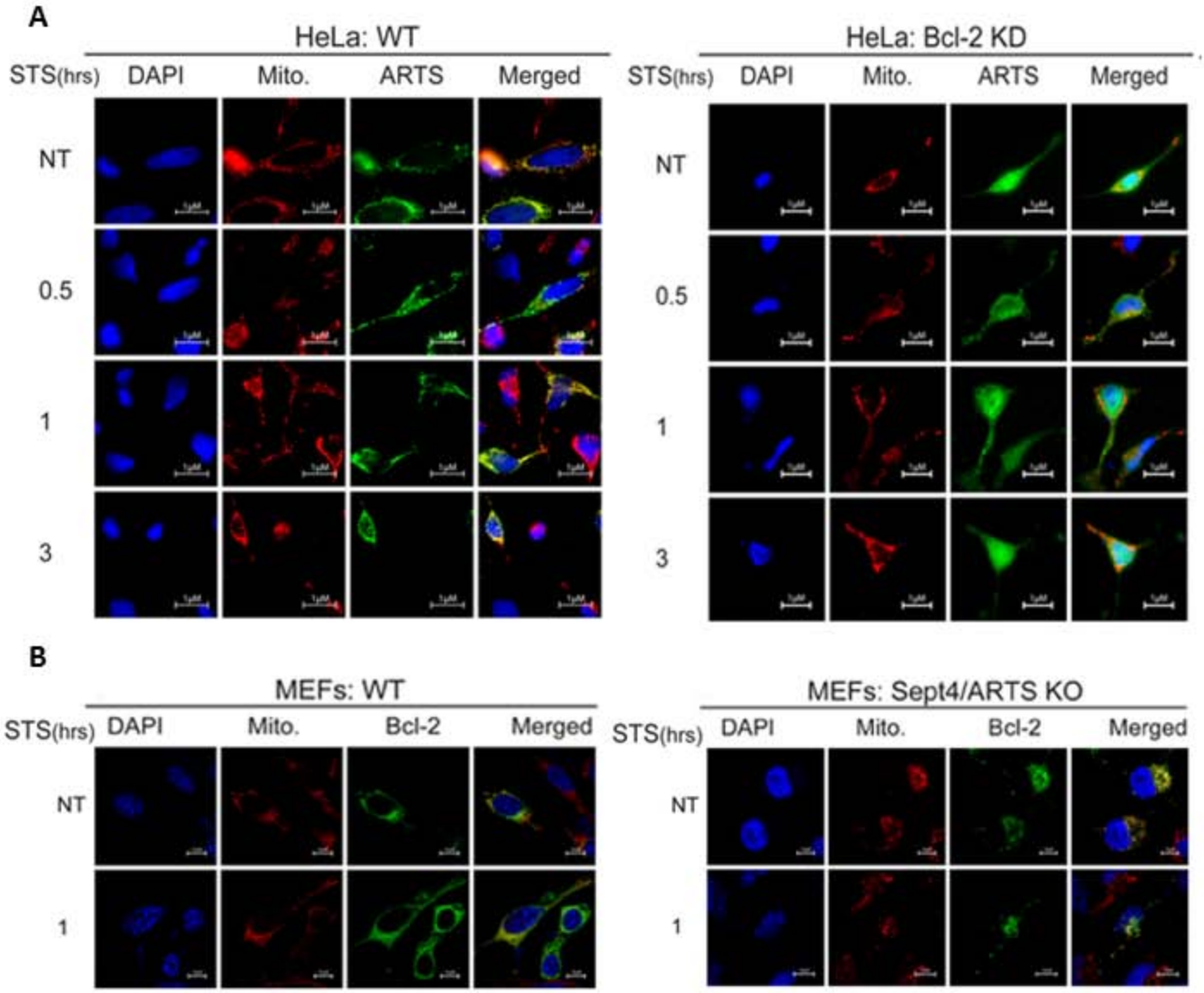
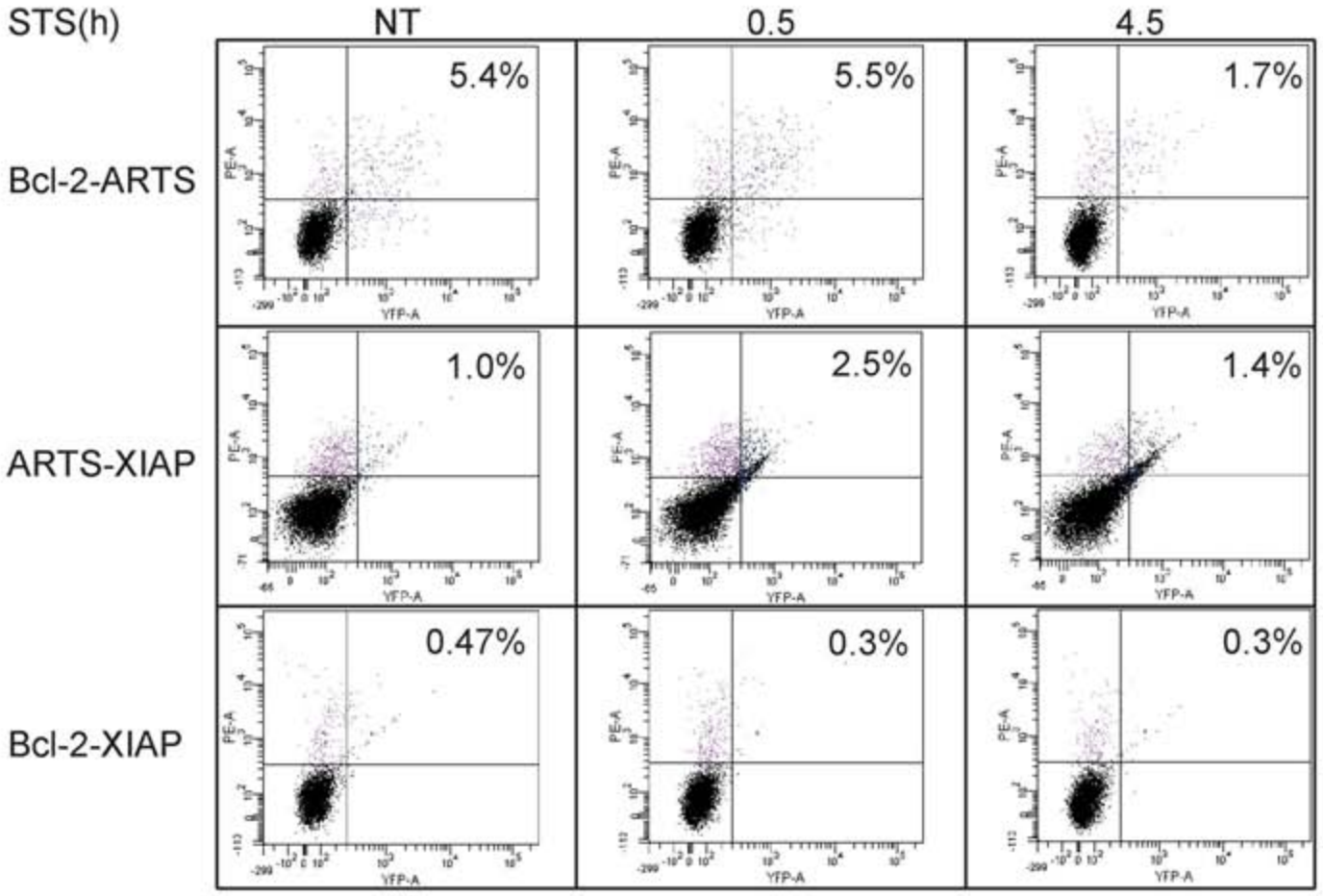
Supplemental Figure S1 related to Figure 2D and 2F:
ARTS is required for down-regulation of Bcl-2 levels in the cytosol



