl-2
- ARTS functions as a distinct Bcl-2 antagonist by binding and leading to its degradation

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Degradation of Bcl-2 by XIAP and ARTS Promotes Apoptosis

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SUMMARY

We describe a mechanism by which the anti-apoptotic B cell lymphoma 2 (Bcl-2) protein is downregulated to induce apoptosis. ARTS (Sept4_i2) is a tumor suppressor protein that promotes cell death through specifically antagonizing XIAP (X-linked inhibitor of apoptosis). ARTS and Bcl-2 reside at the outer mitochondrial membrane in living cells. Upon apoptotic induction, ARTS brings XIAP and Bcl-2 into a ternary complex, allowing XIAP to promote ubiquitylation and degradation of Bcl-2. ARTS binding to Bcl-2 involves the BH3 domain of Bcl-2. Lysine 17 in Bcl-2 serves as the main acceptor for ubiquitylation, and a Bcl-2 K17A mutant has increased stability and is more potent in protection against apoptosis. Bcl-2 ubiquitylation is reduced in both XIAP- and Sept4/ARTS-deficient MEFs, demonstrating that XIAP serves as an E3 ligase for Bcl-2 and that ARTS is essential for this process. Collectively, these results suggest a distinct model for the regulation of Bcl-2 by ARTS-mediated degradation.

INTRODUCTION

Apoptosis plays a major role in development and tissue homeostasis and as a defense mechanism against unwanted and potentially dangerous cells (Fuchs and Steller, 2015; Malin and Shaham, 2015). When deregulated, apoptosis can result in various pathologies, including cancer (Fuchs and Steller, 2011). Caspases, a family of cysteine proteases, are the central executioners of apoptosis (Boyce et al., 2004). In living cells, caspases are inhibited by the inhibitor of apoptosis (IAP) proteins (Gyrd-Hansen and Meier, 2010). To stimulate apoptosis, the function of IAPs needs to be overcome. This is achieved by IAP antagonists such as Smac/Diablo (Du et al., 2000; Verhagen et al., 2000) and ARTS (Gottfried et al., 2004a). ARTS is localized at the mitochondrial outer membrane (MOM) (Edison et al., 2012b). Upon induction of apoptosis, ARTS accumulates in the cytosol and directly binds and antagonizes XIAP, causing activation of caspases and cell death (Bornstein et al., 2011; Edison et al., 2012b; Gottfried et al., 2004a).

XIAP is the only known direct inhibitor of proteolytic caspase activity (Bergmann et al., 2003; Eckelman et al., 2006; Vasudevan and Ryoo, 2015). XIAP can directly bind and inhibit caspases 3, 7, and 9 via its three baculoviral IAP repeat (BIR) domains (Bergmann et al., 2003; Shi, 2002). In addition, it contains an ubiquitin-associated (UBA) domain that enables the binding of polyubiquitin conjugates via lysine 63 (Gyrd-Hansen et al., 2008; Rajalingam and Dikic, 2009) and a RING domain that bestows E3 ligase activity (Schile et al., 2008). XIAP functions as an E3 ligase for several pro-apoptotic proteins such as caspases, SMAC, AIF, and ARTS (Bornstein et al., 2012; Galbán and Duckett, 2010; Schile et al., 2008).

The mitochondrial pathway of apoptosis is regulated by Bcl-2 family members (Cory et al., 2003; Gross et al., 1999a). This family is composed of pro- and anti-apoptotic proteins that share up to four conserved Bcl-2 homology (BH) domains, and form complexes by binding to their common BH3 domain (Adams and Cory, 1998; Youle and Strasser, 2008). Importantly, many cancers are characterized by high levels of Bcl-2 (Adams and Cory, 1998; Castle et al., 1993; Krajewska et al., 1996; Robertson et al., 1996; Youle and Strasser, 2008). Therefore, Bcl-2 antagonists are promising anti-cancer drugs, and Venclexta was recently approved for the treatment of chronic lymphocytic leukemia.

Degradation of intracellular proteins in eukaryotic cells occurs via the ubiquitin proteasome system (UPS). This involves the ordered action of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and E3 ubiquitin ligases, which recognize and transfer ubiquitin to the target proteins (Ciechanover et al., 1980; Glickman and Ciechanover, 2002). It was previously reported that Bcl-2 can be degraded by the ubiquitin proteasome system, but the underlying molecular mechanism has remained obscure (Dimmeler et al., 1999; Kassi et al., 2009; Wang et al., 2008). In this study, we describe a mechanism by which cells are sensitized toward apoptosis through ubiquitin proteasome system-mediated degradation of Bcl-2. We show that the pro-apoptotic XIAP antagonist ARTS is required for downregulation of Bcl-2 levels upon induction of apoptosis. ARTS directly binds to the BH3 domain of Bcl-2 and enables the formation of a XIAP-ARTS-Bcl-2 complex. XIAP functions

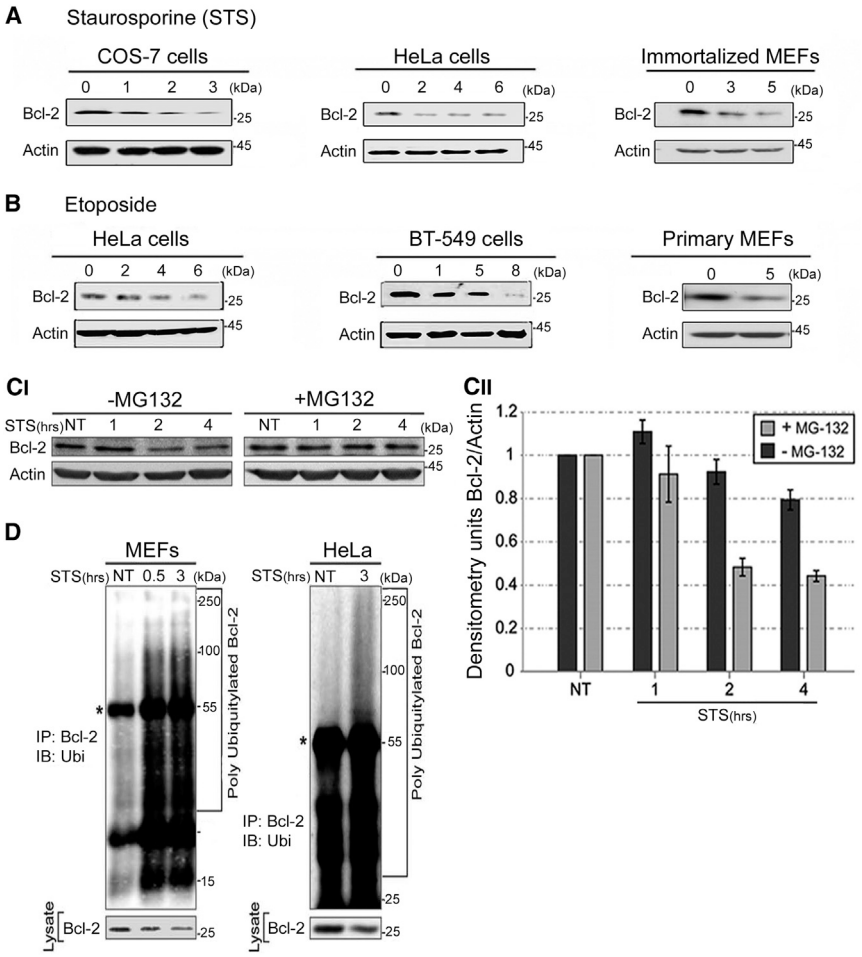

Figure 1. Bcl-2 Protein Levels Are Downregulated by the Ubiquitin Proteasome System during Apoptosis

(A) Apoptosis was induced in COS-7 cells and HeLa cells and immortalized MEFs using STS for the indicated times, and endogenous Bcl-2 was detected by western blot analysis.

(B) Apoptosis was induced in HeLa, BT-549, and COS-7 cells with etoposide. In primary MEFs, apoptosis was induced with 100 μM etoposide for 5 hr. A decrease in endogenous Bcl-2 levels was seen upon treatment with both STS and etoposide. NT, no treatment.

(CI) Apoptosis was induced in HeLa cells using STS in the presence or absence of 20 μM of MG132.

(CII) Western blot and densitometry analyses revealed decreased levels of Bcl-2 with STS treatment and stabilization of Bcl-2 upon MG132 treatment. Error bars represent ± SEM of three biological independent experiments. This suggests that Bcl-2 levels are downregulated via the ubiquitin proteasome system.

(D) WT MEFs and HeLa cells were transiently transfected with Bcl-2, XIAP, and ubiquitin and treated with 20 μM MG-132 for 6 hr and with 1.75 μM STS. Immunoprecipitation with anti-Bcl-2 was followed by western blotting with anti-ubiquitin antibodies. The asterisk represents the immunoglobin (IG) heavy chain. Poly-ubiquitylated forms of Bcl-2 appeared in apoptotic cells and correlated with decreased Bcl-2 levels.

as an E3 ligase for Bcl-2 that binds and ubiquitylates lysine 17 in Bcl-2. Collectively, our data indicate that ARTS acts as a distinct Bcl-2 antagonist that brings Bcl-2 into a ternary complex with XIAP and thereby stimulates ubiquitin proteasome system-mediated degradation of Bcl-2 to promote apoptosis.

