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ORIGINAL PAPER

ARTS binds to a distinct domain in XIAP-BIR3 and promotes apoptosis by a mechanism that is different from other IAP-antagonists

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Abstract ARTS (Sept4_i2), is a pro-apoptotic protein localized at the mitochondria of living cells. In response to apoptotic signals, ARTS rapidly translocates to the cytosol where it binds and antagonizes XIAP to promote caspase activation. However, the mechanism of interaction between these two proteins and how it is regulated remained to be explored. In this study, we show that ARTS and XIAP bind directly to each other, as recombinant ARTS and XIAP proteins co-immunoprecipitate together. We also show that over expression of ARTS alone is sufficient to induce a strong down-regulation of XIAP protein levels and that this reduction occurs through the ubiquitin proteasome system (UPS). Using various deletion and mutation constructs of XIAP we show that ARTS specifically binds to the BIR3 domain in XIAP. Moreover, we found that ARTS binds to different sequences in BIR3 than other IAP antagonists such as SMAC/Diablo. Computational analysis comparing the location of the putative ARTS interface in BIR3 with the known interfaces of SMAC/Diablo and caspase 9 support our results indicating that ARTS interacts with residues in BIR3 that are different from those involved in binding SMAC/Diablo and caspase 9. We therefore suggest that ARTS binds and antagonizes XIAP in a way which is distinct from other IAP-antagonists to promote apoptosis.

Keywords Cell death · Apoptosis · ARTS · XIAP · Mitochondria

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Introduction

Apoptosis is an active and well controlled process of cell death. It plays a major role in development, tissue homeostasis, and as a defense mechanism against unwanted and potentially dangerous cells [1, 2]. When deregulated, apoptosis can result in various pathological conditions, including cancer [2]. The main executioners of apoptosis are caspases, a family of cysteine proteases expressed as inactive zymogens in the cells [3–6]. The apoptotic process is tightly controlled through the action of both activators and inhibitors of caspases [7, 8].

The main inhibitors of caspases are members of the inhibitors of apoptosis proteins (IAPs) family [9–12]. These proteins contain at least one baculoviral IAP repeat (BIR) domain, which can directly interact with caspases and inhibit their apoptotic activity [9, 13, 14]. In addition, some of the IAPs contain an additional RING domain that holds an E3 ubiquitin ligase function [15–17]. In mammals eight different IAP proteins have been described, namely NAIP, cIAP1, cIAP2, X-linked IAP (XIAP), MLIAP, ILP2, survivin, and BRUCE/Apollon [11, 18]. XIAP, the prototype of the IAP family, contains three BIR domains and a RING domain [16, 17]. The BIR domains within XIAP can bind directly to caspases 3, 7 and 9, thereby inhibiting their proteolytic activity [19, 20]. The BIR3 domain of XIAP

selectively inhibits caspase 9, while the BIR2 and the linker preceding BIR2 inhibits both caspase 3 and 7 [20–24].

Several recent reports suggest that the initiation of apoptosis can occur through the release of caspases from their binding to IAPs [25–29]. Therefore, in cells that are doomed to die, inhibition of apoptosis has to be overcome to enable the initiation of apoptosis. This concept was first introduced in Drosophila fruit flies, in which Reaper, Hid and Grim induce apoptosis through their action as IAP-antagonists by binding and inhibiting Drosophila IAP-1 (Diap1) [30–33]. These proteins contain a tetrapeptide domain known as the IAP-binding motif (IBM) which is conserved throughout evolution and found also in mammals [34]. So far, several mammalian XIAP antagonists have been identified, including SMAC/Diablo (here forth referred to as SMAC) [35, 36], Omi/HtrA2, [37–39] and ARTS [40, 41]. Most of the known IAP-antagonists are located in the mitochondria and are released to the cytosol upon apoptotic induction. These proteins are different in sequence and structure, but most of them contain a short, conserved IBM [34]. SMAC and Omi/HtrA2 are both localized in the inter-membrane space of the mitochondria and are released to the cytosol in response to pro-apoptotic signals. Once in the cytosol, they bind BIR2 and BIR3 domains of XIAP via their IBM, disrupting the XIAP-caspases complex [34–36, 38, 42–45]. Interestingly, the IBM of SMAC and Omi/HtrA2 is very similar to the IBM found on the small subunit of caspase 9 [42, 44, 46]. This offers an explanation for the mechanism by which SMAC competes with caspase 9 for binding to BIR3 domain of XIAP [23, 24, 42, 43].

