Background: XIAP E3 ligase induces mitochondrial membrane permeabilization, resulting in intramitochondrial degradation of its inhibitor Smac.

Results: Mitochondrial XIAP action is triggered by drug inducers of apoptosis and occurs before Drp1-mediated cytochrome c release.

Conclusion: Pre-activation of mitochondrial XIAP action can protect against caspase activation.

Significance: Mitochondrial processing by XIAP constitutes a new antagonistic component of intrinsic apoptosis signaling.

Efficient apoptosis requires Bax/Bak-mediated mitochondrial outer membrane permeabilization (MOMP), which releases death-promoting proteins cytochrome c and Smac to the cytosol, which activate apoptosis and inhibit X-linked inhibitor of apoptosis protein (XIAP) suppression of executioner caspasess, respectively. We recently identified that in response to Bcl-2 homology domain 3 (BH3)-only proteins and mitochondrial depolarization, XIAP can permeabilize and enter mitochondria. Consequently, XIAP E3 ligase activity recruits endolysosomes into mitochondria, resulting in Smac degradation. Here, we explored mitochondrial XIAP action within the intrinsic apoptosis signaling pathway. Mechanistically, we demonstrate that mitochondrial XIAP entry requires Bax or Bak and is antagonized by pro-survival Bcl-2 proteins. Moreover, intramitochondrial Smac degradation by XIAP occurs independently of Drp1-regulated cytochrome c release. Importantly, mitochondrial XIAP actions are activated cell-intrinsically by typical apoptosis inducers TNF and staurosporine, and XIAP overexpression reduces the lag time between the administration of an apoptotic stimuli and the onset of mitochondrial permeabilization. To elucidate the role of mitochondrial XIAP action during apoptosis, we integrated our findings within a mathematical model of intrinsic apoptosis signaling. Simulations suggest that moderate increases of XIAP, combined with mitochondrial XIAP preconditioning, would reduce MOMP signaling. To test this scenario, we pre-activated XIAP at mitochondria via mitochondrial depolarization or by artificially targeting XIAP to the intermembrane space. Both approaches resulted in suppression of TNF-mediated caspase activation. Taken together, we propose that XIAP enters mitochondria through a novel mode of mitochondrial permeabilization and through Smac degradation can compete with canonical MOMP to act as an anti-apoptotic tuning mechanism, reducing the mitochondrial contribution to the cellular apoptosis capacity.

The induction of mitochondrial outer membrane permeabilization (MOMP),3 to support activation of executioner caspasess, is paramount for effective cancer cell killing (1, 2). To initiate MOMP, BH3-only proteins bind to inhibit pro-survival Bcl-2 family members or directly bind and activate Bax and Bak (3, 4), which permeabilize the mitochondrial outer membrane. Consequently, pro-apoptotic factors, including Smac (second mitochondria-derived activator of caspasess) (5) and cytochrome c (6), are released to the cytosol to relieve caspase suppression and activate executor caspasess, respectively. X-linked inhibitor of apoptosis protein (XIAP), an endogenous caspase inhibitor, blocks initiator and executioner caspasess by binding to caspasess via its baculoviral inhibitor of apoptosis repeat domain 7–9 and through E3 ligase-mediated caspase degradation (10). Following apoptosis induction, caspase activities are suppressed by XIAP (11). Consequently, cells with high

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3 The abbreviations used are: MOMP, mitochondrial outer membrane permeabilization; XIAP, X-linked inhibitor of apoptosis protein; ROI, region of interest; OMM, outer mitochondrial membrane; IMS, intermembrane space; DN, dominant negative; RFP, red fluorescent protein; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MEF, mouse embryonic fibroblast; BisTris, 2-[(bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; STS, stauro-

sporine; AcD, actinomycin D; PARP, poly(ADP-ribose) polymerase; DKO, dou-

ble-knock out; IF, immunofluorescence; FM, full medium; BH3, Bcl-2 homology domain 3.
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XIAP require MOMP-mediated release of Smac to inhibit XIAP-mediated caspase suppression (12).

In addition, XIAP participates in the upstream and downstream regulation of MOMP, distinct from its direct caspase delay and suppression functions. During anoikis, XIAP can activate Bax/Bak-mediated MOMP, resulting in Smac and cytochrome c release (13). Following MOMP, XIAP has been proposed to directly bind to Smac inside mitochondria and impede its release (14) or to target cytosolic Smac for proteasomal degradation (15). We recently reported that H3-only protein expression or chemical mitochondrial uncoupling triggered rapid XIAP-mediated MOMP and concomitant XIAP entry into inner mitochondrial compartments (16). The action of XIAP at mitochondria was characterized by ubiquitylation of the outer mitochondrial membrane (OMM) and inner mitochondrial compartments and by recruitment of endolysosomal machinery into mitochondria, independently of mitophagy. Importantly, intramitochondrial XIAP induced the degradation of its inhibitor Smac, whereas cytochrome c release was only partially activated, thus suggesting a novel anti-apoptotic role for mitochondrial XIAP.

Here, we elucidated function and regulation of mitochondrial XIAP action within the intrinsic apoptosis signaling pathway. We demonstrate that XIAP entry into mitochondria is mediated by Bax and Bak and antagonized by pro-survival Bcl-2 family proteins. Furthermore, we show that mitochondrial XIAP action prominently occurs in response to drug-induced intrinsic and extrinsic apoptosis and phenotypically results in a reduced pre-MOMP delay, the lag time between the administration of an apoptotic stimulus and the onset of mitochondrial permeabilization. Importantly, we show that XIAP entry and subsequent intramitochondrial targeting of Smac are distinct from Drp1-regulated cytochrome c release during canonical MOMP. To determine whether XIAP action at mitochondria can protect against drug-induced apoptosis, we explored XIAP action at mitochondria versus direct caspase suppression activities using a systems biology approach. Combined mathematical modeling and experimental approaches revealed conditions whereby XIAP suppression of mitochondrial Smac significantly cooperates with downstream XIAP inhibition of caspases.

Based on these findings, we propose that following apoptosis induction XIAP-induced membrane permeabilization 1) is mechanistically and functionally distinct from canonical, pro-apoptotic MOMP, and 2) under increased XIAP levels functions as an anti-apoptotic tuning mechanism capable of reducing the mitochondrial apoptosis capacity via intramitochondrial Smac degradation.