RESULTS

Bcl-2 Protein Levels Are Downregulated by the Ubiquitin Proteasome System during Apoptosis

Levels of Bcl-2 decrease during apoptosis, but the underlying mechanism remains to be elucidated. (Dimmeler et al., 1999; Kassi et al., 2009; Wang et al., 2008). We confirmed that Bcl-2 levels are decreased upon induction of apoptosis as early as 1–2 hr following staurosporine (STS) and etoposide treatment (Figures 1A, 1B, and 2B). To examine whether the observed reduction in Bcl-2 is due to degradation via the ubiquitin proteasome system, HeLa cells were pre-treated with MG132, a potent proteasome inhibitor, and with STS to induce apoptosis. Accumulation of Bcl-2 was observed upon treatment with MG132, suggesting that induction of apoptosis results in ubiquitin proteasome system-mediated degradation of Bcl-2 (Figure 1C). Next we investigated whether in vivo ubiquitylation of Bcl-2 occurs upon induction of apoptosis. Both mouse embryonic fibroblasts (MEFs) and HeLa cells pre-treated with MG132 showed accumulation of poly-ubiquitylated Bcl-2 upon induction of apoptosis (Figure 1D). The appearance of poly-ubiquitylated Bcl-2 was correlated with decreased Bcl-2 levels in apoptotic cells (Figure 1D). This suggests that Bcl-2 is downregulated through ubiquitin proteasome system-mediated degradation during apoptosis.

ARTS Is Required for Downregulation of Bcl-2 Levels in the Cytosol

High levels of ARTS are sufficient to promote apoptosis in a variety of cell lines, and inactivation of ARTS protects against apoptosis (Edison et al., 2012b; García-Fernández et al., 2010; Lotan et al., 2005). To determine whether ARTS is required for reducing Bcl-2 protein levels, we used HeLa cells in which ARTS expression was knocked down with short hairpin RNA (shRNA) (ARTS knockdown (KD) HeLa cells; Edison et al., 2012b) and MEFs from Sept4/ARTS knockout (KO) mice (García-Fernández et al., 2010; Kissel et al., 2005). All of these cells exhibited a significant increase in the steady-state levels of endogenous Bcl-2 (Figure 2A). This indicates that ARTS restricts Bcl-2 levels in vivo and may therefore function as a Bcl-2 antagonist. Similarly, a decrease in Bcl-2 was seen in HeLa cells upon treatment with STS, whereas the levels of Bcl-2 in ARTS KD HeLa cells remained unchanged (Figure 2B). Thus, ARTS is

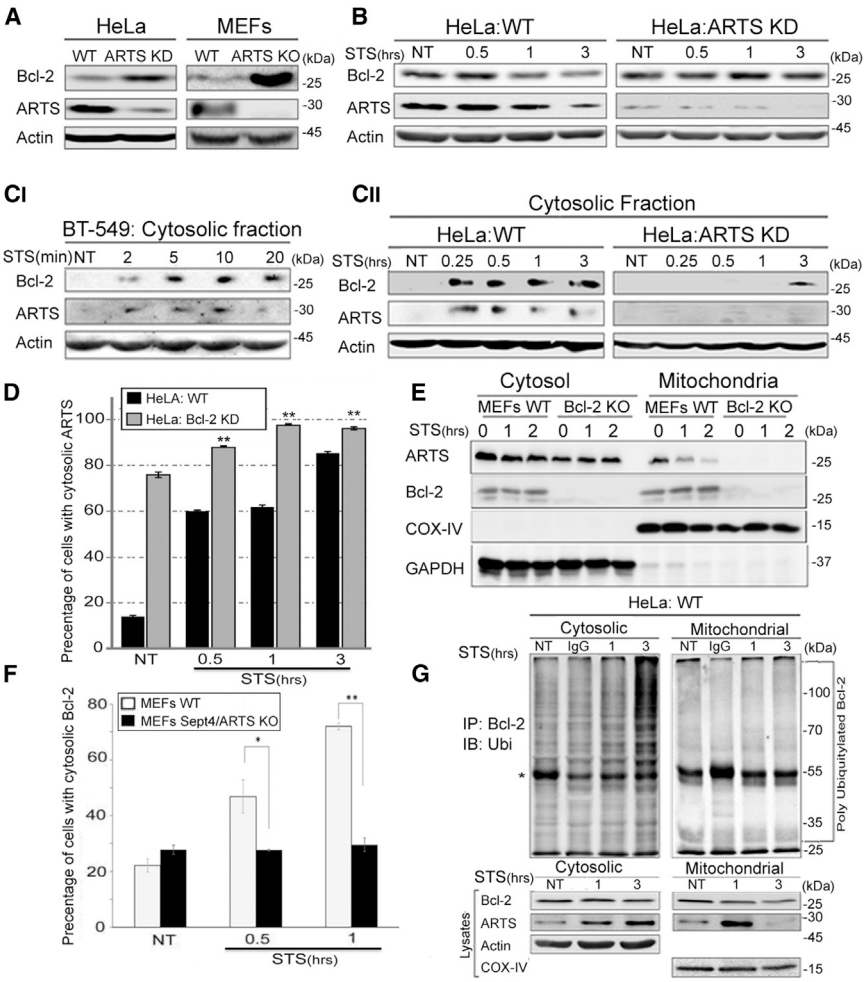

Figure 2. ARTS Is Required for Downregulation of Bcl-2 Levels in the Cytosol

(A) HeLa ARTS knockdown (KD) cells and Sept4/ARTS KO MEFs show significantly higher levels of steady-state Bcl-2 protein compared with WT cells. This suggests that ARTS plays an important role in regulating Bcl-2 levels.

(B) WT and ARTS KD HeLa cells were treated with 1.75 μM STS. Western blot analyses demonstrate that, although decreased Bcl-2 levels were seen in apoptotic WT HeLa cells, Bcl-2 levels in ARTS KD HeLa cells remained unchanged.

(CI and CII) Western blot analyses of cytosolic fractions of BT-549, HeLa WT, and HeLa ARTS KD cells reveal that endogenous Bcl-2 is found in the cytosol of WT STS-treated cells. In contrast, a strong inhibition in translocation of Bcl-2 to the cytosol was seen in ARTS KD HeLa cells. This suggests that ARTS is required for the proper translocation of Bcl-2 from mitochondria to the cytosol upon apoptotic induction.

(D) Immunofluorescence (IF) was performed on HeLa and stable Bcl-2 KD cells. The fraction of cells with cytosolic staining of ARTS is represented in the bar chart. Although only a small portion of WT NT HeLa cells show the presence of ARTS in the cytosol, a significant increase in cells containing cytosolic ARTS was seen following STS treatment. In contrast, the majority of HeLa Bcl-2 KD NT cells exhibit cytosolic ARTS (4-fold higher than WT HeLa cells), and only a slight increase in cells with cytosolic ARTS is seen after STS treatment. Error bars represent ± SEM of three biological independent experiments. (**$p \leq 0.01$). See also Figure S1.

(E) Cytosolic and mitochondrial fractions of WT MEFs and Bcl-2 KO MEFs were analyzed by western blot (WB) analysis with COX IV as a mitochondrial marker and GAPDH as a cytosolic marker. In Bcl-2 KO MEFs, the majority of ARTS was in the cytosol. This suggests that Bcl-2 is involved in localizing ARTS to mitochondria.

(F) Immunofluorescence of WT MEFs and Sept4/ARTS KO MEFs transiently transfected with GFP-Bcl-2. Cellular localization of Bcl-2 was quantified, and the fraction of cells with cytosolic Bcl-2 is shown in the bar charts. Although a significant increase in cytosolic Bcl-2 was seen in apoptotic WT MEFs, the levels of cytosolic Bcl-2 in Sept4/ARTS KO MEFs remained unchanged. Error bars represent ± SEM of three biological independent experiments (*$p \leq 0.05$; **$p \leq 0.01$). See also Figure S1.

(G) Subcellular fractionation of HeLa cells was followed by in vivo ubiquitylation of each fraction. Immunoglobulin G (IgG) represents the control cells incubated with non-specific IgG. Poly-ubiquitylated forms of Bcl-2 were seen only in the cytosolic fraction. The asterisk represents the IG heavy chain. This indicates that ubiquitylation of Bcl-2 occurs in the cytosol and that ARTS is required for translocation of Bcl-2 to the cytosol during apoptosis.

required for downregulation of Bcl-2 upon induction of apoptosis.