Another mitochondrial protein that promotes apoptosis through binding to XIAP is ARTS [40, 41]. ARTS (Sept4_i2) derived by alternative splicing from the *Sept4* gene [41, 47]. Unlike other septins which are localized to actin-rich regions and function during cytokinesis and cellular morphogenesis, ARTS is localized at the outer membrane of the mitochondria [40, 41, 47–49]. Upon apoptotic stimuli ARTS translocates from the mitochondria to the cytosol and antagonizes XIAP, causing activation of caspases and cell death [40, 47, 48]. Importantly, ARTS does not contain the canonical IBM and binds to XIAP via unique sequences [40]. Strong evidence for a physiological role of ARTS as an IAP-antagonist has come from genetic studies in mice [50]. Garcia-Fernandez et al. reported that ARTS/*Sept4* null mice exhibit increased numbers of hematopoietic stem and progenitor cells, develop spontaneous tumors and show accelerated tumor development in an Eμ-Myc background. Importantly, these mice exhibit elevated levels of XIAP protein and increased resistance to cell death, demonstrating a physiological role of ARTS for regulating XIAP levels and apoptosis in vivo. Furthermore, the apoptosis, stem cell and tumor phenotypes of *ARTS*/*Sept4* null mice are suppressed by inactivation of XIAP, providing compelling evidence that the functions of ARTS as a tumor suppressor and regulator of stem cell apoptosis are mediated through its action as an XIAP antagonist in vivo [50].

We have previously showed that ARTS promotes apoptosis, at least in part, through binding and inhibition of XIAP [40, 47]. However, the mechanism of interaction between these two proteins and how it is regulated remained to be explored. Here we report that ARTS is able to bind directly to XIAP and promote its degradation via the ubiquitin proteasome system (UPS). In addition, we show that ARTS interacts with residues in BIR3 that are different from those involved in the binding of SMAC, Omi/HtrA2 and caspase 9. We therefore suggest that ARTS binds and antagonizes XIAP in a way which is distinct from other IAP-antagonists to promote apoptosis.

Materials and methods

Antibodies

Antibodies to the various proteins were purchased from the indicated companies, and used as instructed. The following antibodies were used: anti-CT-ARTS antibody (Sigma, St. Louis) which is the only currently commercially available antibody directed against the unique C-terminus of ARTS; anti-NT-ARTS (#3025, Prosci); anti-XIAP (#610716, BD); anti- cIAP (AF818, R&D systems); anti-GST (B-14, sc-138, Santa Cruz); anti-SMAC (#567365, Calbiochem); anti-His (#8916-1, BD) and anti-Actin (#69100, MP biomedicals).

Mammalian cell cultures and treatments (induction of apoptosis and proteasome inhibition)

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l D-glucose. Media were supplemented with 10% fetal calf serum (FCS), penicillin 100 U/ml, streptomycin 100 mg/ml, Sodium pyruvate 1 mM and glutamine 2 mM (Biological Industries, Israel). To induce apoptosis, COS-7 cells were incubated with STS (1.25 μM) for different time periods. To inhibit the proteasome activity and COS-7 cells were incubated with MG132 (20 μM) for 6 h.

Knockout mice

Immortalized ARTS/*Sept4* null MEFs were prepared from knockout ARTS/*Sept4* mice [51]. Immortalized XIAP null MEFs were originally obtained from David Vaux

and John Silke and were generously provided by Krishnaraj Rajalingam.

Transient transfection of cells

jetPEI™ (Polyplus Transfection) and Transfectol (Gene-Choice) were used according to the manufacturer's protocol.

Constructs

ARTS

pEF1-AU5 and pEF1-AU5-ARTS constructs contain an AU5 tag that is attached to the N-terminus of ARTS [41]. The AU5-ARTS-CTdel construct lacking 68 aa at the C-terminus was generated as described at [40].

The pSC2-6Myc ARTS and pSC2-1Myc ARTS constructs contain Myc tag that is attached to the N-terminus of ARTS. These constructs were generated using PCR with the following forward primer: *Eco*RI 5′-CGAATTCCA TGATCAAGCGTTTC-3′ and the reverse primer: pEG ARTS-R *Xho*I 5′- TACCGCTCGAGCTAGTGGCAGCCC TGCCC-3′.