Supplemental Figure S1: A. Immunofluorescence assays (IF) were performed on HeLa and a stable Bcl-2 knockdown (Bcl-2 KD) HeLa cell line established using shRNAs against Bcl-2. Cells were transiently transfected with ARTS expression plasmids and apoptosis was induced using 1.75 μM staurosporine (STS) for the indicated times. Nuclei were visualized in blue using DAPI stain; Mitochondria were visualized in red using MitoTrackerTM. ARTS is seen in green following incubation with secondary FITC-antibody. Fluorescent images were analyzed and cells containing cytosolic and mitochondrial staining of ARTS were counted (See Experimental Procedures for details). While only a small portion of WT untreated (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 (four fold higher than WT HeLa cells). Only a slight increase in cells exhibiting cytosolic ARTS was seen after 30, 60 and 180 min of STS treatment. **B.** IF assays were performed on WT mouse embryonic fibroblasts (MEFs) and Sept4/ARTS KO MEFs transiently transfected with GFP-Bcl-2 (as described above). While a significant increase in cytosolic Bcl-2 is found in apoptotic WT MEFs, the levels of cytosolic Bcl-2 in Sept4/ARTS KO MEFs remain unchanged in NT and STS treated cells.

Supplemental Figure S2 related to Figure 3C:

ARTS binds to both XIAP and Bcl-2 directly whereas Bcl-2 and XIAP do not bind to each other



Supplemental Figure S2: Representative flow cytometry plots of BiFC assays show the percentage of cells expressing fluorescent YFP (Venus) and PE (Red) signals, indicating close proximity between each pair of proteins. FACS analyses revealed that ARTS can bind to both Bcl-2 and to XIAP directly. Yet, only background levels of fluorescence were seen for XIAP and Bcl-2, suggesting that these two proteins do not bind each other directly. These results indicate that ARTS, XIAP and Bcl-2 form a ternary complex, and that ARTS is required for the formation of this complex.