Experimental Procedures

Plasmids—XIAP was cloned as N-terminally fused to tagRFP and GFP. Intermembrane space (IMS)-RFP-XIAP was constructed by fusing the first 49 amino acids of Smac, responsible for its targeting to the intermembrane space (5) to the N terminus of RFP-XIAP. Wild type pcDNA3-HA-Drp1 and dominant negative (DN) pcDNA3-HA-Drp1(K38A) mutant were obtained from Ref. 17, and WT- and DN-Drp1 were subcloned to pmCherry-Cl. pQCXIP-Vx3K0-mEGFP was obtained from (18). In text and figures, tagRFP and mCherry are both referred to as RFP.

Cell Culture—Human MCF7 breast cancer (CLS Cell Line Service) and HeLa Kyoto cervical cancer cell lines were maintained in DMEM (1 g/liter d-glucose, 0.11 g/liter sodium pyruvate) and embryonic kidney 293T cells in DMEM (4.5 g/liter d-glucose), supplemented with 10% FBS, l-glutamine, nonessential amino acids, and penicillin/streptomycin/ampicillin B. Bax/Bak double-knock out (ATCC® CRL-2913TM) and WT SV40 (ATCC® CRL-2907TM) mouse embryonic fibroblasts (MEFs) were maintained in Iscove’s modified Dulbecco’s medium containing l-glutamine and HEPEs and supplemented with 10% FBS, nonessential amino acids, and penicillin/streptomycin/ampicillin B.

Gene Expression and Drug Treatments—Transient transfections were performed using JetPRIME transfection reagent (Peqlab), and experiments were performed following 24 h of expression, unless stated otherwise. For stable lentiviral gene transfer, pCDH-puro-CMV carrying the super-folding GFP-tagged caspase-3/-7 sensor (GC3A1) (19) was calcium-phosphate-transfected into 293T cells together with pCMVdeltaR8.91 and pMD2.G. MCF7 cells were infected with virus particle-containing supernatants and selected and maintained with puromycin (1 µg/ml), following selection of a stable single clone line. For experiments, cells were plated in puromycin-free medium. Treatments with carbonyl cyanide m-chlorophenyl-hydrazone (CCCP; 20 µM; Merck Millipore), TNF (43 ng/ml; a kind gift of BASF, Mannheim, Germany), actinomycin D (AcD; 1 µg/ml; Calbiochem), and staurosporine (STS, 1 µM; Calbiochem) were performed in fully supplemented cell culture medium (FM) or in glucose-containing Hanks’ balanced salt solution (Life Technologies, Inc.; catalog no. 14025), as indicated.

Immunofluorescence—Cells plated in 8-well microscopy µ-slides (ibidi) were transfected and/or subjected to the indicated drug treatments. Cells were then fixed with paraformaldehyde (4% in PBS, pH 7.4). For immunostaining, fixed cells were permeabilized with 0.3% Triton X-100 in PBS and blocked with 3% BSA. Then the cells were incubated with primary antibodies against Bax (Santa Cruz Biotechnology; catalog no. sc-493), cytochrome c (BD Biosciences; catalog no. 556432), Smac/DIABLO (Santa Cruz Biotechnology; catalog no. sc-22766), Tom20 (Santa Cruz Biotechnology; catalog no. sc-11415 or catalog no. sc-17764), TRAP1 (Novus Biologicals; catalog no. NB300-555), Lys-63 linkage-specific polyubiquitin (Merck Millipore; catalog no. 05-1308), or XIAP (Santa Cruz Biotechnology; catalog no. sc-5551) at room temperature for 1 h. Fluorescent staining was performed for 30 min at room temperature using highly cross-adsorbed Alexa Fluor 350, 488, 546, or 647 secondary antibodies (Life Technologies, Inc.).

Wide Field Fluorescence Imaging—A DeltaVision RT microscope system (Applied Precision) equipped with a ×60 oil immersion objective was employed for wide field fluorescence microscopy. Images of representative cells were captured using the z axis scan function or, when indicated, as z stacks with 0.15-µm step sizes. Acquired images were deconvolved (SoftWoRx, Applied Precision). Image analysis and preparation were performed using ImageJ (rsbweb.nih-
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We recently showed that in response to BH3-only protein expression, XIAP E3 ligase activity leads to prominent mitochondrial Smac, were scored. At least six fields of view were analyzed per condition, from four independent experiments. Here, to determine whether Bax is required for XIAP entry into mitochondria, we examined pathway activation in Bax/Bak double-knock out (DKO) and control SV40 mouse embryonic fibroblasts (MEFs) (3, 21). Treatment with CCCP (20 μM) for 3 h failed to induce translocation of GFP-XIAP to mitochondria in Bax/Bak DKO MEFs (Fig. 1A). In control MEFs, CCCP induced Bax cluster formation at and RFP-XIAP entry into mitochondria of all XIAP-overexpressing cells (Fig. 1B), suggesting that Bax and/or Bak are necessary components of XIAP entry. We therefore reconstituted Bax in DKO MEFs. Indeed, following CCCP treatment, all cells that co-expressed GFP-XIAP displayed GFP-Bax clustering and RFP-XIAP entry into mitochondria (Fig. 1C), demonstrating that Bax reconstitution is sufficient to re-establish pathway activation. Note, in a subset of cells, GFP-XIAP and GFP-Bax localized to mitochondria even in the absence of CCCP, indicating that Bax overexpression alone can be sufficient to activate mitochondrial XIAP entry (data not shown).

We next examined whether, similar to Bax, the OMM-residing Bak (4) is activated during XIAP entry into mitochondria. Under normal growth conditions RFP-Bak, co-expressed with GFP-XIAP in MCF7 cells, localized to elongated mitochondria in the majority of cells homogeneously distributed at the OMM (data not shown). In response to 3-h CCCP treatment, RFP-Bak formed clusters at fragmented mitochondria that contained

Quantifications of Fluorescence Microscopy Data—Images were acquired with identical exposure settings using the z axis scan function to capture total cellular fluorescence within a single image. The number of analyzed fields of view is indicated in plots.

Quantification of Cytochrome c and Smac—Identical cytosolic regions were measured for integrated cytochrome c or Smac immunofluorescence intensity as a measure of protein level, and standard deviation of fluorescence signal, as a measure of mitochondrial cytochrome c/Smac content. Per condition and per image, we calculated the ratio of cytochrome c/Smac levels and standard deviations in cells expressing XIAP cells versus cytochrome c/Smac levels and standard deviations in nontransfected cells. Cells within at least four fields of view were analyzed per condition, each from at least three independent experiments.

Quantification of XIAP Levels—RFP-XIAP was expressed for 24 h in MCF7 cells, fixed, and immunostained for XIAP. Overexpression of XIAP was calculated from the immunofluorescence levels of transfected versus nontransfected cells.