Recombinant 6-His-ARTS was generated by cloning ARTS into the pHLTParallel2-6-His-ARTS construct.

XIAP

The mammalian expression construct encoding Myc tagged wild-type XIAP in pcDNA3 and the pEBG mammalian expression constructs encoding N-terminus GST fusion proteins together with XIAP or XIAPΔRING or XIAP-Δloop or XIAP-BIR1+2 or XIAP-BIR1 or XIAP-BIR2 or XIAP-BIR3 were a kind gift from Colin Duckett [52].

The GST-XIAP BIR3 C-terminus deletion constructs were generated using PCR with the following forward primer: F-BIR3-*Bam*HI–ATAGGATCCGCAGATTATGAA GCACGG and the reverse primers: RBIR-21aa *Cla*I–AATATCGATTTATATTTGCACCCTGGAT, R-BIR-31aa *Cla*I–AATATCGATTTATTGTTCCCAAGGGTCTT and R-BIR-41aa *Cla*I–AATATCGATTTAATCAGTTAGCCC TCCTC.

The GST-XIAP BIR3 N-terminus deletion constructs were generated using PCR with the following forward primer: F-5aa *Bam*HI–ATAGGATCCCGGATCTTTAC TTTTGGG, F-10aa *Bam*HI–ATAGGATCCCGGGACAT GGATATACTCAGTT, F-15aa *Bam*HI–ATAGGATCCTC AGTTAACAAGGAGCAGCT, F-30aa *Bam*HI-ATAGGA TCCGTGAAGGTGATAAAGTAAAGC and the reverse primer: R-BIR-*Cla*I-AATATCGATTTAATGAATATTGT TTATATA.

The pEF expression constructs encoding C-terminal Flag-tagged XIAP mutants: W310A, E314S and H343A were a kind gift from John Silke [53].

The pCDNA3-GFP-XIAP construct was a kind gift from Herman Steller [16].

Western blot analysis

Proteins were separated on 12% SDS PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 3% nonfat milk in 1×PBS containing 0.05% Tween (PBS-T). Then, the membrane was incubated with the appropriate antibodies. The membrane was washed three times with 1×PBS-T followed by incubation with secondary antibody. The membrane was developed using the enhanced chemiluminescence detection system (Pierce). To quantify the protein levels, the blots were analyzed using a densitometer and compared with the level of a housekeeping gene (β-actin). Calculations were carried out using densitometry-analysis software (Total lab, TL100, nonlinear).

Binding assays

Pull-down binding

GST pull down COS-7 cells were co-transfected with ARTS and GST-tagged constructs expressing different domains of XIAP. The cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8), 1% NP-40, 0.5% deoxycholate acid, containing protease inhibitors (mini Complete, Roche). The samples were left rotating for 4 h at 4°C with glutathione beads (Amersham Biosciences). Samples were centrifuged at 4,000 rpm at 4°C for 5 min and washed five times in lysis buffer. Proteins were eluted from beads following 5 min of boiling in sample buffer. Proteins were separate on 12.5% SDS-PAGE gel, followed by Western blot analysis. Myc and Flag pull-down: COS-7 cells were co-transfected with 6Myc-ARTS and different GST-XIAP constructs; Myc-XIAP and different ARTS constructs; or 6Myc-ARTS and different Flag-XIAP constructs. The cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8), 1% NP-40, 0.5% deoxycholate acid, containing protease inhibitors (mini Complete, Roche). The samples were left rotating for 16 h at 4°C with anti-Myc (Santa Cruz) or anti-Flag (Sigma) agarose beads. Samples were centrifuged at 4,000 rpm at 4°C for 5 min and washed three times in PBS. Proteins were eluted from beads following 5 min of boiling in sample buffer. Proteins were separate on 12.5% SDS-PAGE gel, followed by Western blot analysis.