Supplemental tables S1, S2 related to Figure 4B:

Peptide array: Table S1- Bcl-2 peptides

Table S2- Bcl-2 peptides that binds ARTS

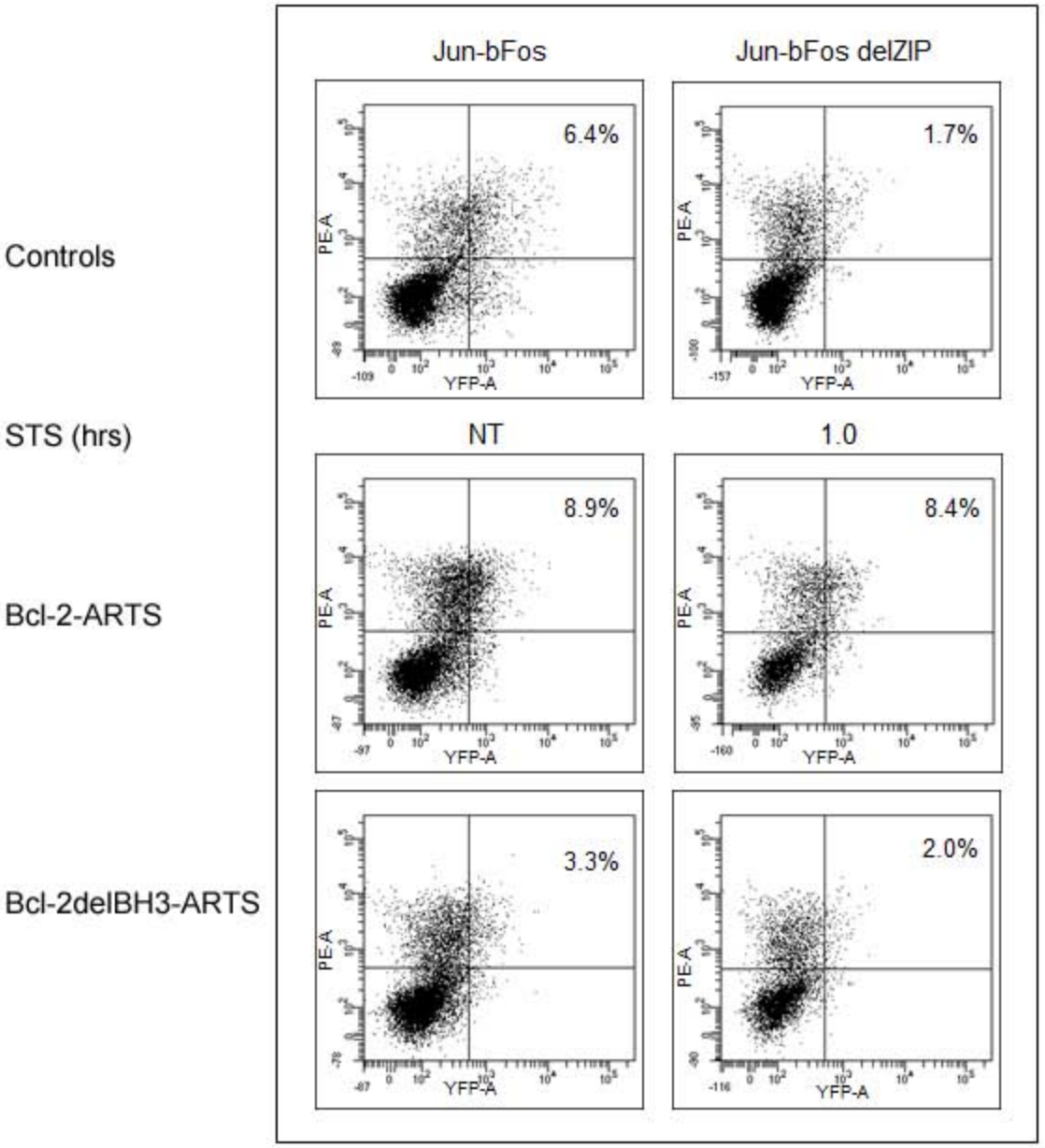
Table 1: Total Bcl-2 peptides synthesized on the peptide array:

Well #	Residue	Length	sequence
B18	1-9	9	MAHAGRTGY
B19	1-15	15	MAHAGRTGYDNREIV
B20	10-24	15	DNREIVMKYIHYKLS
B21	10-30	21	DNREIVMKYIHYKLSQRGYEW
B22	16-30	15	MKYIHYKLSQRGYEW
B23	25-39	15	QRGYEWDAGDVGAAP
B24	31-45	15	DAGDVGAAPPGAAPA
C 1	40-54	15	PGAAPAPGIFSSQPG
C 2	46-60	15	PGIFSSQPGHTPHPA
C 3	55-69	15	HTPHPAASRDPVART
C 4	61-75	15	ASRDPVARTSPLQTP
C 5	70-84	15	SPLQTPAAPGAAAGP
C 6	76-91	16	AAPGAAAGPALSPVPP
C 7	85-99	15	ALSPVPPVVHLTLRQ
C 8	92-108	17	VVHLTLRQAGDDFSRRY
C 9	100-113	14	AGDDFSRRYRRDFA
C10	109-122	14	RRDFAEMSSQLHLT
C11	114-129	16	EMSSQLHLTPFTARGR
C12	123-138	16	PFTARGRFATVVEELF
C13	130-143	14	FATVVEELFRDGVN
C14	136-155	20	ELFRDGVNWGRIVAFFEFGG
C15	144-163	20	WGRIVAFFEFGGVMCVESVN
C16	156-170	15	VMCVESVNREMSPLV
C17	164-178	15	REMSPLVDNIALWMT
C18	171-186	16	DNIALWMTEYLNRHL
C19	179-193	15	EYLNRHLHTWIQDNG
C20	186-202	17	HTWIQDNGGWDAFVELY
C21	194-207	14	GWDAFVELYGPSMR
C22	203-217	15	GPSMRPLFDFSWLSL
C23	208-223	16	PLFDFSWLSLKTLLSL
C24	218-232	15	KTLLSLALVGACITL
D 1	224-239	16	ALVGACITLGAYLGHK
D 2	233-239	7	GAYLGHK