Quantification of Caspase Activation in MCF7 Cells Expressing a GFP-based Caspase-3/7 Activity Sensor (19)—Identical cytosolic areas within control and treated cells were analyzed for GFP fluorescence. The impact of expressed proteins on GFP fluorescence was calculated for each image, based on relative changes (transfected − nontransfected fluorescence)/nontransfected fluorescence). The fold change induced by CCCP or TNF/AcD, relative to control FM, is reported. Cells within at least four fields of view were analyzed per condition, from each of at least three independent experiments.

Quantification of MOMP Activation—At 24 h of expression of RFP or RFP-XIAP, MCF7 cells were treated for 3 h with STS or TNF, fixed, and immunostained for Smac. The numbers of RFP- or RFP-XIAP-positive cells with mitochondrial Smac, and with loss of mitochondrial Smac, were scored. At least six fields of view were analyzed per condition, from four independent experiments.

Western Blotting—MCF7 whole cell lysates were prepared with RIPA lysis buffer containing protease inhibitors. Dosed samples were electrophoresed using BisTris NuPAGE gels (Invitrogen), and proteins were transferred to nitrocellulose using the iBlot dry blotting system (Invitrogen). Immunodetection was performed using antibodies against β-actin (GeneTex; catalog no. GTX26276), cleaved PARP (Cell Signaling; catalog no. 5625), cytochrome c (Santa Cruz Biotechnology; catalog no. sc-131566), GAPDH (Santa Cruz Biotechnology; catalog no. sc-25778), GFP (Chromotek; catalog no. 3H9), and Smac (Santa Cruz Biotechnology; catalog no. sc-22766). Horseradish peroxidase-conjugated secondary antibodies were used for digital chemiluminescence detection. Blots shown are representative of three independent experiments.

Statistical Analyses—The probability of statistically significant increases or decreases between conditions of at least three independent experiments was determined using Student’s t test. Two-tailed, unpaired t tests were performed for imaging experiments; one-tailed, unpaired t test was performed for Western blot analysis. Bar graphs represent values as mean ± S.D. Box plots show individual data points and median values, and whiskers indicate range of data points. Scatter plots show individual data points and the linear fit, and the shaded area indicates confidence intervals. Asterisks indicate a statistically significant changes (p < 0.05).

Mathematical Modeling—Deterministic simulations of the SMBL file “BIOMD0000000220” from Albeck et al. (11) were performed using Copasi software (20). Simulations were run with a 50% decrease to initial Smac concentrations and with a 3-fold increase of initial XIAP concentrations. Relative PARP cleavage is plotted over time.

Results

XIAP Translocation into Depolarized Mitochondria Requires Bax or Bak—We recently showed that in response to BH3-only protein expression, XIAP E3 ligase activity leads to prominent mitochondrial ubiquitylation, associated with entry of XIAP and endolysosomal machinery into inner mitochondrial compartments, independently of mitophagy and Parkin (16). We further showed that in cells transiently overexpressing XIAP, mitochondrial uncoupling with CCCP is sufficient for induction of these events and triggers Bax translocation to and clustering at the OMM.

Here, to determine whether Bax is required for XIAP entry into mitochondria, we examined pathway activation in Bax/Bak double-knock out (DKO) and control SV40 mouse embryonic fibroblasts (MEFs) (3, 21). Treatment with CCCP (20 μM) for 3 h failed to induce translocation of GFP-XIAP to mitochondria in Bax/Bak DKO MEFs (Fig. 1A). In control MEFs, CCCP induced Bax cluster formation at and RFP-XIAP entry into mitochondria of all XIAP-overexpressing cells (Fig. 1B), suggesting that Bax and/or Bak are necessary components of XIAP entry. We therefore reconstituted Bax in DKO MEFs. Indeed, following CCCP treatment, all cells that co-expressed GFP-XIAP displayed GFP-Bax clustering and RFP-XIAP entry into mitochondria (Fig. 1C), demonstrating that Bax reconstitution is sufficient to re-establish pathway activation. Note, in a subset of cells, GFP-XIAP and GFP-Bax localized to mitochondria even in the absence of CCCP, indicating that Bax overexpression alone can be sufficient to activate mitochondrial XIAP entry (data not shown).
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GFP-XIAP (Fig. 1D). To test whether, in the absence of Bax, Bak was sufficient to mediate XIAP entry into mitochondria, we co-expressed RFP-Bak with GFP-XIAP in Bax/Bak DKO MEFs. In response to 3-h CCCP, Bak formed clusters at the OMM and, similar to Bax reconstitution, alone permitted mitochondrial XIAP localization.
Mitochondrial Action of XIAP Is Suppressed by Bcl-2 and Bcl-xL. Inhibition of Bax/Bak—Given the obligatory role for the presence of either Bax or Bak in XIAP entry into mitochondria, we speculated that inhibition of Bax/Bak via pro-survival Bcl-2 family proteins (22, 23) would block the CCCP-induced XIAP action at mitochondria. Indeed, CCCP treatment of Bcl-xL-overexpressing cells did not trigger mitochondrial translocation of XIAP (Fig. 1E). Moreover, Bcl-xL overexpression prevented mitochondrial Smac degradation (Fig. 1, E and F), a hallmark consequence of CCCP treatment in combination with XIAP overexpression (16). In contrast, the Bcl-xL(G138E/R139L/I140N) mutant that does not bind Bax or Bak (24) did not inhibit XIAP entry into mitochondria or degradation of Smac (Fig. 1, E and F), indicating that Bcl-xL blockage of mitochondrial XIAP action is dependent on its inhibition of Bax/Bak. Similarly, wild type GFP-Bcl-2 suppressed XIAP targeting of mitochondria, whereas the GFP-Bcl-2(G145A) mutant with impaired binding to Bax (25) and Bak (26) permitted CCCP-induced mitochondrial entry of XIAP (results not shown) and Smac degradation (Fig. 1F). Taken together, Bax/Bak-mediated XIAP-induced MOMP permits XIAP entry into depolarized mitochondria and, similar to canonical MOMP, is suppressed by pro-survival Bcl-2 signaling.