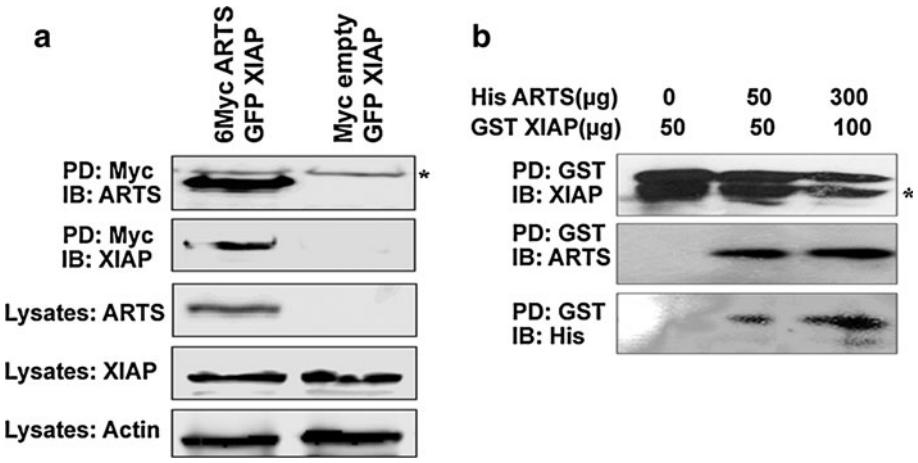

Fig. 1 ARTS binds directly to XIAP. **a** Exogenous ARTS binds to exogenous XIAP protein. COS-7 cells were transiently transfected with GFP-XIAP construct together with 6Myc-ARTS (*left lane*) or empty 6Myc-vector (*right lane*) constructs. Pull down (*PD*) assays with anti Myc beads were carried out, followed by Western blot analysis with anti-ARTS, anti-XIAP and anti-actin antibodies. **b** Recombinant ARTS and XIAP bind directly to each other. In vitro binding assays with different concentrations of GST-XIAP and purified recombinant His-ARTS were used. PD assays with glutathione beads were carried out, followed by Western blot analysis with anti-XIAP, anti-ARTS and anti-His antibodies. *Asterisk* a non specific IgG band

In vitro binding of recombinant proteins

Recombinant 6His-ARTS protein was generated with the TNT-Quick Coupled Transcription/Translation System (Promega) and incubated over-night at 4°C with recombinant GST-XIAP bound to glutathione beads. Samples were centrifuged at 4,000 rpm at 4°C for 5 min and washed five times with PBS. SDS-PAGE analysis and Western blot was performed on the eluted proteins.

Immunofluorescence assay

COS-7 cells were seeded in 24-well plates on cover slips previously coated with fibronectin (5 μg/ml, Biological Industries). The cells were transfected with AU5-ARTS or AU5-ARTS-C-del constructs. Following induction of apoptosis with 1.25 μM STS for two hours, the cells were stained with MitoTracker® Red (M-7512, Molecular Probes) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Image analysis was carried out using fluorescent microscope Zeiss AxioPhot.

Results

ARTS binds directly to XIAP

ARTS is an IAP antagonist localized at the outer membrane of mitochondria in living cells [40, 41, 49]. Upon induction of apoptosis, ARTS translocates from mitochondria to the cytosol, where it binds XIAP and promotes caspase activation [40]. Myc pull down assay of exogenous ARTS and XIAP showed that ARTS binds to XIAP in vivo (Fig. 1a). In order to investigate whether the binding between ARTS and XIAP is direct or whether it requires a third party, we performed an in vitro binding assay. For this purpose recombinant proteins (6His-ARTS and GST-XIAP) were used. GST pull down assay revealed that these two proteins were able to bind directly to each other (Fig. 1b). Altogether, these results indicate that ARTS binds directly to XIAP, both in vivo and in vitro.

ARTS promotes apoptosis mainly through binding and antagonizing XIAP

The unique C-terminal sequences in ARTS are necessary for its binding to XIAP [40]. Transfection of cells with an ARTS construct which lack its C-terminal part (ARTS-CTdel) failed to bind XIAP [40]. To confirm that the pro-apoptotic activity of ARTS is mainly mediated through binding and antagonizing XIAP, we transfected XIAP null MEFs with either full length ARTS or ARTS-CTdel constructs. As shown in Fig. 2a, expression of ARTS (and ARTS-CTdel) did not induce apoptosis in cells lacking XIAP. Yet, over-expression of XIAP together with full length ARTS rescued the ability of ARTS to induce apoptosis in these cells. In contrast, over-expression of XIAP with ARTS-CTdel did not restore apoptosis, suggesting that the apoptotic function of ARTS is mainly executed through its binding to XIAP (Fig. 2a). To exclude the possibility that inability of ARTS-CTdel to bind XIAP is due to different cellular localization as compared to full length ARTS, we performed an immunofluorescence assay. COS-7 cells were transfected with either full length ARTS