Table 2: Bcl-2 peptides that bind HLT-ARTS

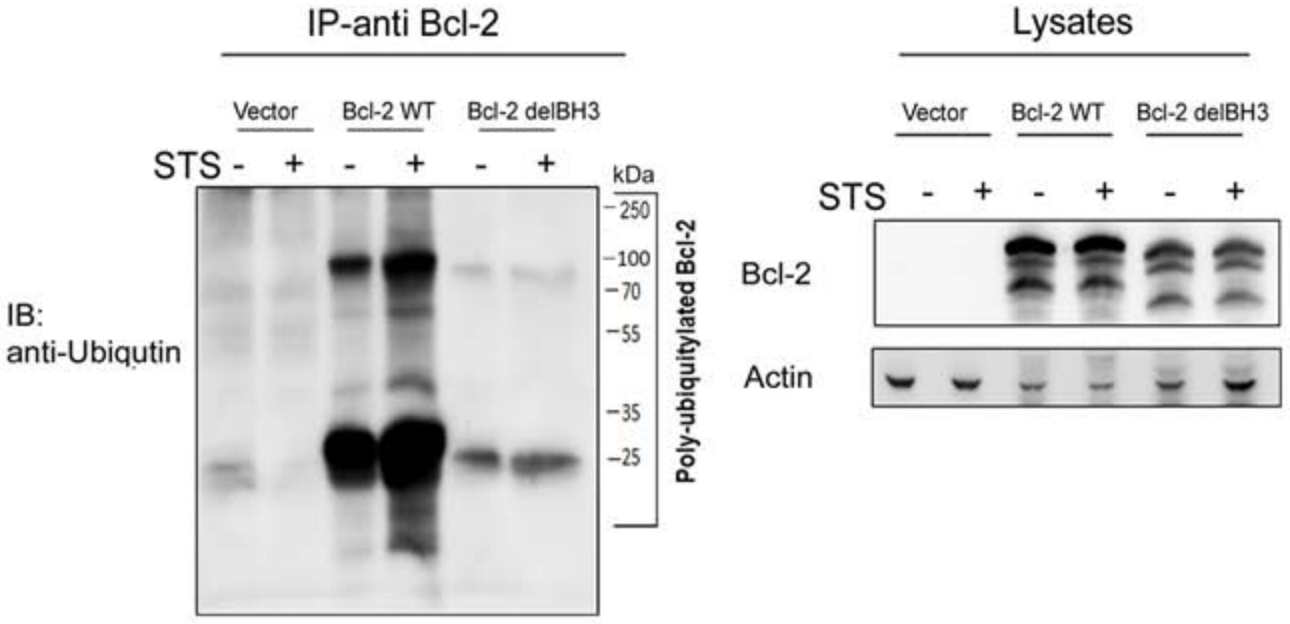
Well #	Residues	Length	Sequence	ARTS Binding
C 7	85-99	15	ALSPVPPVVHLTLRQ	weak
C 8	92-108	17	VVHLTLRQAGDDFSRRY	medium/strong
C11	114-129	16	EMSSQLHLTPFTARGR	medium/strong
C12	123-138	16	PFTARGRFATVVEELF	medium/strong
C14	136-155	20	ELFRDGVNWGRIVAFFEFGG	medium/strong
C21	194-207	14	GWDAFVELYGPSMR	weak
C22	203-217	15	GPSMRPLFDFSWLSL	weak

Supplemental Figure S3 related to Figure 4C:
ARTS binds to the BH3 domain in Bcl-2