Both ARTS and Bcl-2 are localized at the mitochondrial outer membrane (Edison et al., 2012b; Volkmann et al., 2014). Shortly following induction of apoptosis, ARTS translocates to the cytosol, where it binds XIAP and initiates apoptosis (Edison et al., 2012b). This translocation occurs prior to mitochondrial outer membrane permeabilization (MOMP), an event that allows the release of other pro-apoptotic factors such as Smac/Diablo and cytochrome c from the inner membrane space of the mitochondria (Edison et al., 2012b). Cellular fractionation assays showed a concomitant appearance of Bcl-2 and ARTS in the cytosol of apoptotic cells (Figure 2C). This translocation of both Bcl-2 and ARTS to the cytosol occurred very rapidly after 15 min of addition of STS in HeLa cells (Figure 2C). ARTS is localized at the mitochondrial outer membrane, but it does not contain a trans-membrane domain (Edison et al., 2012b). We investigated whether Bcl-2 plays a role in the localization of ARTS to the mitochondrial outer membrane. Immunofluorescence (IF) staining showed that, although ARTS was seen in the cytosol in 18% of wild-type (WT) HeLa cells, ARTS was cytosolic in the majority of Bcl-2 KD cells (78%; Figure 2D; Figure S1A). Similarly, the vast majority of ARTS in Bcl-2 KO MEFs was found in the cytosol, with no detectable levels in mitochondria (Figure 2E). This suggests that the mitochondrial localization of ARTS depends, at least in part, on Bcl-2. Furthermore, ARTS seems to be important for the translocation of Bcl-2 to the cytosol during apoptosis. Although a significant increase in cytosolic Bcl-2 was found in STS-treated WT MEFs, the levels of cytosolic Bcl-2 in apoptotic Sept4/ARTS KO MEFs remained unchanged (Figure 2F;

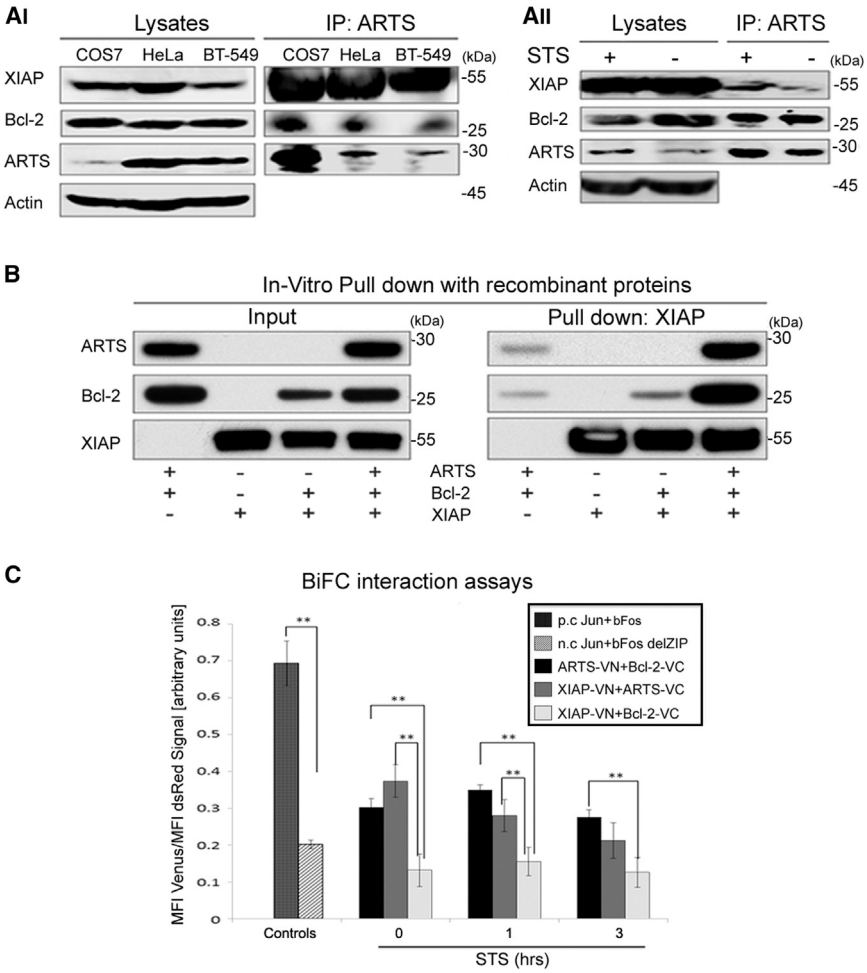

Figure 3. ARTS Is Required for Formation of a Ternary Complex with Bcl-2 and XIAP

(AI) Immunoprecipitation of ARTS in COS-7 cells transiently transfected with 6-Myc-ARTS using a monoclonal anti-ARTS antibody (Sigma-Aldrich). Endogenous ARTS was immunoprecipitated from HeLa and BT-549 cells. Western blot analyses show that, in COS-7, HeLa, and BT-549 cells, Bcl-2 and XIAP co-precipitate with ARTS.

(AII) HeLa cells were treated with 1.75 μM STS, and immunoprecipitation of ARTS was performed as described in (AI). Although binding of ARTS to Bcl-2 was seen in NT cells, binding of ARTS to XIAP and Bcl-2 was increased in STS-treated cells.

(B) Recombinant His-ARTS, Bcl-2, and GST-XIAP were incubated overnight at 4°C. Pull-down of GST-XIAP shows that binding of Bcl-2 to XIAP depends on ARTS.

(C) To assess the proximity of proteins, consistent with complex formation, we used BiFC. HeLa cells were transiently transfected with ARTS, Bcl-2, and XIAP fused to parts of YFP-Venus. Jun and bFos, known to form heterodimers, served as a positive control (p.c.). Jun and bFosdelZIP lacking the C-terminal half of bFos leucine zipper domain were used as a negative control (n.c.). The fluorescent signal indicating the proximity of each pair of proteins was measured by flow cytometry. MFI, mean fluorescence intensity. Fluorescence-activated cell sorting (FACS) results were normalized to the readings of the transfection efficiency reporter (pdsRED). Error bars represent ± SEM of three biological independent experiments (**$p \leq 0.01$). The y axis represents the ratio between YFP fluorescence (reflecting binding of a pair of proteins) and red fluorescence (marking transfected cells). FACS analyses reveal that ARTS can bind to both Bcl-2 and XIAP. However, only background levels of fluorescence were seen with XIAP and Bcl2, suggesting that these two proteins do not bind each other. These results indicate that ARTS, XIAP, and Bcl-2 form a ternary complex and that ARTS is required for the formation of this complex.

See also Figure S2.

Figure S1B). Finally, we observed accumulation of poly-ubiquitylated Bcl-2 in the cytosol, with a concomitant decrease of Bcl-2 protein in the mitochondrial fraction (Figure 2G). Taken together, these results indicate that ARTS is required for the ubiquitylation, translocation, and degradation of Bcl-2.

ARTS Is Required for the Formation of a Ternary Complex with Bcl-2 and XIAP

Because ARTS binds directly to XIAP, we examined the possibility that ARTS can bind to both XIAP and Bcl-2 and form a ternary complex. Immunoprecipitation (IP) experiments with an anti-ARTS monoclonal antibody suggest that ARTS can indeed form a complex with both XIAP and Bcl-2 (Figures 3AI and 3AII). Furthermore, although strong binding of ARTS to Bcl-2 was seen both in untreated and apoptotic cells, an increase in binding to XIAP was observed upon induction of apoptosis (Figure 3AII). This implies that, although ARTS and Bcl-2 bind to each other under normal conditions at the mitochondrial outer membrane, upon induction of apoptosis, they can form a ternary complex with XIAP that leads to ubiquitylation and degradation of Bcl-2. Moreover, using an in vitro binding assay with recombinant proteins, we observed a significant and strong formation of a ternary complex only when recombinant ARTS was added (Figure 3B).

Next we used the bimolecular fluorescence complementation (BiFC) split-Venus assay (Li et al., 1998). The proximity between each pair of proteins was measured by flow cytometry. These results confirm our in vitro binding data (Figure 3B), showing that, although ARTS can bind directly to both XIAP and Bcl-2, the latter two proteins did not bind each other directly (Figure 3C; Figure S2). Together, these results show that ARTS binds directly to XIAP and that Bcl-2 enables the formation of a ternary complex in which ARTS serves as an adaptor to bring XIAP and Bcl-2 into close proximity to allow degradation of Bcl-2 by XIAP.

ARTS Interacts with the BH3 Domain of Bcl-2

To more precisely define the interaction of ARTS and Bcl-2, we used peptide array binding assays and structure-function analysis with deletion mutants of Bcl-2. First, to identify the