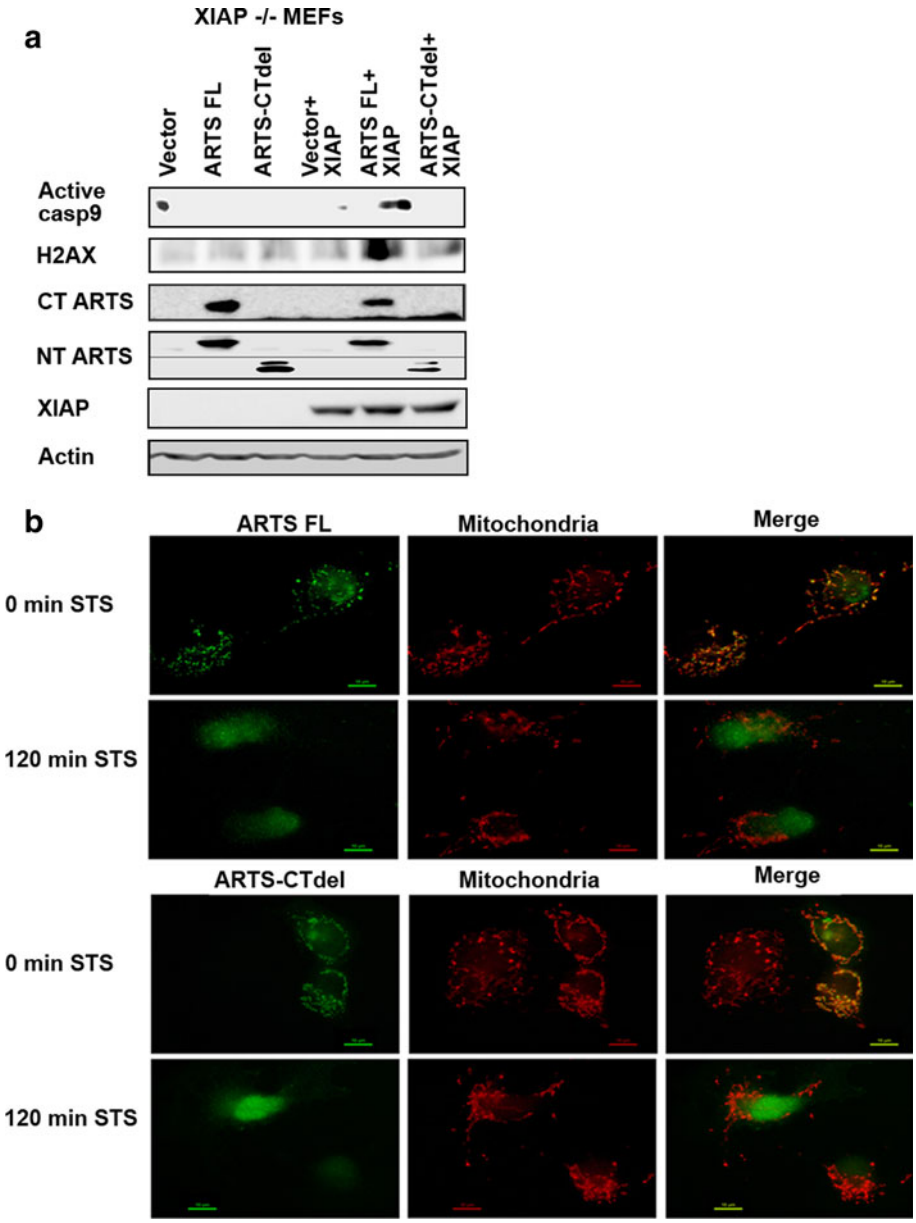

Fig. 2 ARTS promotes apoptosis mainly through antagonizing XIAP. **a** ARTS-induced apoptosis is mainly mediated through binding to XIAP. XIAP null MEFs were transiently transfected with full length ARTS (ARTS FL), mutated ARTS lacking it unique C-terminus (ARTS-CTdel), or empty vector with or without co-transfection with XIAP. Western blot analysis was performed with anti-active caspase 9, anti-H2AX, antibodies directed against the C-terminus of ARTS (CT ARTS, Sigma), antibodies directed against the N-terminus of ARTS (NT ARTS, Prosci), anti-XIAP and anti-actin antibodies. XIAP null MEFS over-expressing full length ARTS showed induction of apoptosis only when co-transfected with XIAP. In contrast, MEFS co-trasfected with ARTS-CTdel and XIAP did not exhibit apoptosis. **b** Mutant ARTS lacking its unique C-terminus localizes to mitochondria in living cells similar to full length ARTS. COS-7 cells were transfected with full length-ARTS (ARTS FL) or mutated ARTS (ARTS-CTdel). Cells were treated with 1.25 μM STS for 2 h, stained with MitoTracker (*red*), and incubated with anti ARTS antibody (*green*). ARTS and ARTS-CTdel showed similar cellular localization both in living cells, localizing to mitochondria, and in apoptotic cells. (*Bar* 10 μm) (Color figure online)