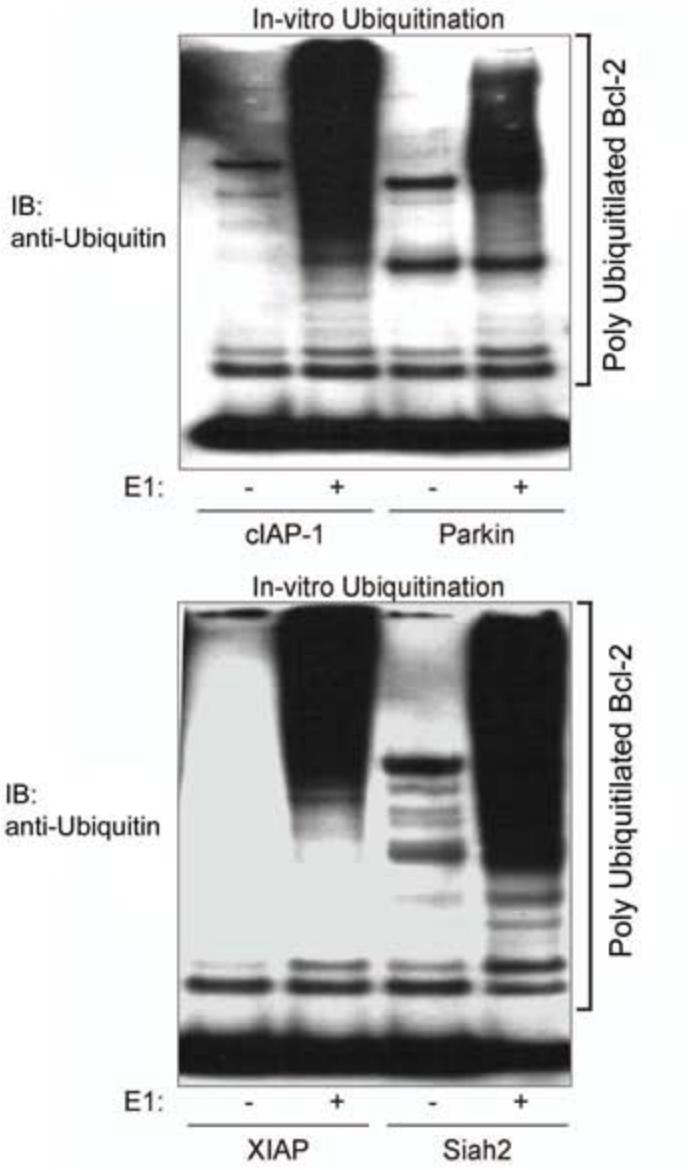
Supplemental Figure S3: Representative flow cytometry plots of BiFC assays show the percentage of cells expressing fluorescent YFP (Venus) and PE (Red) signals, indicating close proximity between each pair of proteins. While strong binding was seen between ARTS and WT Bcl-2, only background levels of fluorescence were observed between ARTS and Bcl-2delBH3 construct. This suggests that ARTS binding to Bcl-2 involves the Bcl-2 BH3 domain.

Supplemental Figure S4 related to Figure 4D:
BH3 domain in Bcl-2 is important for binding to ARTS and ubiquitylation of Bcl-2



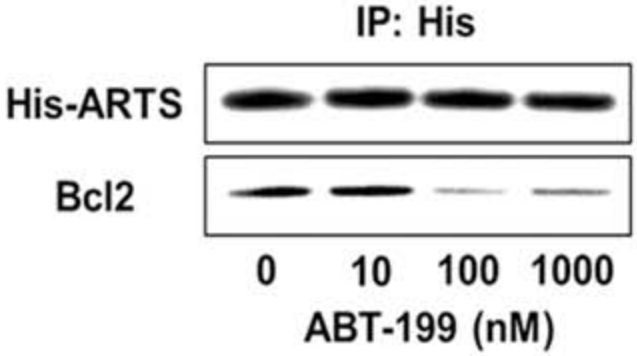
Supplemental Figure S4: In vivo ubiquitylation of Bcl-2 wt and Bcl-2delBH3. Bcl-2-KO MEFs were transfected with Flag-WT Bcl-2 or Flag-Bcl-2delBH3. Cells were treated with 20 μM MG132 for six hours and with 1.75 μM STS for one hour or left untreated. Proteins were extracted and Bcl-2 was immuno-precipitated using an anti-Bcl-2 antibody. The poly-ubiquitylated forms of Bcl-2 were detected using an antibody against ubiquitin. These results show that the BH3 domain of Bcl-2 is required for ubiquitylation of Bcl-2 in vivo.

Supplemental Figure S5 related to Figure 5D:
E3 ligases purified from bacteria are catalytically active



Supplemental Figure S5: E3 ligases purified from bacteria are catalytically active. Ubiquitylation activity of the E3-ligases cIAP-1, Parkin, XIAP and Siah 2 expressed in E.coli were tested in an in vitro assay. Each E3-ligase was incubated with E1, E2, Ubiquitin or ATP at 37 °C for one hour. Formation of Ub-conjugates was assayed by Western Blotting with anti-ubiquitin antibodies. In the negative controls E1 was not included. All tested E3-ligases showed ubiquitylation activity.

Supplemental Figure S6:
The BH3 mimetic ABT-199 attenuates binding of Bcl-2 to ARTS



Supplemental figure S6: The BH3 mimetic ABT-199 interferes with binding of Bcl-2 to ARTS. Bacterially expressed His-ARTS and Bcl-2 were mixed in the presence of increasing concentrations of ABT-199 (0, 10, 100 or 1000 nM). His-ARTS and associated Bcl-2 were pulled down using Ni-NTA resin, electrophoresed and detected by Western Blot with anti-ARTS (Sigma A4471) or anti-Bcl-2 (Santa Cruz sc-7382, C-2). BH3 mimetic ABT-199 disrupts the binding of Bcl-2 to ARTS. These results suggest that the BH3-binding motif in Bcl-2 which binds to ABT-199 is important for the interaction with ARTS.