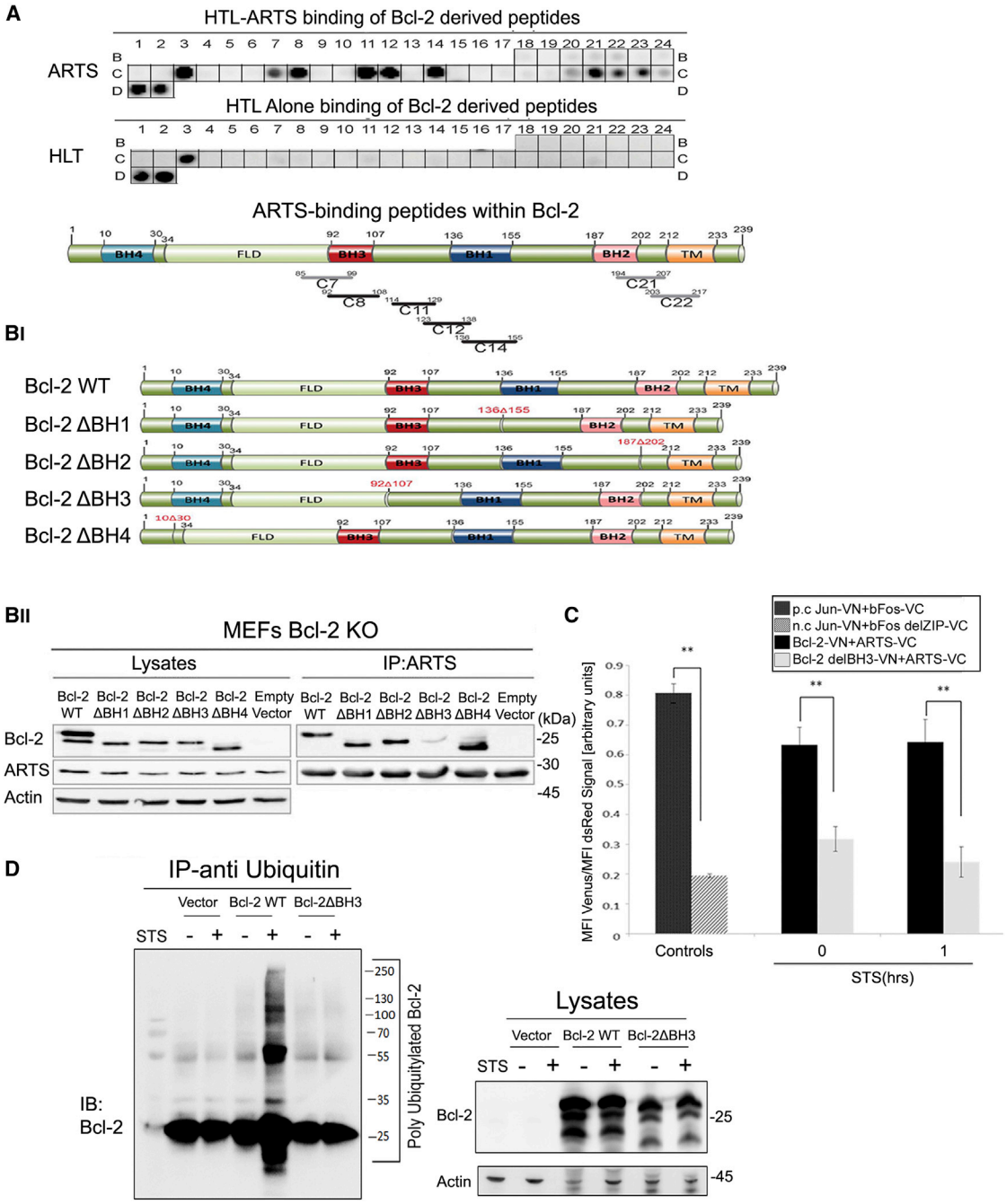

Figure 4. ARTS Binds to the BH3 Domain of Bcl-2

(A) A custom designed Bcl-2 derived peptide array (CelluSpot) was incubated with recombinant (His)6-Lipo-TEV (HLT)-tagged ARTS. The peptide array was also incubated with a recombinant HLT tag alone, which served as a negative control. The arrays were probed with the indicated antibodies. Bottom: the Bcl-2 peptides that bind to ARTS. These peptides are schematically represented across the Bcl-2 domains. The black lines indicate strong binding and the gray lines weak to moderate binding.

(B) Bcl-2 expression vectors. Full-length and four constructs deleting each BH domain in Bcl-2 are illustrated at the top (FLD, flexible loop domain; TM, transmembrane domain). These vectors were co-transfected with 6myc-ARTS into Bcl-2 KO MEFs. Immunoprecipitation of ARTS was performed in MEFs transfected with Bcl-2-BH deletions, WT Bcl-2 (positive control), and empty vector (negative control). A significant reduction in binding of ARTS to Bcl-2delBH3 was seen. See also Tables S1 and S2.

(C) BiFC assays were performed as described in Figure 3C. HeLa cells were co-transfected with vectors expressing ARTS, Bcl-2, and Bcl-2delBH3 fused to either VN or VC parts of the YFP Venus fragment (ARTS-VN, Bcl-2-VC, Bcl-2delBH3 –VC). A 47% decrease in the proximity between ARTS and Bcl-2delBH3 was seen

(legend continued on next page)

ARTS-binding motif in Bcl-2, we used the peptide array method. The array was screened for binding with recombinant $(His)_6$-Lipo-TEV (Tobacco Etch Virus) (HLT)-tagged ARTS protein. Bcl-2 peptides that showed binding to HLT-ARTS were concentrated around the BH3 binding cleft, the same binding region that binds to the BH3 domain of other Bcl-2 family members (Figure 4A; Tables S1 and S2). Next, immunoprecipitation experiments were performed with Bcl-2 WT and four constructs with deletions of different BH domains (BH-1, 2, 3, and 4). Immunoprecipitation assays revealed a significant decrease in binding of ARTS to the Bcl-2 mutant lacking the BH3 domain (Bcl-2delBH3) (Figure 4B). Moreover, BiFC assays also showed strongly reduced interaction of Bcl-2delBH3 with ARTS compared with WT Bcl-2 (Figure 4C; Figure S3). Additionally, Bcl-2delBH3 showed a major reduction in its ability to undergo ubiquitylation compared with WT Bcl-2 upon induction of apoptosis (Figure 4D; Figure S4). Furthermore, the BH3-mimetic ABT-199 can attenuate the interaction of Bcl-2 and ARTS, indicating that the BH3-binding motif to which ABT-199 binds in Bcl-2 is important for the interaction with ARTS (Figure S6). Taken together, these results indicate that the BH3 domain of Bcl-2 is important for the interaction with ARTS and that this interaction is required to promote ubiquitylation and degradation of Bcl-2 during apoptosis.

XIAP Serves as an E3-Ligase for Bcl-2

Because ARTS, XIAP, and Bcl-2 can form a complex, we investigated whether the decrease in Bcl-2 levels upon induction of apoptosis depends on the catalytic activity of XIAP. For this purpose, we overexpressed either XIAP or a mutant plasmid lacking its RING domain (XIAPdelRING) (Schile et al., 2008), which abolishes its E3-ligase function, together with Bcl-2 in HeLa cells. Although exogenous XIAP reduced the levels of Bcl-2, Bcl-2 levels remained high in cells overexpressing XIAPdelRING (Figure 5AI). Furthermore, increased endogenous Bcl-2 levels were seen in primary XIAPdelRING MEFs compared with WT MEFs (Figure 5AII). This suggests that the E3-ligase catalytic activity of XIAP is required for regulating the levels of Bcl-2 and that XIAP functions as the physiological E3 ligase for Bcl-2. We also used a mutant version of ARTS deleted at its unique C-terminal part (Cdel-ARTS) that cannot bind to XIAP (Gottfried et al., 2004a). Sept4/ARTS KO MEFs expressing WT ARTS exhibited decreased levels of Bcl-2 upon apoptotic induction, whereas cells transfected with Cdel-ARTS had increased levels of Bcl-2 (Figure 5B). Next, in vitro ubiquitylation assays were performed with recombinant Bcl-2 and XIAP together with E1, E2-UbcH5b, and ubiquitin. These experiments showed that ubiquitylation of Bcl-2 occurred only when XIAP was present (Figure 5C). Furthermore, in vitro ubiquitylation assays with Bcl-2 and three other E3 ligases (cIAP1, Parkin, or Siah2) revealed that only XIAP was capable of ubiquitylating Bcl-2 (Figure 5D; Figure S5). Finally, we compared in vivo ubiquitylation of Bcl-2 in WT and XIAPdelRING MEFs (Figure 5E) and in WT and XIAP KO MEFs (Figure 5F). A strong reduction in accumulation of poly-ubiquitylated forms of Bcl-2 was seen in XIAP delRING and XIAP KO MEFs (Figures 5E and 5F, respectively). These results indicate that XIAP ubiquitylates Bcl-2 in a direct and specific manner. Furthermore, the catalytic activity of XIAP is necessary for ubiquitin proteasome system-mediated degradation of Bcl-2 upon induction of apoptosis. In these in vitro assays, the presence of recombinant XIAP and Bcl-2 is sufficient to ubiquitylate Bcl-2, presumably because of the high concentration of these proteins. In contrast, in living cells, ARTS is required for bringing XIAP and Bcl-2 into sufficiently close proximity to allow for XIAP-mediated ubiquitylation of Bcl-2 (see below). Collectively, these data show that XIAP serves as a physiological E3 ligase for Bcl-2.

ARTS Is Required for Ubiquitin Proteasome System-Mediated Degradation of Bcl-2 by XIAP

To investigate the requirement of ARTS for XIAP-mediated ubiquitylation of Bcl-2, we performed several in vitro and in vivo ubiquitylation assays. We observed a significant increase in the appearance of ubiquitylated forms of Bcl-2 upon addition of recombinant ARTS (Figure 6A). To examine whether ARTS is required for the early, pre-MOMP ubiquitylation and degradation of Bcl-2, we examined in vivo ubiquitylation of Bcl-2 as early as 30 min following STS treatment, which is several hours prior to MOMP (Adrain et al., 2001; Edison et al., 2012b; Gao et al., 2001). Significant accumulation of poly-ubiquitylated forms of Bcl-2 was seen 30 min after induction of apoptosis (Figure 6B). This indicates that ubiquitylation of Bcl-2 starts prior to MOMP and release of cytochrome *c* and Smac/Diablo.

To further establish that Bcl-2 ubiquitylation and degradation are dependent on the presence of ARTS, in vivo ubiquitylation assays were performed using Sept4/ARTS KO MEFs. Although no ubiquitylation of Bcl-2 was observed in the Sept4/ARTS KO MEFs, transfections of ARTS into these MEFs restored their ability to ubiquitylate and degrade Bcl-2 upon induction of apoptosis (Figure 6C). These results demonstrate that ARTS is required for the ubiquitylation of Bcl-2.