or ARTS-CTdel construct and their cellular localization was determined in the presence or absence of pro-apoptotic treatment, STS for 120 min. This assay shows that there is no difference in the cellular localization of ARTS or ARTS-CTdel (Fig. 2b). Both constructs localize to mitochondria in living cells (as seen by their co-localization with MitoTracker), and translocate to the cytosol (as seen by their diffused staining) following STS induction. Thus, it seems that the mutation in ARTS which affects its binding to XIAP and the ability of ARTS to promote apoptosis [40](Fig. 2a) are not influenced by its cellular localization.

ARTS promotes rapid and specific degradation of XIAP but not cIAP1 protein upon induction of apoptosis

Yang et al. reported that upon induction of apoptosis, XIAP protein is degraded via the UPS [17]. Taking into consideration the direct binding of ARTS to XIAP, we tested whether this binding may affect degradation of XIAP. Figure. 3 demonstrates that over-expression of ARTS alone in COS-7 cells is sufficient for strongly reducing the levels of XIAP. Moreover, this reduction was specific to XIAP, as cIAP1 levels remained intact (Fig. 3a). In addition, binding of the unique C-terminal domain of ARTS to

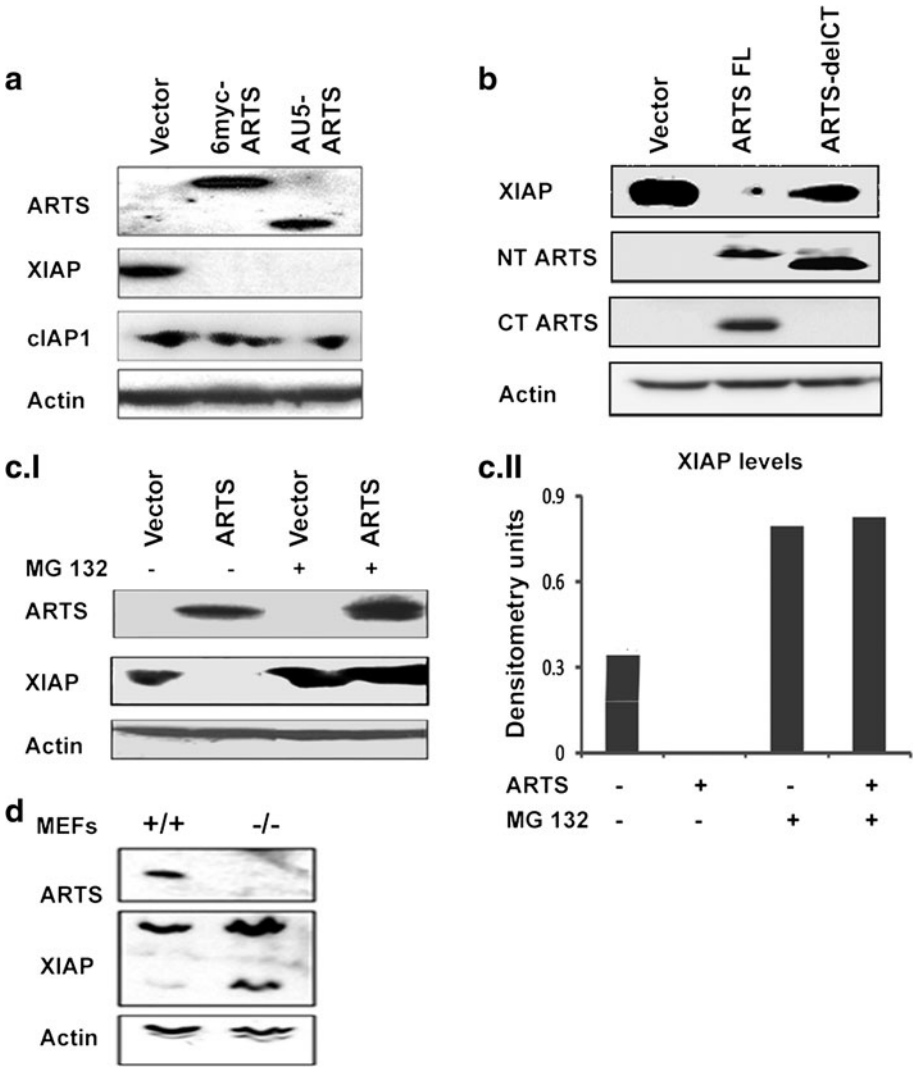

Fig. 3 ARTS promotes rapid and specific degradation of XIAP but not cIAP1 upon induction of apoptosis. **a** High levels of ARTS are sufficient to promote reduction of XIAP but not cIAP1 protein levels. COS-7 cells were transiently transfected with 6myc-ARTS (*middle lane*), AU5-ARTS (*right lane*) or 6myc empty vector (*left lane*) constructs. Western blot analysis was performed with anti-ARTS, anti-XIAP, anti-cIAP1 and anti-actin antibodies. High levels of ARTS alone induce down regulation of XIAP, but not of cIAP1 