Mitochondrial XIAP Action Is Part of the Cell Intrinsic Response to Stauroporine- and TNF-induced Apoptosis—In response to the overexpression of BH3-only proteins Bimel, tBid, Bik, and Bad, endogenous XIAP levels are sufficient to induce ubiquitylation and endolysosomal targeting of mitochondria (16). We therefore speculated that the mitochondrial XIAP pathway might be endogenously engaged during drug-induced apoptosis. Wild type MCF7 cells were treated with the intrinsic apoptosis inducer STS or the death receptor ligand tumor necrosis factor (TNF), both of which trigger activation of endogenous Bid (27, 28). Indeed, at 6 h of treatment, both STS- and TNF-treated cells exhibited the characteristic phenotypes of XIAP-mediated mitochondrial processing (16), including XIAP entry into (Fig. 2A) and Lys-63-linked polyubiquitylation of (Fig. 2B) mitochondria. Moreover, Smac protein levels were markedly reduced in cells treated with staurosporine for 6 h, while cytochrome c levels were less impacted (Fig. 2C).

Because a hallmark of the combination of CCCP treatment and XIAP overexpression is the rapid activation of MOMP, as indicated by Bax (16) and Bak (Fig. 1C) cluster formation at the outer mitochondrial membrane and, loss of mitochondrial Smac (16), we hypothesized that high XIAP levels might similarly promote MOMP activation in response to drug-initiated apoptosis induction. Therefore, we compared the activation of MOMP in MCF7 cells expressing either RFP or RFP-XIAP in response to 3 h of treatment with STS or TNF, using loss of mitochondrial Smac as the indicator of MOMP. Intriguingly, at this early time point, at which only a small percentage of TNF- or STS-treated RFP control cells had undergone MOMP, in cells expressing RFP-XIAP the percentage of cells with loss of mitochondrial Smac was significantly increased 3-fold in response to both STS and TNF (Fig. 2, D and E).

Taken together, XIAP action at mitochondria is activated by common drug inducers of apoptosis under endogenous protein levels, and during drug treatment high XIAP levels can sensitize cells to activation of MOMP. As high XIAP expression protects against TNF (10) as well as STS-mediated cell death (14), and XIAP-induced MOMP of depolarized mitochondria results in Smac degradation (16), the above findings further support the possibility that mitochondrial action of XIAP can function as a novel cellular pro-survival mechanism.

Increasing XIAP Reveals Mitochondrial XIAP Action in Cells with High Executioner Caspase Levels—We initially observed XIAP action at BH3-only protein-targeted mitochondria in MCF7 cells (16), which have low executioner caspase activity due to lost caspase-3 expression (29). In HeLa cells, which typically undergo caspase-mediated death within minutes following MOMP (11), overexpression of activator BH3-only proteins tBid and Bim resulted in toxicity prior to detection of transgene fluorescence (data not shown). We thus speculated that high executioner caspase activity may outperform, and effectively mask basal mitochondrial action of XIAP. We therefore verified pathway existence in HeLa cells under XIAP overexpression, in the absence of an apoptotic stimulus. In accordance with our findings in MCF7 cells (16), CCCP-induced mitochondrial depolarization, in combination with XIAP overexpression, was sufficient to rapidly trigger mitochondrial XIAP action also in HeLa cells, as indicated by Bax clustering at OMMs, translocation of XIAP to mitochondria, and mitochondrial Lys-63 ubiquitylation (Fig. 3A). Likewise, cellular Smac levels were significantly decreased (Fig. 3, A and B). Of note, in a subpopulation of HeLa cells cellular Smac levels were not reduced, as a sign of cell-to-cell variability visible in the single-cell analysis (Fig. 3B). Furthermore, as observed with MCF7 cells (Fig. 1E), both Bcl-2 and Bcl-xL suppressed XIAP action at mitochondria also in HeLa cells in a Bax suppression-dependent fashion (Fig. 3C).

Next, we explored whether XIAP-mediated caspase inhibition would be sufficient to allow for tBid-mediated activation of the mitochondrial XIAP pathway in HeLa cells. Cells were transfected with GFP-XIAP, followed 6 h later by transfection with tBid-RFP, and analyzed at 24 h. In the absence of GFP-XIAP pre-expression, HeLa cells did not survive tBid-RFP expression, even when co-expressing GFP-XIAP (results not shown). HeLa cells pre-transfected with GFP-XIAP allowed for
detectable tBid-RFP expression at 24 h. tBid-RFP-targeted mitochondria contained GFP-XIAP and prominent Lys-63-linked polyubiquitin chains and displayed clustered Bax (Fig. 3D). Furthermore, tBid-RFP-targeted mitochondria with intramitochondrial XIAP were depleted of Smac, but they often retained cytochrome c (Fig. 3E and F), similar to the observed differential effects of XIAP-induced MOMP on Smac and cytochrome c in CCCP-treated MCF7 cells (16). These results indicate that XIAP action at mitochondria is enabled under conditions of reduced executioner caspase activities, either through caspase 3 deletion in MCF7 cells or XIAP overexpression in HeLa cells. Furthermore, CCCP- and tBid-induced mitochondrial XIAP action was associated with Bax clustering, reduced Smac levels, and mostly mitochondria-maintained cytochrome c.

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**FIGURE 2. Endogenous engagement of mitochondrial XIAP action in response to staurosporine- and TNF treatment.** A, wild type MCF7 cells in full medium (FM) alone, or with STS (1 μM) or TNF (43 ng/ml) for 6 h. Immunofluorescence (IF) detection of endogenous XIAP (green) and OMM protein Tom20 (red). B, cells treated as in A and immunostained for endogenous Lys-63-linked ubiquitin chains (green) and OMM protein Tom20 (red). Scale bar, 10 μm; ROIs, 8.0 × 8.0 μm. C, MCF7 cells were treated with STS for 0, 0.5, 3, or 6 h, and whole cell lysates were analyzed by Western blotting for Smac and cytochrome c (Cyto c) content. D, MCF7 cells expressing either RFP or RFP-XIAP (green) treated with TNF or STS for 3 h in FM. IF of endogenous Tom20 (blue) and Smac (red). Scale bar, 10 μm. E, RFP or RFP-XIAP-expressing MCF7 cells were treated with STS or TNF for 3 h in FM and immunostained for endogenous Smac. Then cells were scored based on loss of mitochondrial Smac IF. Bar graphs represent the percentage of cells with mitochondrial membrane permeabilization. *, p < 0.05 versus RFP expressing control cells.
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A

HeLa + CCCP
Tom20 IF X:AP
Bax IF
HeLa + CCCP
Tom20 IF Vx3K0
X:AP
Smac IF
HeLa + CCCP
Tom20 IF X:AP