Lysine 17 Is the Main Ubiquitin Acceptor in Bcl-2

Bcl-2 contains four lysine residues (Figure 7A). To specifically identify the lysine in Bcl-2 that is ubiquitylated by XIAP, we performed mass spectrometry analysis on lysates of HeLa cells transfected with a GFP-Bcl-2 construct. Immunoprecipitation experiments with an anti-GFP antibody showed specific ubiquitylation of lysine 17 in Bcl-2 (Figure 7B). We conclude that K17 of Bcl-2 is an acceptor for XIAP-mediated ubiquitylation. To examine the role of K17, we mutated it to alanine (K17A). In addition, we also generated a Bcl-2 mutant in which all lysines were mutated (no K). Protein levels of both mutant forms of Bcl-2 were increased compared with the WT under apoptotic conditions

compared with WT Bcl-2. Jun/bFos served as a positive control and Jun/bFosdel ZIP as a negative control. Error bars represent ± SEM of three biological independent experiments (**$p \leq 0.01$). See also Figure S3.

(D) In vivo ubiquitylation of Bcl-2 WT and Bcl-2delBH3. MEF Bcl-2 KO cells were transfected with FLAG-Bcl-2WT or FLAG-Bcl-2delBH3. Cells were treated with MG132 and STS for 1 hr or left untreated. Proteins were immunoprecipitated with anti-ubiquitin. Poly-ubiquitylated forms of Bcl-2 were detected using an anti-Bcl-2 antibody. Although WT Bcl-2 underwent ubiquitylation following treatment with STS, no ubiquitylation of Bcl-2delBH3 was seen.
See also Figure S4.

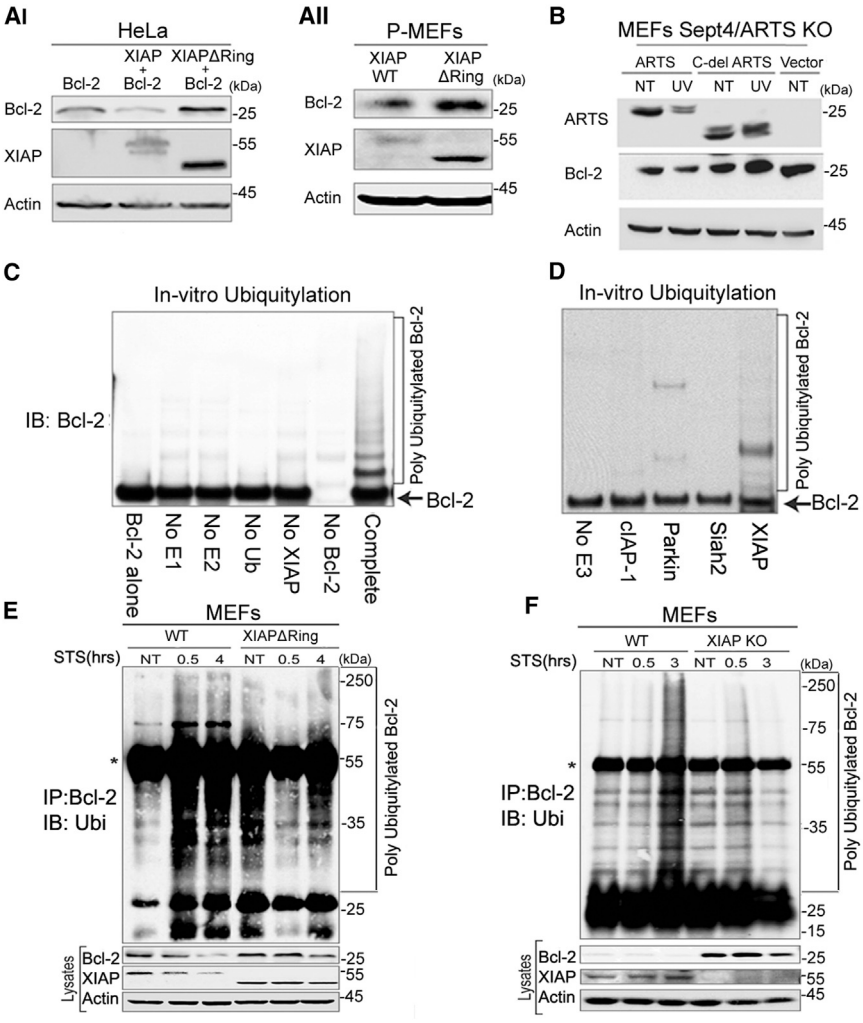

Figure 5. XIAP Serves as the E3 Ligase of Bcl-2

(AI) HeLa cells were transiently transfected with Bcl-2 or co-transfected with Bcl-2 and XIAP or with Bcl-2 and XIAPdelRING.

(AII) MEFs were produced from WT mice and from XIAPdelRING 14-day-old mouse embryos. WB and densitometry analyses revealed a significant accumulation of Bcl-2 in HeLa cells co-transfected with XIAPdelRING and in XIAPdelRING MEFs.

(B) Sept4/ARTS KO MEFs were transfected with WT ARTS and a mutant version of ARTS lacking its unique C terminus (Cdel-ARTS) that cannot bind to XIAP. Sept4/ARTS KO MEFs transfected with WT ARTS exhibit decreased levels of Bcl-2 following apoptotic induction (UV), whereas MEFs transfected with Cdel-ARTS have increased levels of Bcl-2.

(C) In vitro ubiquitylation assays were performed by incubating recombinant Bcl-2 with recombinant XIAP, E1, E2-UbcH5b, and ubiquitin. In the control reactions, the indicated components were excluded. Ubiquitylation of Bcl-2 was seen only upon addition of XIAP.

(D) In vitro ubiquitylation assays with purified Bcl-2 and recombinant XIAP, cIAP1, Parkin, or Siah2. Only addition of XIAP resulted in the ubiquitylation of Bcl-2. See also Figure S5.

(E) In vivo ubiquitylation in WT and XIAPdelRING MEFs. MEFs were co-transfected with ARTS, Bcl-2, and HA-ubiquitin. Cells were treated with 20 μM MG-132 for 6 hr and concomitantly treated with 1.75 μM STS, and immunoprecipitation was performed with an anti-Bcl-2 antibody. The asterisk represents the heavy chain of the antibody. Although significant ubiquitylation of Bcl-2 occurred after treatment with STS in WT MEFs, no ubiquitylation of Bcl-2 was seen in XIAPdelRING MEFs.

(F) In vivo ubiquitylation assays using WT and XIAP KO MEFs. No ubiquitylation of Bcl-2 was seen in XIAP KO MEFs. Collectively, these results show that XIAP serves as the specific E3-ligase for Bcl-2 and is required for its degradation.

(Figure 7C). Furthermore, three different markers of apoptosis (cleaved PARP, cleaved caspase-9, and cleaved caspase-3) showed a consistent decrease in cells expressing both Bcl-2 mutant forms compared with WT Bcl-2 (Figure 7C). Finally, expression of either Bcl-2 K17A or Bcl-2 No K was more effective in preventing caspase-3 cleavage than WT Bcl-2 upon induction of apoptosis (Figure 7D). Because mutation of either K17 or all lysines showed similar results, we conclude that lysine 17 is the main acceptor for ubiquitylation of Bcl-2. Collectively, these results suggest a distinct model for the regulation of Bcl-2 by ARTS-mediated degradation. According to this model, ARTS brings XIAP and Bcl-2 into a ternary complex that stimulates ubiquitylation and degradation of Bcl-2, thereby lowering the threshold of cells to undergo apoptosis.

DISCUSSION

Bcl-2 is a key cell death regulator (Vaux et al., 1988). Most efforts to understand the regulation of this protein have focused on interactions with other Bcl-2 family members (Adams and Cory, 2007; Chipuk et al., 2010; Youle and Strasser, 2008). Here we investigated the mechanism by which Bcl-2 protein levels are regulated at the onset of apoptosis. We show that XIAP serves as an E3 ligase for Bcl-2 to promote its degradation and that ARTS brings XIAP into a ternary complex with Bcl-2. This study focuses specifically on the mechanism by which ARTS and XIAP mediate proteasomal degradation of Bcl-2. It is possible that other Bcl-2 family members are regulated in a similar fashion, but this point remains to be investigated. This work reveals that ARTS functions as a distinct Bcl-2 antagonist that directly binds to the BH3 cleft of Bcl-2. By reducing Bcl-2 levels, ARTS acts upstream of MOMP to promote initiation of caspase activation and cell death.