levels. **b** ARTS lacking it C-terminus fails to down regulates XIAP protein levels. COS-7 cells were transiently transfected with full length ARTS (ARTS FL), ARTS lacking its C-terminus (ARTS-CTdel) or empty vector constructs. Western blot analysis was performed with anti- C-terminus of ARTS (CT ARTS, Sigma), anti- N-terminus of ARTS (NT ARTS, Prosci), anti-XIAP and anti-actin antibodies. Full length ARTS but not ARTS-CTdel induced down regulation of XIAP. **c.I** ARTS induces degradation of XIAP by the UPS. COS-7 cells were transiently transfected with 6myc-ARTS construct or an empty vector construct. Cells were treated with 20 μM of the proteasome inhibitor MG-132 for 6 h. Western blot analysis was performed with anti-ARTS, anti-XIAP and anti-actin antibodies. This analysis indicates that ARTS promotes degradation of XIAP through the UPS. **c.II** Densitometry analyses of XIAP protein levels of the experiment shown in ***b.I***. **c** MEFs from ARTS/*Sept4* null mice exhibit elevated steady-state levels of XIAP. ARTS/*Sept4* null MEFs were lysed and subjected to Western blot analysis with anti-ARTS, anti-XIAP and anti-actin antibodies

XIAP is essential for XIAP degradation, as ARTS-CTdel has a much lower ability to degrade XIAP when compared to the full length ARTS (Fig. 3b). Next, we assessed whether the observed ARTS-induced reduction of XIAP occurs via the UPS. COS-7 cells were transiently transfected with ARTS construct and treated with the proteasome inhibitor MG132. This assay revealed that treatment with MG132 blocked ARTS-induced reduction of XIAP levels, indicating that ARTS promotes degradation of XIAP by the UPS (Fig. 3cI, 3cII). Moreover, MEFs from ARTS/*Sept4* null mice express elevated levels of XIAP (Fig. 3d), indicating that ARTS serves as a specific XIAP antagonist in vivo.