B

+ CCCP

Relative Smac levels

1.25
1.00
0.75
0.50
n=30
n=29
n=20
n=27
WT Mut WT Mut
Bcl-xL Bcl-2

C

D

HeLa tBid XIAP
K63 IF
HeLa tBid XIAP
Bax IF

E

HeLa tBid XIAP
Smac IF
Cyto c IF
HeLa tBid XIAP

F

Relative levels of total protein

1.0
0.5
0.5 1.0
Relative mitochondrial content

Smac (n=23)
Cytochrome c (n=25)

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often associated with mitochondrial fragmentation involving dynamin-related protein 1 (Drp1), a GTPase that induces physiological and stress-associated mitochondrial fission (17, 30). Importantly, suppression of Drp1 activity can delay apoptotic MOMP-mediated release of cytochrome c, without altering Smac release (31, 32). We therefore sought to explore the impact of Drp1 inhibition on mitochondrial XIAP action. MCF7 cells co-expressing GFP-XIAP and the K38A dominant negative mutant of Drp1 (DN-Drp1) (17) were treated with CCCP for 3 h. In contrast to the complete fragmentation of mitochondria by CCCP in XIAP-expressing cells (e.g. Fig. 4), mitochondria in CCCP-treated GFP-XIAP and DN-Drp1 co-expressing cells displayed large, swollen, and often elongated morphologies (Fig. 4, A and B). Intriguingly, DN-Drp1 co-expression did not block entrance of XIAP into mitochondria. Also, intramitochondrial Lys-63-linked polyubiquitylation, a previously determined marker for mitochondrial activity of XIAP (16), was prominently present as indicated by Vx3K0-GFP (Fig. 4B) (18). Thus, XIAP/CCCP-induced mitochondrial permeabilization and processing still progress even under conditions of Drp1 inhibition.

To test whether XIAP-mediated degradation of Smac was likewise permitted under Drp1 inhibition, we quantified both mitochondrial and total cellular levels of endogenous Smac in response to XIAP/CCCP under co-expression of wild type (WT) RFP-Drp1 or RFP-DN-Drp1 in MCF7 cells (Fig. 4C). Indeed, Smac was degraded also in the presence of RFP-DN-Drp1, as mitochondrial Smac levels were reduced to a similar extent in both WT Drp1 and DN-Drp1 co-expressing cells. Moreover, DN-Drp1 significantly increased XIAP/CCCP-induced reduction of total Smac levels in MCF7 cells, compared with WT Drp1 (Fig. 4C). Also in HeLa cells RFP-DN-Drp1 co-expression did not block XIAP-induced mitochondrial permeabilization, as indicated by mitochondrial translocation of GFP-XIAP and loss of mitochondrial Smac (Fig. 4D). Quantification of total cellular Smac levels in HeLa cells further demonstrates unaltered XIAP/CCCP-induced degradation of Smac in cells that co-express either RFP-WT-Drp1 or RFP-DN-Drp1 (Fig. 4E). Note, unlike in MCF7 cells, DN-Drp1 did not further increase the degradation of total Smac in HeLa cells, indicating a degree of cell type-specific regulation.

We previously described that cells with XIAP/CCCP-induced MOMP displayed heterogeneous cytochrome c phenotypes, with subpopulations of cells displaying either mitochondrial retention or released and/or degraded cytochrome c (16). Unlike Smac degradation, these cytochrome c phenotypes occurred in a manner independent of XIAP E3 ligase activity (16). Thus, we next sought to determine whether DN-Drp1, which did not prevent mitochondrial XIAP-mediated mitochondrial permeabilization and Smac degradation (Fig. 4, C–E), but has been shown to inhibit cytochrome c release during canonical MOMP (17, 30), could inhibit cytochrome c release in response to XIAP/CCCP. Indeed, when co-expressing DN-Drp1, the majority of cells that underwent XIAP/CCCP-induced mitochondrial processing displayed mitochondrially retained cytochrome c (Fig. 4, F and G). Quantification of total cellular and mitochondrial cytochrome c levels further demonstrates that although RFP-WT-Drp1 co-expressing cells exhibit pronounced cytochrome c release and degradation, RFP-DN-Drp1 co-expressing cells significantly decreased both cytochrome c release and degradation (Fig. 4H).

Collectively, these findings demonstrate that XIAP-induced permeabilization of depolarized mitochondria can occur in the absence of Drp1-mediated mitochondrial fragmentation. Furthermore, following XIAP entry, XIAP targeting of Smac and cytochrome c release are two distinct events that can be separated by Drp1 inhibition. Together with our previous findings (16), we conclude that XIAP E3 ligase-mediated intramitochondrial processing via endolyssosomal entry into and ubiquitylation of mitochondria can be uncoupled from canonical MOMP.

**Activation of Mitochondrial XIAP Action Can Protect against TNF-induced Apoptosis**—Our work reveals that XIAP function during apoptosis signaling is more complex than previously understood. Because of XIAP multifunctionality, it is not readily possible to discriminate between the apoptosis-regulatory contributions via cytosolic caspase suppression (7–9) and the possible protective role for mitochondrial XIAP-mediated Smac degradation. Therefore, we utilized a previously established mathematical model developed for MOMP analysis (11) to explore the relationship between XIAP impact on mitochondrial Smac levels and XIAP control of post-MOMP signaling. We simulated mitochondrial XIAP action as a reduction of mitochondrial Smac prior to death receptor activation of canonical MOMP. Death receptor activation was simulated for combinations of reduced Smac (up to a 50% reduction; corresponding to Fig. 3B) and up to 3-fold increased XIAP levels, corresponding to experimental XIAP overexpression (Fig. 5B). PARP cleavage, as a readout for MOMP-activated executioner caspases (33), was plotted for time points following MOMP (Fig. 5). Under standard conditions, pre-activation of Smac degradation in combination with WT (= 1X) XIAP levels
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resulted in a reduction to maximal PARP cleavage by only 6%. PARP cleavage, as readout for MOMP-activated executioner caspases (33), was plotted for time points following MOMP (Fig. 5A). As expected, increasing XIAP expression by itself suppressed PARP activation (Fig. 5A, dotted lines). At higher XIAP levels, caspases were almost fully suppressed by cytosolic XIAP action alone, and consequently, reduced Smac had no appreciable further effect. Thus, mathematical modeling supports that reducing mitochondrial Smac prior to activation of canonical MOMP can potentiate XIAP-mediated caspase suppression, in a XIAP concentration-dependent manner.