Mitochondrial Regulation of Apoptosis

Mitochondrial apoptosis involves MOMP and the release of cytochrome *c* (Adrain et al., 2001; Edison et al., 2012b; Gao et al., 2001; Rehm et al., 2006; Strasser et al., 2011; Youle and

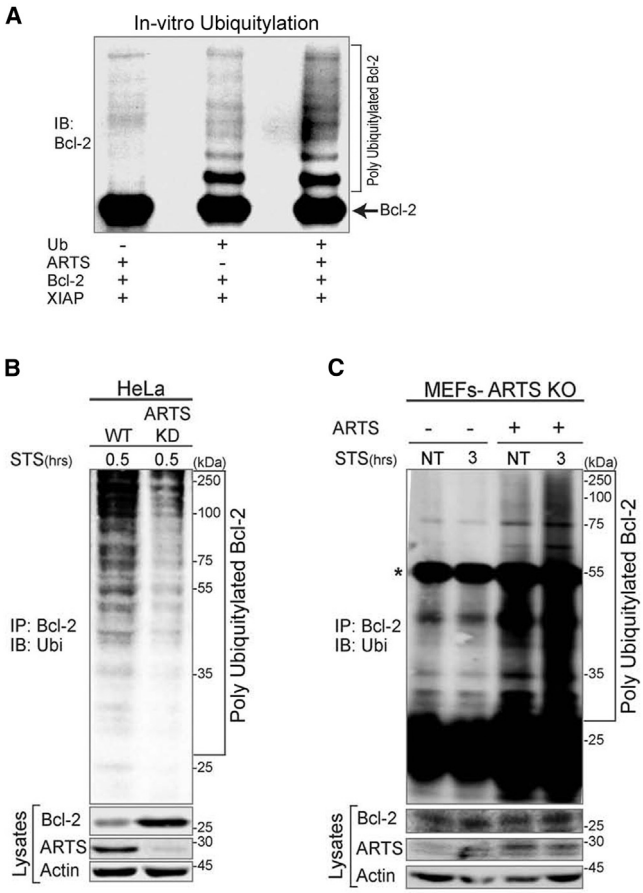

Figure 6. ARTS Is Required for Ubiquitylation of Bcl-2 by XIAP

(A) In vitro ubiquitylation assays were performed by incubating recombinant Bcl-2 with XIAP and ARTS, E1, E2-UbcH5b, and ubiquitin in the presence of ATP at 37°C for 1 hr. A significant increase in the appearance of ubiquitylated forms of Bcl-2 was seen in the presence of ARTS.

(B) HeLa WT or HeLa ARTS KD cells were transiently transfected with Bcl-2, XIAP, and ubiquitin and treated with 20 μM MG132 for 6 hr and STS for 0.5 hr. Bcl-2-ubiquitin conjugates were seen in apoptotic WT HeLa cells but not in ARTS KD HeLa cells.

(C) In vivo ubiquitylation assays of Sept4/ARTS KO MEFs transfected with either empty vector or ARTS expression vector, followed by treatment with MG132 and STS for 3 hr. The asterisk represents the IgG heavy chain. Although no ubiquitylation of Bcl-2 was observed in the Sept4/ARTS KO MEFs, transfection of ARTS restored their ability to generate poly-ubiquitylated forms of Bcl-2 upon induction of apoptosis.

Strasser, 2008). Bcl-2 family members can control apoptosis through their pore-forming abilities, which enable MOMP. During MOMP, mitochondrial pro-apoptotic proteins, including Smac/Diablo and cytochrome *c*, are released into the cytosol and stimulate caspase activation (Edison et al., 2012b; Strasser et al., 2011; Youle and Strasser, 2008).

We previously reported that ARTS is important for the initiation of apoptosis upstream of MOMP and that ARTS is required for the proper release of cytochrome *c* and Smac/Diablo (Edison et al., 2012b). The current study extends these results and further suggests a mechanism by which ARTS can promote MOMP by stimulating ubiquitin proteasome system-mediated degradation of Bcl-2. Additionally, this study also explains several other previous observations. First, ARTS resides in the mitochondrial outer membrane in living cells, but it does not contain any known trans-membrane domain. Because Bcl-2 is inserted into the mitochondrial outer membrane, and ARTS can bind directly to Bcl-2, this association offers a possible explanation for the localization of ARTS to the mitochondrial outer membrane. Consistent with this idea, ARTS is mostly in the cytosol in Bcl-2 KD cells. Upon induction of apoptosis, both ARTS and Bcl-2 translocate to the cytosol (Figures 2C and 2F) and are subsequently degraded. Importantly, we show that XIAP acts as an E3 ligase for Bcl-2 to promote its degradation. On the surface, these results are unexpected because they suggest a pro-apoptotic activity of XIAP. The general accepted view is that XIAP has anti-apoptotic function. This is based on the ability of XIAP to inhibit caspases in vitro because overexpression of XIAP can inhibit cell death and because genetic inactivation of XIAP in mice facilitates apoptosis in at least some paradigms (García-Fernández et al., 2010; Jost et al., 2009; Schile et al., 2008). However, none of these observations are inconsistent with the proposed pro-apoptotic function of XIAP via targeting Bcl-2. First, the available genetic evidence reveals only the net effect of XIAP overexpression/inactivation, and any pro-apoptotic roles of this protein may be masked by a more prominent anti-apoptotic function of XIAP. Second, dual functions have been reported for other proteins regulating cell death, including Bak, Bax, and Bcl-2 (Darding and Meier, 2012; Kirsch et al., 1999; Lewis et al., 1999). We reconcile these diverse findings by proposing that XIAP has two opposing functions for the regulation of apoptosis. First, in its "classic" function, XIAP behaves as an anti-apoptotic protein by binding to and inhibiting caspases. However, upon induction of apoptosis, when ARTS promotes the formation of a ternary complex with Bcl-2, the function of XIAP is "switched" to become pro-apoptotic because it promotes degradation of Bcl-2. According to this model, ARTS acts as a switch to change the activity of XIAP from anti-apoptotic to pro-apoptotic. Because complete loss of XIAP function sensitizes at least certain cells toward apoptosis, we conclude that, in the paradigm studies so far, the anti-apoptotic function of XIAP is more dominant than its pro-apoptotic function (Edison et al., 2012b; Fuchs et al., 2013; García-Fernández et al., 2010). However, our work raises the intriguing possibility that there may be conditions where the net effect of eliminating XIAP may produce the opposite outcome.

Several key observations support this model. First, down-regulation of Bcl-2 already occurs 30–60 min after induction of apoptosis by STS, whereas the release of cytochrome *c* and Smac/Diablo occurred only after 3 hr (Figures 1D and 2B). Furthermore, translocation of Bcl-2 to the cytosol and ubiquitylation of Bcl-2 were observed as early as 30 min after STS induction (Figures 2CII and 2F). Moreover, ARTS KD HeLa cells exhibit a significant inhibition in MOMP and the release of cytochrome *c* and Smac/Diablo (Edison et al., 2012b). Finally, inactivation of ARTS by either KD in HeLa cells or deletion of Sept4/ARTS in mice can inhibit apoptosis (Edison et al., 2012b; Fuchs et al., 2013; García-Fernández et al., 2010).

It appears that ARTS can promote MOMP through more than one mechanism. First, ARTS stimulates cleavage of Bid, which is

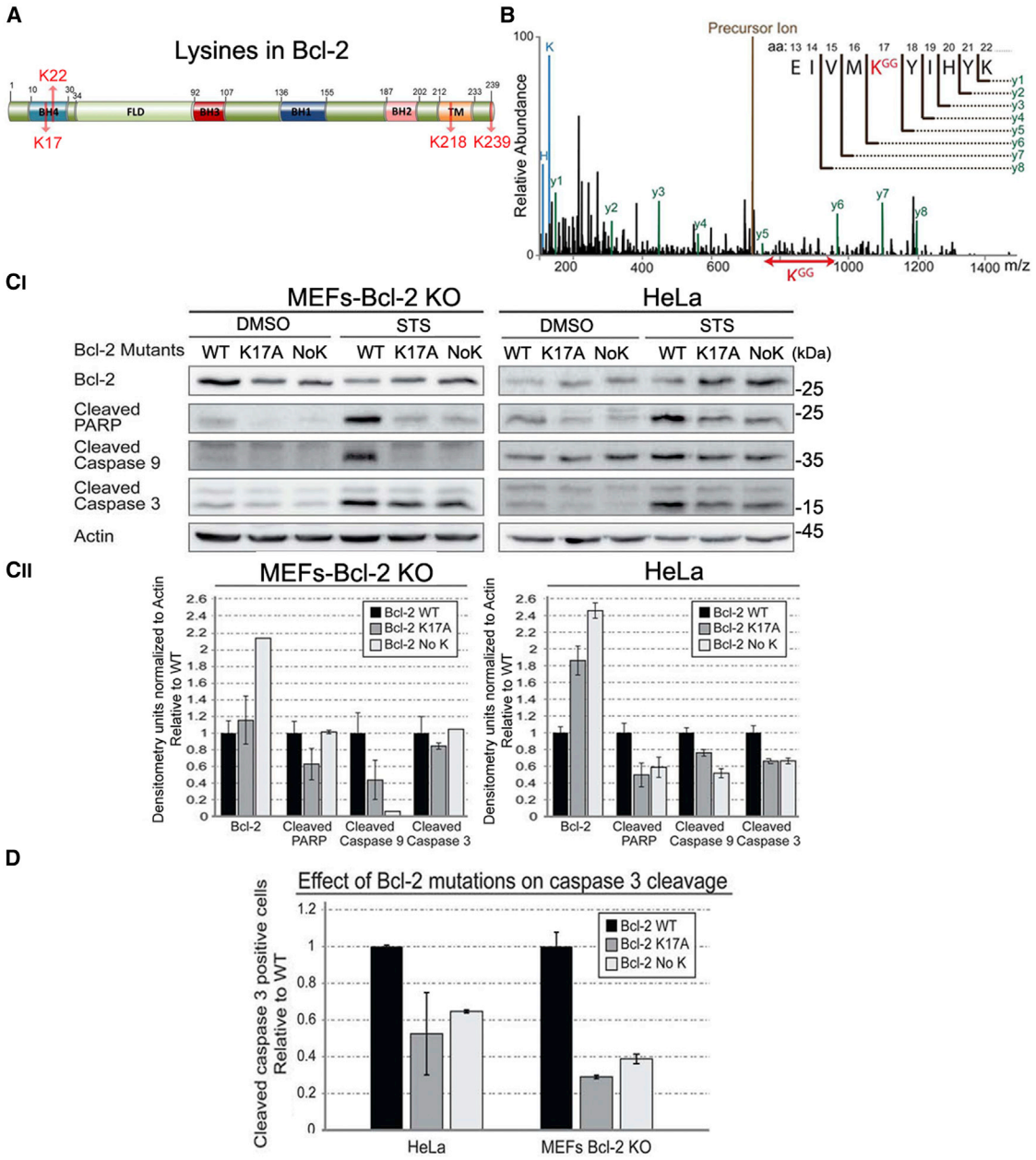

Figure 7. Lysine 17 Is the Main Ubiquitin Acceptor in Bcl-2

(A) Schematic of Bcl-2, highlighting its four lysine residues.