Supplemental Information

Supplemental experimental procedures

Antibodies

Antibodies used in all experiments: mouse monoclonal anti Unique C-term of ARTS (Sigmaanti-ARTS (A4471, Sigma-Aldrich), rabbit anti-Bcl2 (ab32124, Abcam), mouse anti-Bcl-2 (sc-7382, Santa Cruz Biotechnology used for in vitro binding assays), mouse anti-Bcl2 (610538, BD Biosciences), mouse anti-ubiquitin (P4D1, sc-8017, Santa Cruz Biotechnology), rabbit anti-Ubiquitin (BML-PW0755-0025, Enzo Life Sciences), mouse anti-XIAP (#610716, BD Biosciences), mouse anti-Flag (F1804, Sigma-Aldrich), mouse anti-Actin (c4, #691001, MP Biomedicals), rabbit anti-GAPDH (sc-25778, Santa Cruz Biotechnology), mouse anti-Myc (#2278, Cell Signaling Technology), mouse anti-His (#8916-1, BD Biosciences), mouse anti-VDAC (sc-8828, Santa Cruz Biotechnology), mouse anti-COX IV (#11967, Cell Signaling Technology), mouse anti-HA (NB600-363, Novus Biologicals). Matching secondary antibodies goat anti-rabbit HRP or goat anti-mouse HRP (Jackson Laboratories) were used.

Constructs

pEF1-AU5 and pEF1-AU5-ARTS constructs containing an AU5 tag that is attached to the N-terminus of ARTS were designed as described in (Larisch et al., 2000). The pSC2-6Myc ARTS was generated using PCR as described in (Edison et al., 2012). The pcDNA3-Bcl-2 and pcDNA3-flag-Bcl-2 constructs were kindly provided by Reuven Stein. On the back bone of these constructs the Bcl-2 Lysine to Alanine (K-to-A) mutations were generated by GeneCust LTD. The schematic representation of these mutants is shown in Figure 7A. The pEBG mammalian expression constructs encoding N-terminus GST fusion proteins together with XIAP or XIAPdelRing were a kind gift from Colin Duckett. The pcDNA3-N-myc-XIAP construct was a kind gift from Herman Steller. The pEF1-HA-Ubiquitin construct was a kind gift from Mia Horowitz. All plasmids used in BiFC experiments were cloned by standard PCR strategies. The coding sequences for human ARTS, XIAP and Bcl2 were subcloned into pBiFC plasmids containing Venus fragments: pBiFC.Flag/VN1-173 (VN) and pBiFC.HA/VC155-238 (VC) (a kind gift from Douglas Green, with permission from Chang-Deng Hu, Purdue University, Indiana). Appropriate restriction sites (EcoRI/XbaI or EcoRI/SalI for pBiFC.VN173 and EcoRI/BglII for pBiFC.VC155) were added by PCR to ARTS, XIAP and Bcl-2 for in frame cloning. Bcl-2 mutant lacking the BH3 domain (Bcl-2delBH3) was cloned into pBIFC.VC and pBiFC.VN by Syntezza Bioscience Ltd. In all constructs VN and VC Venus fragments were fused to the C-terminal part of ARTS, XIAP,

Bcl2 and Bcl-2delBH3. The accuracy of all generated plasmids was verified by gene sequencing. The expression of fusion proteins was confirmed by Western blot and by immunofluorescence. GC3AI is a switch-on GFP-based indicator of caspase 3 cleavage, a kind gift from Prof. Binghui Li laboratory (Zhang et al., 2013). Four Bcl-2 mutants containing deletions of different domains were prepared by Syntezza BioScience Ltd. Bcl-2delBH1 domain mutant lacks amino acids 136-156, Bcl-2delBH2 mutant lacks amino acids 187-202, Bcl-2delBH3 mutant lacks amino acids 92-107, Bcl-2delBH4 mutant lacks amino acids 10-30, were cloned into pcDNA3 plasmid in frame with N-terminal flag tag. The schematic representation of these mutants is shown in Figure 4B.

Fluorescence assays

Cells were seeded in 24-well plates on cover slips coated with fibronectin (5 μg/ml, Biological Industries). Following apoptotic induction (1.75 μM STS), cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed with PBS. For fluorescence assay, HeLa and a stable Bcl-2 knockdown (Bcl-2 KD) HeLa cell line which was established using shRNAs decreasing Bcl-2 levels cells were transiently transfected with AU5-ARTS and stained with DAPI (#157574, MP). Image analysis was carried out using fluorescent microscopy (Nikon 50i, Kawasaki, Kanagawa, Japan). For mitochondria visualization, HeLa cells were stained with MitoTracker™ Red CMXRos (M-7512, Molecular Probes, Eugene, OR, USA). IF staining was performed with an anti-ARTS antibody followed by a FITC conjugated fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Visualization was carried out with Zeiss AxioPhot (Carl Zeiss AG, Feldbach, Switzerland). 200 transfected cells of each condition were analyzed for cellular localization of ARTS. Diffused staining of ARTS, not co-localized with mitochondria was determined as cytosol-localized. Spotted staining of ARTS, co-localized with mitochondria was determined as mitochondrial localized. Statistical significance of the values representing the differences between the cell lines at each time point was assessed using Student's t-test.