We first explored this predicted protective effect of pre-activated Smac degradation experimentally in the setting of TNF-induced apoptosis in MCF7 cells expressing GFP or GFP-XIAP. To trigger mitochondrial XIAP action prior to death receptor induction of apoptosis, we pre-treated cells with CCCP for 2 h to activate XIAP at mitochondria, and subsequently we exposed the cells to TNF for 16 h. We monitored caspase activation via immunoblot detection of the cleaved form of PARP (Fig. 5B). As expected, overexpression of XIAP decreased TNF-induced caspase activation. Moreover, CCCP pre-treatment suppressed caspase activation in wild type cells and to a greater extent in XIAP-overexpressing cells, supporting a pro-survival role of mitochondrial XIAP action. These data suggest that when mitochondrial depolarization precedes BH3-only protein-activated MOMP, XIAP-mediated mitochondrial processing can antagonize mitochondrial apoptosis signaling.

Alternatively, we constitutively targeted XIAP to the mitochondrial IMS via expression of RFP-XIAP, N-terminally fused to the IMS-targeting sequence of Smac. IMS-RFP-XIAP-targeted mitochondria contained unaltered Smac (Fig. 6A). Upon treatment with CCCP for 3 h, ~50% of IMS-RFP-XIAP-expressing cells displayed a loss of mitochondrial Smac (Fig. 6B), and within activated cells Smac levels were significantly decreased (Fig. 6C). In addition, following activation of apoptosis with TNF/AcD for 3 h, IMS-RFP-XIAP-expressing cells exhibited significantly reduced Smac levels, compared with nontransfected cells that had undergone Smac release.

To measure the downstream impact on apoptosis, we utilized a GFP-based caspase sensor that fluoresces upon executioner caspase cleavage (Fig. 6, C–E) (19). Importantly, 6 h of exposure to CCCP had no impact on caspase activity in XIAP-overexpressing cells, indicating that XIAP-induced mitochondrial permeabilization does not per se result in caspase activation. TNF/AcD treatment significantly increased caspase sensor GFP fluorescence in control cells by 2-fold. In contrast, RFP-XIAP and IMS-RFP-XIAP maintained GFP fluorescence at basal levels, indicating that both constructs equivalently suppress executioner caspase activation.

Discussion

In addition to its well known function in caspase suppression (9), XIAP can localize at mitochondria (13, 14, 16) and activate Bax-mediated mitochondrial permeabilization (13, 16). Moreover, we previously reported that XIAP can enter BH3-only protein-targeted or CCCP-depolarized mitochondria, resulting in the degradation of its antagonist, Smac, through endolysosomal and proteasomal actions (16). As MOMP-mediated release of Smac, and consequent inhibition of XIAP, is essential for activation of apoptosis (12), this suggests an additional pro-survival role for XIAP during apoptosis. Here, we further investigated the function and regulation of XIAP at mitochondria during apoptosis signaling.

Importantly, we report that mitochondrial XIAP action is cell-intrinsically activated by TNF and STS, common drug inducers of apoptosis, and thus constitutes a previously undescribed component of apoptosis signaling. Mechanistically, we demonstrate that XIAP entry into depolarized mitochondria requires the presence of Bax or Bak, and was inhibited by pro-survival Bcl-2 and Bcl-xL. In this way both apoptotic MOMP and XIAP-activated mitochondrial permeabilization appear comparably regulated by these essential Bcl-2 protein family mediators of the intrinsic apoptotic pathway (23).

Furthermore, our findings suggest that XIAP-activated mitochondrial permeabilization can selectively target Smac uncoupled from cytochrome c release. This is in contrast to canonical MOMP, which causes the simultaneous release of Smac and cytochrome c (34, 35). We previously reported that following CCCP-induced recruitment of XIAP, mitochondrial Smac was degraded at a time point when only a fraction of cells had undergone cytochrome c release (16). Here, we report that when activating MOMP via tBid expression in cells with high XIAP, Smac was degraded, whereas cytochrome c was maintained within tBid-targeted mitochondria. Furthermore, XIAP(W310A), a BIR3 mutant that lacks Smac binding (36), entered the mitochondria without inducing Smac degradation.
but permitted Smac release instead (16). Together, these results suggest that XIAP can selectively and bi-directionally permeabilize the OMM, permitting XIAP entry and subsequent Smac degradation.

We explored Drp1 as a candidate regulator of selective OMM permeability, as loss of Drp1 activity can delay cytochrome c release without affecting Smac release in response to apoptosis induction (31, 32). We found that suppressing Drp1 activity by expression of dominant negative Drp1 blocked XIAP-mediated cytochrome c release, but permitted Smac degradation. Moreover, we found that apoptotic triggers TNF and STS activated mitochondrial XIAP action and that increasing XIAP levels sensitized mitochondria to mitochondrial membrane permeabilization, indicating that XIAP action at mitochondria can occur parallel to or even precede MOMP. These findings provoke the immediate questions of whether XIAP-mediated membrane permeabilization is a distinct “selective mode” of MOMP or a “transition state” toward MOMP, and whether Drp1 drives entry to the “point-of-no-return” canonical MOMP during apoptosis. To help elucidate these questions, it remains to be determined whether XIAP inhibition of tBid-induced cytochrome c release is related to a direct effect of XIAP on Drp1 activity, to an effect on OPA1-mediated mitochondrial cristae retention of cytochrome c (37), or to the latency period between Bax activation and Bax pore formation (38).