(B) Tandem mass spectrometry (MS/MS) spectrum spanning the ubiquitylation site in Bcl-2. The trypsin-cleaved peptides were enriched for Gly-Gly peptides using the K-ε-GG kit and subjected to liquid chromatography-mass spectrometry separation. The precursor ion (brown) represents the uncleaved GFP-Bcl-2. Lines represent the relative abundance of detected peptides. Black lines represent peptides that are not cleaved products of the precursor ion (y1-y6) and the precursor ion of the un-fragmented peptide (brown). Green lines (y1-y8) represent the cleaved GFP-Bcl-2 peptides and their corresponding amino acid sequences. K-GG (red) represents an ubiquitylated lysine. This analysis identified lysine 17 in Bcl-2 as the acceptor for XIAP-mediated ubiquitylation.

(C) MEFs Bcl-2 KO and HeLa cells were transfected with expression vectors for WT Bcl-2 (WT), Bcl-2 containing a substitution mutation of lysine 17 into alanine (K17A), and Bcl-2, in which all lysines were changed to alanine (No K). Increased levels of mutant Bcl-2 (K17A, No K) were seen upon apoptotic induction. This was accompanied by a decrease in apoptosis, as shown with three different apoptotic markers. Densitometry analyses are shown at the bottom.

(D) Bcl-2 KO MEFs and HeLa cells were transfected with Bcl-2 as in (C) together with a GFP-cleaved caspase-3 reporter. Bcl-2 KO MEFs and HeLa cells expressing lysine mutants had significantly less cleaved caspase 3-positive cells compared with WT Bcl-2. Error bars represent ± SEM of three biological independent experiments. These results suggest that lysine 17 is the main acceptor for ubiquitylation of Bcl-2.

known to promote MOMP (Edison et al., 2012b; Gross et al., 1999b; Li et al., 1998; Lovell et al., 2008). Second, reduced levels of Bcl-2 protein are expected to facilitate MOMP through de-repression of Bax and Bak. Furthermore, upon apoptotic induction, components of the fission machinery co-localize with Bax, suggesting that MOMP is linked to mitochondrial fragmentation (Delivani et al., 2006; Martinou and Youle, 2011). We have shown that the BH3 domain of Bcl-2 is important for binding to ARTS (Figures 4A–4D; Figure S4). This is surprising because the BH3 domain in Bcl-2 is thought to mainly interact with BH3 domains of other Bcl-2 members. Using a peptide array method, we found that ARTS binds to the same Bcl-2-BH3 binding pocket as the pro-apoptotic Bax protein (Figure 4A). These results are further supported by our finding that the BH3 mimetic ABT-199 can attenuate the interaction of Bcl-2 and ARTS (Figure S6).

Intriguingly, it was previously suggested that "in addition to the post-mitochondrial apoptosome pathway, there are initiator caspases perhaps controlled fairly directly by Bcl-2, which act upstream of organelle damage to activate Bax and Bak, perhaps via cleavage of a Bid-like protein that interacts with them" (Cory et al., 2003). Our results support this postulate and provide evidence that ARTS acts upstream of MOMP by releasing pre-apoptosome active caspases through de-repression. This non-lethal amount of active caspase-9 can cleave Bid and promote MOMP (Edison et al., 2012b). In addition, ARTS also directly antagonizes Bcl-2 by promoting its degradation. Support for the importance of controlling Bcl-2 stability in the regulation of apoptosis comes from the identification of lysine 17 as the main ubiquitin acceptor in Bcl-2. Because mutating either K17 or all lysines gave virtually identical results (Figures 7C and 7D), we conclude that lysine 17 is the main acceptor for ubiquitylation of Bcl-2.

Implications for Cancer

Bcl-2 is overexpressed in different hematological malignancies and solid tumors (Kelly and Strasser, 2011; Youle and Strasser, 2008) Given the oncogenic potential of Bcl-2, its antagonists are expected to act as tumor suppressors (Cory and Adams, 2002). Here we show that ARTS can antagonize Bcl-2 by promoting the degradation of this protein. Significantly, ARTS functions as a tumor suppressor protein because ARTS expression is frequently lost in acute lymphoblastic leukemia patients, lymphoma, and hepatocellular carcinoma, and deletion of Sept4/ARTS in mice accelerates tumorigenesis (Elhasid et al., 2004; García-Fernández et al., 2010). Our study points to a distinct connection between ARTS and Bcl-2 in malignancies because silencing of ARTS may contribute to increased levels of Bcl-2.

Anticancer agents such as ABT-737, ABT-263, and ABT-199 that directly target Bcl-2-like pro-survival proteins by mimicking the BH3 domain have been developed (Baell and Huang, 2002; Fesik, 2005; Oltersdorf et al., 2005; Rutledge et al., 2002) Interestingly, we found that the BH3 mimetic ABT-199 can disrupt the interaction of Bcl-2 and ARTS, indicating that the BH3-binding motif to which ABT-199 binds in Bcl-2 is important for the interaction with ARTS (Figure S6). This activity of ABT-199 is expected to reduce degradation of Bcl-2, which has the potential to cause increased resistance toward apoptosis. However, because ABT-199 causes cell killing, it is clear that this compound potently inactivates the anti-apoptotic activity of Bcl-2, even when present at somewhat elevated levels. Because ARTS antagonizes both XIAP and Bcl-2, these features may be useful for developing compounds that target both Bcl-2 and XIAP for cancer therapy.

EXPERIMENTAL PROCEDURES

Antibodies

In all of our assays, we used the monoclonal anti-ARTS antibody (A4471, Sigma-Aldrich) which is the only currently commercially available antibody directed against the unique C terminus of ARTS binding specifically to ARTS (Sept4_i2) and not to the other Septin 4 protein product, Sept4_i1 (H5, PNUTL). The entire antibody list is detailed in the Supplemental Experimental Procedures.

Mammalian Cell Cultures and Treatments

COS-7 monkey fibroblast-like kidney cells and HeLa human cervical carcinoma cells were grown in DMEM with 4.5 g/L D-glucose. Media were supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 2 mM glutamine (Biological Industries). The KD ARTS HeLa stable cell line and Bcl-2-HeLa KD lines were established by using shRNAs as described by Edison et al. (2012a). Cells were grown in the presence of 0.5 mg/mL G418 (Sigma). The WT and XIAPdelRING-deficient MEFs were prepared from 14-day-old WT and XIAPdelRING-deficient mouse embryos as described by Schile et al. (2008). To induce apoptosis, COS-7, HeLa, MEF, and BT-549 (human breast epithelial carcinoma) cells were incubated with STS (Sigma-Aldrich) (1.75 μM for HeLa cells, 0.6 μM for BT-549 cells, 1.75 μM for MEFs, and 1.25 μM for COS-7 cells) for different time periods or with etoposide (Sigma-Aldrich) (200 μM for HeLa, BT-549, COS-7, and immortalized MEF cells and 100 μM for primary MEFs) for different time periods. For proteasome inhibition, the cells were incubated with 20 μM MG-132 for 6 hr.

Constructs and Transient Transfection of Cells

The entire constructs list is detailed in the Supplemental Experimental Procedures. For transient transfections, the following reagents were used according to the manufacturer's instructions: jetPEI (Polyplus), Transfectol (GeneChoice), Turbofect (Thermo Fisher Scientific), and PolyJet (SignaGen).

Western Blot Analysis

Western blot analysis was performed as described by Lotan et al. (2005). Visualization was performed using an LAS4000 luminescent image analyzer (Fujifilm), and densitometry analysis was performed with TotalLab TL100 graphic software.

Cell Fractionation Assay

Subcellular fractionation was done using a digitonin-based method as described by Adrain et al. (2001). Briefly, cells were harvested and centrifuged at 300 × *g* for 10 min, washed in Tris-buffered saline (TBS, 2.5 mM, pH 7.5), and re-pelleted. Cells were permeabilized for 5 min on ice with cytosolic extraction buffer (CEB) (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.2], and 1× complete protease inhibitor cocktail [Roche]) containing freshly prepared digitonin (200 μg/mL, D-5628, Sigma-Aldrich). The cytosolic fraction was isolated by collecting the supernatant after centrifugation at 1,000 × *g* for 5 min at 4°C. The mitochondrial fraction was washed in CEB with digitonin, CEB, and twice with PBS and resuspended in whole-cell extraction (WCE) buffer (25 mM HEPES [4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid] [pH 7.7], 0.3 M NaCl, 1.5 mM Mg $Cl2_2$, 0.2 mM EDTA, 0.1% Triton X-100, 100 μg/mL PMSF, and 1× complete protease inhibitor cocktail [Roche]).

IF Assays

Immunofluorescence assays were performed as detailed in the Supplemental Experimental Procedures. Briefly, cells were seeded in 24 wells. Following apoptotic induction (1.75 μM STS), cells were fixed with 4% paraformaldehyde.