Quantitation of Bcl-2 in WT MEFs compared to MEFs Sept4/ARTS KO were done as follows: Nuclei were stained with DAPI, mitochondria with Mitotracker™ Red and Bcl-2 was stained with an anti-Bcl2 antibody followed by FITC conjugated fluorescent secondary antibody. 24-well plates were measured in triplicates usingIncell microscopy (GE Incell Analyzer 2000). 100 fields were imaged in a X40 magnification and cells from each treatment performed in triplicates were counted manually. For statistical analysis Student's t-test analysis was performed.

BiFC assay

The BiFC experiments were performed as described in (Kerppola, 2013). All plasmids used in BiFC experiments were cloned as described above. In all constructs VN and VC Venus fragments were fused to the C terminal part of ARTS, XIAP, Bcl-2 and Bcl-2delBH3. HeLa cells grown to at least 70% confluence were transfected with 200 ng of each vector using PolyJet (SignaGen Laboratories) according to the instructions of the manufacturer. Co-transfection with 20 ng of the vector pdsRedN-2, was performed to assess transfection efficiency and relative fluorescence intensities. We used transcription factors Jun and bFos known to form heterodimers as a positive control and Jun and bFosdelZIP lacking the carboxyl-terminal half of the bFos leucine zipper domain, as a negative control for protein-protein interaction in BiFC assays. Transfected cells were cultured at 37 °C for 24 h. Apoptosis was induced by 1.75 µM STS for indicated time points and BiFC complex formation was analyzed by flow cytometry. Venus (BiFC) and dsRed signals in cells were quantified in a FACSCantoII flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser emitting at 488 nm. Analysis was restricted to live cells. Results were analyzed using FACSDIVA software (BD Biosciences). To normalize the results per transfection efficiency, the mean fluorescence intensities of the BiFC complexes were normalized to the mean fluorescence intensity of dsRED. The ratio between YFP and Red fluorescence was calculated for each time point. At least 10,000 cells were analyzed in each experiment. For statistical analysis Student's t-test was used.

Protein expression and purification

Proteins for peptide array incubation: Full-length ARTS was expressed and purified using pRSET based plasmid supplemented with a His6-Lipoyl-TEV (HLT)-tag (Rotem et al., 2008). HLT-ARTS was expressed in *Escherichia coli* BL21 (DE3) Rosetta 2 pLysS cells (Novagen). Cells were grown in Luria Broth (LB) media, at 37 °C to an optical density at 600 nm of 0.6 - 0.8. The cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 21°C for 4 h. Cell pellets were lysed by sonication and HLT-tagged ARTS was purified using a His Trap HP 1 ml nickel column (GE Healthcare), using an FPLC system (AKTA Avant; GE Healthcare) equilibrated with buffer A (20 mM Tris pH 7.4, 250 mM NaCl, 10 mM Imidazole, 5 mM β-mercaptoethanol, 0.1% Triton X). Proteins were eluted with buffer B composed of buffer A with 250 mM Imidazole. 1 mM EDTA was added to the proteins upon elution. The protein purification was confirmed by Western Blot analysis using mouse anti-His antibody for the N-terminal (His) 6 part of the HLT-tagged ARTS and mouse anti-ARTS C-term for the ARTS C-terminal domain.

Protein preparation for *in vitro* ubiquitylation: E1 was purified from *E. coli* using Ub-agarose. His-UbcH5b and GST-E3s were purified using either Ni-NTA column or glutathione-

Sepharose 4B column accordingly. GST-Bcl-2 was purified using glutathione-Sepharose 4B and the GST-tag was removed by thrombin treatment. From the eluted Bcl-2 solution, thrombin was removed by using benzamidine-sepharose. Ubiquitin was purchased from Boston Biochem (MA, USA).

In vitro binding assay**:** Bacterially expressed His-ARTS (5μg) and Bcl-2 (2μg) were mixed as indicated and incubated at 4˚C overnight in binding buffer (Tris 20mM, pH7.6, DTT 1mM) in the presence of increasing concentration of ABT-199 (0, 10, 100 or 1000 nM). His-ARTS was pulled down after incubation (at 4 ˚C for 2 h) with Ni-NTA resin (10μL) in the same buffer. After centrifugation to remove unbound proteins, the resin was washed three times with binding buffer. His-ARTS and associated Bcl-2 were electrophoresed and detected by Western Blot with anti-ARTS (A4471, 1/10000, Sigma-Aldrich) or anti-Bcl-2 (sc-7382 C-2, Santa Cruz Biotechnology,)

In vivo ubiquitylation of Bcl-2 wt and Bcl-2delBH3.