**FIGURE 5.** Systems biology approach reveals pro-survival potential of mitochondrial XIAP action during TNF-induced apoptosis. A, time course simulations of the SBML file BIOMD0000000220 from Ref. 11. Relative PARP cleavage is plotted over time, beginning prior to MOMP induction. Fold increases of XIAP concentrations are indicated in color code. For each XIAP condition, the dashed line indicates the standard 1× Smac concentration, and the solid line indicates Smac concentration reduced to 0.5×. Color-coded arrows indicate the percent decrease in PARP cleavage resulting from Smac reduction. B, quantification of XIAP overexpression. MCF7 cells expressing RFP-XIAP were immunostained for XIAP, and XIAP immunofluorescence of transfected and nontransfected cells was quantified. Fold increases for single cell RFP-XIAP expression levels are represented as a binned histogram, color-coded according to A, and a Gaussian fit. C, MCF7 cells expressing GFP or GFP-XIAP were pre-treated with 20 μM CCCP for 2 h in FM, followed by treatment with 43 ng/ml TNF for 16 h in Hanks’ balanced salt solution. Cellular levels of cleaved PARP (PARP*) were detected via Western blotting. Representative blot is shown (left panel). Quantified (n = 3) values were normalized to GAPDH and are represented relative to TNF treatment in GFP-expressing cells. p values for one-tailed, unpaired t test are indicated.
FIGURE 6. Forced localization of XIAP to the IMS is sufficient for drug-induced Smac degradation and reduction of caspase activation. A, MCF7 cells expressing mitochondrial IMS-targeted RFP-XIAP and IMS-RFP-XIAP (red) and immunostained for Smac (green). B, MCF7 cells expressing IMS-RFP-XIAP were treated with CCCP for 3 h and immunostained for Smac. The fraction of cells expressing IMS-RFP-XIAP and demonstrating loss of mitochondrial Smac was scored. C, IF of endogenous Smac was quantified in IMS-RFP-XIAP-expressing MCF7 cells subjected to 3 h of CCCP or TNF/AcD in FM. Box plots represent cellular Smac IF levels, normalized to FM control (indicated by dashed line). *, p < 0.05 compared with FM. D, MCF7 cells stably expressing a GFP-based caspase sensor and transfected with IMS-RFP-XIAP were subjected to FM/TNF/AcD for 6 h. Scale bar, 10 μm. E, quantification of GFP fluorescence in MCF7 cells stably expressing a GFP-based caspase sensor, which was transfected with RFP, RFP-XIAP, or IMS-RFP-XIAP and subjected to FM ± TNF/AcD or CCCP for 6 h. Bar graphs represent fold increase of GFP fluorescence relative to control-treated cells. The dashed line represents basal GFP fluorescence under nonapoptotic conditions. *, p < 0.05 comparing FM with CCCP and TNF/AcD.

FIGURE 7. Proposed model of mitochondrial XIAP action in relation to the canonical MOMP pathway. During intrinsic apoptosis signaling, mitochondria contribute to the progression of apoptosis through the release of pro-apoptotic proteins, including cytochrome c and Smac, from the intermembrane space to the cytosol. This is accomplished via Bcl-2 family of protein-regulated MOMP (indicated by solid lines). In the cytosol, cytochrome c promotes caspase activation through apoptosisosome complex formation, and Smac inhibits XIAP to relieve caspase suppression. In parallel, BH3-only protein activity or mitochondrial bioenergetics stress in combination with high XIAP levels induced a pro-survival XIAP-mediated mitochondrial processing pathway (indicated by dashed lines and yellow nodes). XIAP translocates to and enters into mitochondria, where its E3 ligase activity results in ubiquitylation (Ub) of OMM and inner mitochondrial compartments. Consequently, the XIAP inhibitor Smac is degraded through concerted endolysosomal and proteasomal action (16). XIAP entry into mitochondria depends on Bax/Bak and is opposed by Bcl-2/-xL. Intramitochondrial XIAP-associated Smac degradation occurs independently of Drp1-regulated cytochrome c release, i.e., uncoupled from canonical MOMP.
To functionally analyze XIAP action at mitochondria, we employed a mathematical model designed to quantitatively analyze MOMP dynamics (11). Thereby we were able to gain insight into conditions that would discriminate XIAP action at mitochondria from XIAP suppression of caspases. Our simulations predict that increasing XIAP levels more than 2-fold overwhelms Smac antagonism capacity, while normal levels of XIAP are only slightly sensitive to reduced Smac. However, for up to 2-fold XIAP increases, which correspond to levels of XIAP overexpression tested here, modeling and experimental findings demonstrate potent mitochondrial XIAP-mediated protection against caspase activation.

Of note, our findings indicate that to engage a pro-survival role, XIAP is required to enter mitochondria and degrade Smac, prior to BH3-only protein-activated MOMP. As the period of MOMP-mediated protein release from all mitochondria can occur within 10 min (35, 39, 40) and caspase-mediated cell death can be activated within minutes following MOMP (11, 34), we speculate that XIAP action at mitochondria is not efficient during rapid-onset apoptosis. While we demonstrate here the potential of mitochondrial XIAP to modulate apoptosis via pre-activation of mitochondrial depolarization or artificial targeting of XIAP to the IMS, the understanding of its in vivo role necessitates the identification of the mechanisms targeting XIAP to mitochondria. Although these upstream regulatory components of XIAP translocation to mitochondria remain undetermined, it has been identified that mitochondrial targeting by XIAP occurs under conditions of mitochondrial depolarization and in response to apoptosis induction by drugs and loss of cell-extracellular matrix interactions (13, 14, 16). Here, we demonstrate that the initial event of XIAP translocation to mitochondria requires the presence of Bax and/or Bak. Furthermore, mitochondrial XIAP action is independent of Parkin and autophagy (16), and PINK1 is not involved in its activation (results not shown). However, we envisage that the screening approaches used in recent years to delineate the PINK1/Parkin pathway are fully applicable for further investigating mechanisms of XIAP regulation.

In summary, our data characterize a novel pro-survival function for XIAP, as a concentration-dependent responder to apoptotic and bioenergetic stresses, upstream of caspase activation. As XIAP is increased in cancer (41–43), and hypoxia and metabolic stresses decrease mitochondrial membrane potential (44), future work will aim to identify mechanisms targeting XIAP to mitochondria, as well as conditions under which mitochondrial XIAP actions contribute to cancer cell chemoresistance.

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**References**

1. Ni Chonghaille, T., Sarosiek, K. A., Vo, T. T., Ryan, J. A., Tammareddi, A., Moore Vdel, G., Deng, J., Anderson, K. C., Richardson, P., Tai, Y. T., Mitsuides, C. S., Matulonis, U. A., Drapkin, R., Stone, R., Deangelo, D. J., et al. (2011) Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science* **334**, 1129–1133

2. Vo, T. T., Ryan, J., Carrasco, R., Neuberg, D., Rossi, D. J., Stone, R. M., Deangelo, D. J., Frattini, M. G., and Letal, A. (2012) Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell* **151**, 344–355

3. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsokopoulou, V., Ross, A. J., Roth, A. K., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727–730

4. Shamas-Din, A., Brahmbhatt, H., Leber, B., and Andrews, D. W. (2011) BH3-only proteins: orchestrators of apoptosis. *Biochim. Biophys. Acta* **1813**, 508–520

5. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33–42

6. Bao, Q., and Shi, Y. (2007) Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ.* **14**, 56–65

7. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388**, 300–304

8. Takahashi, R., Deveraux, Q., Tammi, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* **273**, 7787–7790

9. Eckelman, B. P., Salvesen, G. S., and Scott, F. L. (2006) Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep.* **7**, 988–994

10. Schile, A. J., García-Fernández, M., and Steller, H. (2008) Regulation of apoptosis by XIAP ubiquitin-ligase activity. *Genes Dev.* **22**, 2256–2266