Nuclei were stained with DAPI (157574, MP Biomedicals), mitochondria were visualized by MitoTracker Red CMXRos (M-7512, Thermo Fisher Scientific), and immunofluorescence staining was performed with anti-ARTS or anti Bcl-2 antibodies, followed by fluorescein isothiocyanate (FITC)-conjugated fluorescent secondary antibody. Image analysis was carried out using a fluorescence microscope (Nikon 50i). 300 cells from each sample were analyzed for cellular localization of ARTS or Bcl-2. Diffused staining of the protein not co-localized with MitoTracker was determined as cytosolic. Dotted staining of the protein co-localized to the MitoTracker staining was determined as localized to the mitochondria.

GFP-Caspase-3 Cleavage Reporter Assay

MEFs, Bcl2 KO, and HeLa cells were seeded on 13-mm round slides previously coated with fibronectin (5 mg/mL, Biological Industries). Cells were co-transfected with 0.25 μg DNA of the GC3AI plasmid and 0.25 μg DNA of Bcl-2 WT, Bcl-2 K17A, and Bcl-2 No K for 40 hr. GC3AI is a switch-on GFP-based reporter for caspase-3 cleavage (a kind gift from Prof. Binghui Li). Apoptosis was induced with 0.9 μM STS for 2 hr. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS, and stained with DAPI (157574, MP Biomedicals). 300 cells were counted in each slide; the percentage of positive GFP cells exhibiting cleaved caspase-3 was calculated of Bcl-2-transfected cells.

Binding Assays

In Vitro Pull-Down Binding Assays

Bacterially expressed His-ARTS, Bcl-2, and GST-XIAP (5 μg each) were co-incubated at 4°C overnight in 60 μL binding buffer (20 mM Tris [pH 7.6] and 1 mM DTT). GST-XIAP was pulled down after incubation (at 4°C for 2 hr) with 20 μL glutathione Sepharose 4B in the same buffer. After removal of unbound proteins, the resin was washed three times with the binding buffer. XIAP and associated proteins were electrophoresed and detected by western blotting with anti-ARTS (A4471, Sigma-Aldrich), anti-Bcl-2 (sc-492, N-19, Santa Cruz Biotechnology), and anti-XIAP (610763, BD Transduction Laboratories).

Co-Immunoprecipitation

Co-immunoprecipitation was performed as described previously (Gottfried et al., 2004a). Briefly, lysates were prepared in WCE buffer. 10 μg of anti-ARTS antibody or 2 μg of anti-Bcl-2 or anti-XIAP antibody was added to the protein lysate, and the samples were left rotating overnight at 4°C. The next day, agarose beads conjugated to protein A/G (Santa Cruz Biotechnology) were added for 4 hr. Samples were centrifuged at 4,000 rpm at 4°C for 5 min and washed five times with WCE buffer. Proteins were eluted from beads following 5 min of boiling in sample buffer and separated on 12.5% SDS-PAGE gel, followed by western blot analysis.

BiFC Assay

The split-Venus BiFC system (Li et al., 1998) was used to evaluate close proximity indicating possible direct binding between pairs of proteins. The assay was performed as detailed in the Supplemental Experimental Procedures. Briefly, the proteins were fused either to the N-terminal part of the Venus-YFP (yellow fluorescence protein) (VN) or the C-terminal part (VC). All Venus fragments were fused to the C-terminal sequences of these proteins. The Jun and bFos pair was used as a positive control (p.c.), and the Jun and bFosdeltaZIP pair was used as a negative control (n.c.). A vector encoding dsRed was used as a transfection efficiency marker. Venus and dsRed fluorescence were analyzed by flow cytometry as detailed in the Supplemental Experimental Procedures.

Protein Expression and Purification

The protein expression procedures for both the peptide array and in vitro ubiquitylation assays are detailed in the Supplemental Experimental Procedures.

Peptide Array Screening

Peptide arrays (CelluSpots) were custom-made and purchased from INTAVIS Bioanalytical Instruments (Köln, Germany) based on the sequence of human Bcl-2 and ARTS. The designed arrays included 33 Bcl-2 peptides and 41 ARTS peptides (Table S1) that are 9–21 residues long, partially overlapping, acetylated at their N termini, and attached to a cellulose membrane via their C termini through an amide bond. The array was screened for binding following incubation with the recombinant HLT-ARTS protein and Bcl-2 protein minus the C terminus (827-BC-050, R&D Systems). First, the peptide array was washed with HEPES buffer (HB) (25 mM HEPES [pH 7.4] and 150 mM NaCl) and HB supplemented with 0.05% Tween 20 (HB-T). The array membrane was incubated for 4 hr in blocking solution (BS) containing 2.5% BSA (Sigma-Aldrich) in HB-T. Blocking was followed by two washing steps in HB-T and two washing steps with HB. Purified HLT-tagged ARTS protein and His-tagged Bcl-2 protein were diluted in BS to final concentrations of ~2 μM and incubated with the array overnight at 4°C in the presence of 2 mM DTT. The binding was detected by incubation with either anti-ARTS or anti-Bcl-2 and anti-His antibodies in BS. Binding of antibodies to the peptide array was visualized as described in Western Blot Analysis.

In Vivo Ubiquitylation Assay

Cells were transiently transfected with different constructs as indicated in the text and figure legends and treated with 20 μM MG132 (Sigma-Aldrich) for 6 hr. The medium was aspirated, and the cells were washed with 1× PBS at room temperature. The cells were scraped in 200 μL of lysis buffer (1% SDS and 1 mM EDTA in PBS) containing protease inhibitor cocktail (Complete, Roche), 5 mM N-ethylmaleimide (NEM), and 5 mM iodoacetamide to preserve ubiquitin chains. The cells were homogenized by passing through a 25G needle (20 times) and boiled for 10 min after vigorous vortexing. 200 μL of renaturation buffer (2% Triton X-100, 0.5% Na deoxycholate, 1% BSA, and 1 mM EDTA in PBS) containing protease inhibitor cocktail (Complete, Roche), 5 mM NEM, and 5 mM iodoacetamide was added to the lysate. Following 15 min of centrifugation (10,000 × *g*, 4°C), the supernatant was transferred into a clean Eppendorf tube. In vivo ubiquitination assay using immunoprecipitation with anti-ubiquitin was performed as described above with 2 μg anti-ubiquitin antibody (sc-8017, P4D1, Santa Cruz Biotechnology). Poly-ubiquitylated forms of Bcl-2 were detected using anti-Bcl2 antibody (ab32124, Abcam).

In Vitro Ubiquitylation Assay

In vitro ubiquitylation was assayed as described previously (Kim et al., 2007). Briefly, purified Bcl-2 was incubated with E1, UbcH5b, ubiquitin (Ub), and the appropriate E3 in conjugation buffer (20 mM Tris-Cl [pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, and 1 mM DTT) containing 2 mM ATP at 37°C for 1 hr. In the control reactions, one component at a time was omitted, as specifically indicated in the legend. Changes of the molecular weight of Bcl-2 by ubiquitylation were detected by western blotting using anti-Bcl2 antibody (sc-492 N-19, Santa Cruz Biotechnology).

Mass Spectrometry Analysis of Ubiquitylated Bcl-2

Ubiquitylated Bcl-2 Isolation

HeLa cells were transfected with 3 μg of Bcl-2-GFP together with 3 μg of hemagglutinin (HA)-ubiquitin. 24 hr after transfection, the cells were treated with 20 μM MG132 for 6 hr. Apoptosis was induced using 1.75 μM STS for 30 min. The cells were counted and then lysed with 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) buffer (10 mM HEPES [pH 7.5], 1% CHAPS, and 150 mM NaCl) containing protease inhibitor cocktail (Roche). The lysates were precipitated with α-GFP magnetic beads (MBL International) for 18 hr. For eight million cells, 40 μL antibody-bead slurry was used. Beads were washed three times with CHAPS buffer. Detection of ubiquitylated Bcl-2 lysines is detailed in the Supplemental Experimental Procedures. Briefly, the anti-GFP bead-GFP-Bcl2 conjugates were digested with trypsin (Promega). Digested peptides were enriched for glycine-glycine peptides with the PTMScan Pilot Ubiquitin Remnant Motif (lysine-ε−glycine glycine [K-ε-GG]) kit (Cell Signaling Technology) according to the manufacturer's instructions. Peptide sequences were identified by liquid chromatography followed by mass spectrometry. The analysis of the mass spectrometry data detected the KGG-containing peptides.

Statistical Analysis

Densitometry analyses of the western blot results were performed using TotalLab TL100 graphic software. Image analysis was performed using the Imaris image analysis software. At least 300 cells were counted for each immunofluorescence sample. SE was obtained from three to four biologically

independent experiments. The indicated p values were calculated using Student's t test (*$p \leq 0.05$, **$p \leq 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.052.

AUTHOR CONTRIBUTIONS

Conceptualization, N.E. and S.L.; Methodology, H.T.K. and S.L.; Investigation, N.E., Y.C., N.P., D. Mamriev, N.K., N.C., D. Morgenstern, M.K., T.H.-R., J.K., and H.T.K.; Writing – Original Draft, S.L.; Writing – Review & Editing, H.T.K. and S.L.; Funding Acquisition, H.T.K. and S.L.; Resources, H.T.K. and S.L; Supervision, S.L.

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