Bcl-2-KO MEFs were transfected with Flag-WT Bcl-2 or Flag-Bcl-2delBH3 as described in "Experimental procedures". Cells were treated with 20 μM MG132 or with DMSO for six hours and concomitantly with 1.75 μM STS for one hour or left untreated. Proteins were extracted and Bcl-2 was immunoprecipitated using anti-flag magnetic beads (M185, MBL International). The poly-ubiquitylated forms of Bcl-2 were detected by western blot analysis using an antibody against Ubiquitin (ab7780, abcam).

In vitro ubiquitylation assay

Purified Bcl-2 was incubated with 100 nM E1, 500 nM UbcH5b, 50 nM Ub and 700 nM XIAP and other appropriate E3 in the conjugation buffer (20 mM Tris-Cl, pH7.6, 100 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT) containing 2 mM ATP at 37 ºC for 1 h. In a control reaction, a component indicated was excluded. 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).

Ubiquitylation identification mass-spectrometry

HeLa cells were transfected with GFP-Bcl-2 and HA-Ubiquitin. 24 h post transfection cells were treated with 15 μM MG-132 for 6 h and apoptosis was induced by treatment with 1.75 μM STS for 30 min. Cells were lysed and Bcl-2 was precipitated by anti GFP magnetic beads. Beads were washed and digested by trypsin overnight at 37 °C, in order to cleave the ubiquitylated Lysines to the Lysine Glycine-Glycine (KGG) form.

On beads digestion: All reagents were purchased from Sigma, unless noted otherwise. The immunoprecipitation beads were washed twice with 50 mM ammonium bicarbonate pH 8.0 followed by the addition of 8 M urea for 30 minutes at room temperature. DTT was added to a final concentration of 5 mM and incubated for an additional hour at room temperature. The sample was alkylated by a 45 minute incubation with 10 mM iodoacetamide and then diluted 1:5 with 50 mM ammonium bicarbonate pH 8.0. Trypsin (Promega) was added to an estimated ratio of 50:1 protein:trypsin and incubated overnight at 37 °C. The reaction was quenched by acidification with 1% TFA. The digested peptides were desalted using HBL Oasis (094225, Waters Israel), vacuum centrifuged to dry and resuspended in Immunoaffinity Purification (IAP) buffer from the PTMScan® Pilot Ubiquitin Remnant Motif (K-ε-GG) Kit (Cell Signaling Technology) for GlyGly enrichment.

GlyGly enrichment: The GlyGly enrichment was performed using the PTMScan® Pilot Ubiquitin Remnant Motif (K-ε-GG) Kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, the resuspended samples were centrifuged and the supernatant loaded onto motif antibody beads that were washed and resuspended in 1 x PBS. The peptides were incubated with the beads rotating at 4˚C for 2 h, followed by three washes with IAP buffer and three with water. The bound peptides were eluted by adding 55µl of 0.15% TFA to the beads, incubating for 10 min, and mixing every 2-3 min. The elution step was repeated once again and the two eluates combined. The eluate was vacuum centrifuged to dry and resuspended in 96:4 Water + 0.1% formic acid: ACN.

Liquid chromatography: ULC/MS grade solvents were used for all chromatographic steps. Each sample was loaded using split-less nano-Ultra Performance Liquid Chromatography (10 kpsi nanoAcquity; Waters, Milford, MA, USA). The mobile phase was: A) H_2O + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online using a reversed-phase C18 trapping column (180µm internal diameter, 20mm length, 5µm particle size; Waters Israel). The peptides were then separated using a T3 HSS nano-column (75µm internal diameter, 250mm length, 1.8µm particle size; Waters Israel) at 0.35µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 30%B in 50 min, 30% to 90%B in 5 min, maintained at 90% for 5 min and then back to initial conditions.

Mass Spectrometry: The nanoUPLC was coupled online through a nano ESI emitter (10µm tip; New Objective; Woburn, MA, USA) to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) using a Flex Ion nanospray apparatus (Proxeon). Data was acquired in parallel reaction monitoring (PRM) mode, where MS2 resolution was set to 35,000

and the maximum injection time set to 150 ms. Full scan MS was also acquired at a resolution of 70,000 and a maximum injection time of 50 ms.

Data processing and analysis: Raw data were processed using Proteome Discoverer v1.41. MS/MS spectra were searched using Mascot v2.5.1 (Matrix Sciences) and Sequest HT. Data were searched against the human sequences UniprotKB, last updated on November 25th, 2015 and appended with 125 common laboratory contaminant proteins. Fixed modification was set to carbamidomethylation of cysteines and variable modifications were set to oxidation of methionines, deamidation of asparagine and glutamine and addition of 2 glycines on lysine residues (K-GG). Search results were filtered such that the global false discovery rate was maximum of 1%. The spectra corresponding the K-GG containing peptides were manually inspected to verify the identification.

Supplemental references

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