11. Albeck, J. G., Burke, J. M., Aldridge, R. B., Zang, M., Lauffenburger, D. A., and Sorger, P. K. (2008) Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol. Cell* **30**, 11–25

12. Host, P. J., Grabow, S., Gray, D., McKenzie, M. D., Nachbur, U., Huang, D. C., Bouillet, P., Thomas, H. E., Bornier, C., Silke, J., Strasser, A., and Kaufmann, T. (2009) XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature* **460**, 1035–1039

13. Owens, T. W., Foster, F. M., Valentinj, A., Gilmore, A. P., and Streuli, C. H. (2010) Role for X-linked inhibitor of apoptosis protein upstream of mitochondrial permeabilization. *J. Biol. Chem.* **285**, 1081–1088

14. Flanagan, L., Sébastià, J., Tuffy, L. P., Spring, A., Lichawiska, A., Devocelle, M., Prehn, J. H., and Rehn, M. (2010) XIAP impairs Smac release from the mitochondria during apoptosis. *Cell Death Dis.* **1**, e49

15. MacFarlane, M., Merrison, W., Bratton, S. B., and Cohen, G. M. (2002) Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. *J. Biol. Chem.* **277**, 36611–36616

16. Hamacher-Brady, A., Choe, S. C., Krijnse-Locker, J., and Brady, N. R. (2014) Intramitochondrial recruitment of endolysosomes mediates Smac degradation and constitutes a novel intrinsic apoptosis antagonizing function of XIAP E3 ligase. *Cell Death Differ.* **21**, 1862–1876

17. Smirnova, E., Shurland, D. L., Ryazantsev, S. N., and van der Bliek, A. M. (1998) A human dynamin-related protein controls the distribution of mitochondria. *J. Cell Biol.* **143**, 351–358

18. Sims, J. J., Scavone, F., Cooper, E. M., Kane, L. A., Youle, R. J., Boeke, J. D., and Cohen, R. E. (2012) Polyubiquitin-sensor proteins reveal localization and linkage-type dependence of cellular ubiquitin signaling. *Nat. Methods* **9**, 303–309

19. Zhang, J., Wang, X., Cui, W., Wang, W., Zhang, H., Liu, L., Zhang, Z., Li, Z., Ying, G., Zhang, N., and Li, B. (2013) Visualization of caspase-3-like activity in cells using a genetically encoded fluorescent biosensor activated by protein cleavage. *Nat. Commun.* **4**, 2157

20. Hoops, S., Sahle, S., Gauges, R., Lee, C., Pahle, J., Simus, N., Singhal, M., Xu, L., Mendes, P., and Kummer, U. (2006) COPASI—a CComplex Pathway Simulator. *Bioinformatics* **22**, 3067–3074
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21. Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahur, P., Faucewirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., et al. (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell 6, 1389–1399

22. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13, 1899–1911

23. Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47–59

24. Cheng, E. H., Levine, B., Boise, L. H., Thompson, C. B., and Hardwick, J. M. (1996) Bax-independent inhibition of apoptosis by Bcl-XL. Nature 379, 554–556

25. Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369, 321–323

26. Kawanati, M., and Imoto, M. (2003) Deletion of the BH1 domain of Bcl-2 accelerates apoptosis by acting in a dominant negative fashion. J. Biol. Chem. 278, 19732–19742

27. Slee, E. A., Keogh, S. A., and Martin, S. J. (2000) Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. Cell Death Differ. 7, 556–565

28. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94, 491–501

29. Jänicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. 273, 9357–9360

30. Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L., and Youle, R. J. (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. 159, 931–938

31. Estaquier, J., and Arnould, D. (2007) Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. Cell Death Differ. 14, 1086–1094

32. Ishihara, N., Nomura, M., Jofuku, A., Kato, H., Suzuki, S. O., Masuda, K., Otera, H., Nakashii, Y., Nonaka, I., Goto, Y., Taguchi, N., Morinaga, H., Maeda, M., Takayanagi, R., Yokota, S., and Mihara, K. (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat. Cell Biol. 11, 958–966

33. Chaitanya, G. V., Steven, A. J., and Babu, P. P. (2010) PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell Commun. Signal. 8, 31

34. Rehm, M., Düssmann, H., and Prehn, J. H. (2003) Real-time single cell analysis of Smac/DIABLO release during apoptosis. J. Cell Biol. 162, 1031–1043

35. Muñoz-Pinedo, C., Guio-Carrion, A., Goldstein, J. C., Fitzgerald, P., Newmeyer, D. D., and Green, D. R. (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. Proc. Natl. Acad. Sci. U.S.A. 103, 11573–11578

36. Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000) Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature 408, 1004–1008

37. Olichon, A., Baricault, L., Gas, N., Guillois, E., Valette, A., Belenguer, P., and Lenaers, G. (2003) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J. Biol. Chem. 278, 7743–7746

38. Kushnareva, Y., Andreyev, A. Y., Kuwana, T., and Newmeyer, D. D. (2012) Bax activation initiates the assembly of a multimeric catalyst that facilitates Bax pore formation in mitochondrial outer membranes. PLoS Biol. 10, e1001394

39. Bhola, P. D., Mattheyes, A. L., and Simon, S. M. (2009) Spatial and temporal dynamics of mitochondrial membrane permeability waves during apoptosis. Biophys. J. 97, 2222–2231

40. Goldstein, J. C., Muñoz-Pinedo, C., Ricci, J. E., Adams, S. R., Kelek, A., Schuler, M., Tsien, R. Y., and Green, D. R. (2005) Cytochrome c is released in a single step during apoptosis. Cell Death Differ. 12, 453–462

41. Ferreira, C. G., van der Valk, P., Span, S. W., Jonker, J. M., Postmus, P. E., Kruyt, F. A., and Giaccone, G. (2001) Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. Ann. Oncol. 12, 799–805

42. Krajewska, M., Krajewski, S., Banares, S., Huang, X., Turner, B., Bubenr, L., Kallioniemi, O. P., Shabaik, A., Vitiello, A., Peehl, D., Gao, G. J., and Reed, J. C. (2003) Elevated expression of inhibitor of apoptosis proteins in prostate cancer. Clin. Cancer Res. 9, 4914–4925

43. Tam, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qu, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000) Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin. Cancer Res. 6, 1796–1803

44. Frezza, C., Zheng, L., Tennant, D. A., Papkovsky, D. B., Hedley, B. A., Kalna, G., Watson, D. G., and Gottlieb, E. (2011) Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival. PLoS ONE 6, e24411