ARTS binds to the BIR3 domain of XIAP

Several studies have established that BIR3 is the domain to which SMAC binds to XIAP [24, 42, 43]. In order to identify the binding site of ARTS within XIAP, we performed co-immunoprecipitation assays with Myc-tagged ARTS and different GST-tagged XIAP expression constructs (full length XIAP, XIAPΔRING, XIAPΔLoop, BIR1+2, BIR1, BIR2, BIR3) [52]. GST pull down assays indicated that ARTS binds to the full length XIAP, XIAPΔRING and XIAPΔLoop and BIR3 domain (Fig. 4). Therefore, the RING and loop regions of XIAP are not important for ARTS-XIAP binding while BIR3 domain is essential for binding of these two proteins. Occasionally we observed a weak binding of ARTS to XIAP-BIR1 (data not shown). However, we decided to concentrate on exploring the binding of ARTS to XIAP-BIR3. Several reports have pointed to amino acid E314 in BIR3 to be essential for binding of IBM bearing proteins SMAC, Omi/HtrA2 and caspase 9 [23, 42, 53, 54].In addition, amino acid W310 in BIR3 was found to be important for binding of SMAC and caspase 9 to BIR3 9 [23, 42, 53]. Amino acid H343 in BIR3 has been shown to bind only to caspase 9 but not to SMAC [23]. Therefore, if ARTS binds to the same sequences in BIR3 as SMAC, Omi/HtrA2 and caspase 9 we would expect that mutations in these specific amino acids (E314, W310 and H343) would prevent binding of ARTS to BIR3. Surprisingly, we found that ARTS could still bind to these three mutation constructs (Fig. 5b). To further investigate the exact binding site of ARTS within BIR3 we used a set of deletion constructs lacking 21, 31 and 41 amino acids from the C-terminal part of BIR3, and 5, 10, 15, 30 amino acids from its N-terminus (Fig. 5a). We found that while ARTS could bind to all C-terminal BIR3 deletion constructs, SMAC did not bind to any of them (Fig. 5c). Further analysis of the ARTS binding site using the N terminal BIR3 deletions allowed us to map it to amino acids 272–292 in BIR3 (Fig. 5d). These are different

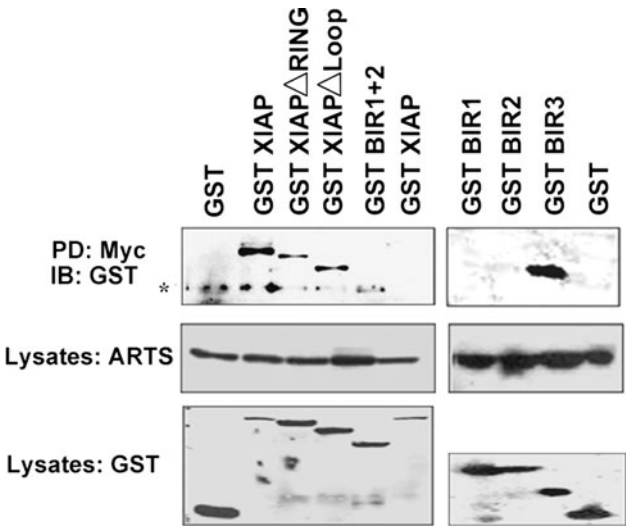

Fig. 4 ARTS binds specifically to the BIR3 domain of XIAP. COS-7 cells were transiently transfected with 6myc-ARTS construct together with various mammalian GST-tagged-XIAP and XIAP mutation constructs. The following constructs were used: GST-XIAP, GST-XIAPΔRING, GSTXIAPΔloop, GST-XIAP-BIR1+2, GST-XIAP-BIR1, GST-XIAP-BIR2 and GST-XIAP-BIR3. Cells were treated with 1.25 μM STS for 1 h. PD assays with anti-Myc beads were carried out, followed by Western blot analysis with anti-ARTS and anti-GST antibodies. ARTS binds to GST-XIAP, GST-XIAPΔRING and GST-XIAPΔloop. In addition ARTS demonstrates specific binding to the BIR3 domain alone. Whole-cell lysates (input) showed that all constructs were expressed in the transfected cells. *Asterisk* nonspecific bands

sequences than those required for SMAC and caspase 9 binding. Importantly, amino acids 272–277 in BIR3 seem to contain residues which are of particular importance for binding to ARTS, since a BIR3 mutant containing a 15aa deletion from its N- terminus, could no longer bind ARTS, while deleting the first 10aa in BIR3 still showed binding (Fig. 5d). This indicates that ARTS interacts with a distinct motif within BIR3.

To learn more about the structural implications of our findings, we applied a series of computational methods to compare the location of the putative ARTS interface in BIR3 with that of SMAC and caspase 9 (CASP9) interfaces. For this purpose we superimposed the known structures of the BIR3-CASP9 complex (code 1nw9 from the Protein Data Bank (PDB)) [23, 55] with that of the BIR3-SMAC complex (PDB code 1g73) [42, 43]. Since the BIR3 domain is almost identically folded in both cases (RMSD 0.323 Å), this model allows us to study simultaneously both SMAC, CASP9, and ARTS interfaces on BIR3. Our model predicts that ARTS interacts with residues in BIR3 (amino acids 277–292, in green) that are distinct from those involved in binding SMAC and CASP9 (Fig. 6a, b). This model is consistent with the idea that ARTS binds to a distinct domain in XIAP-BIR3.

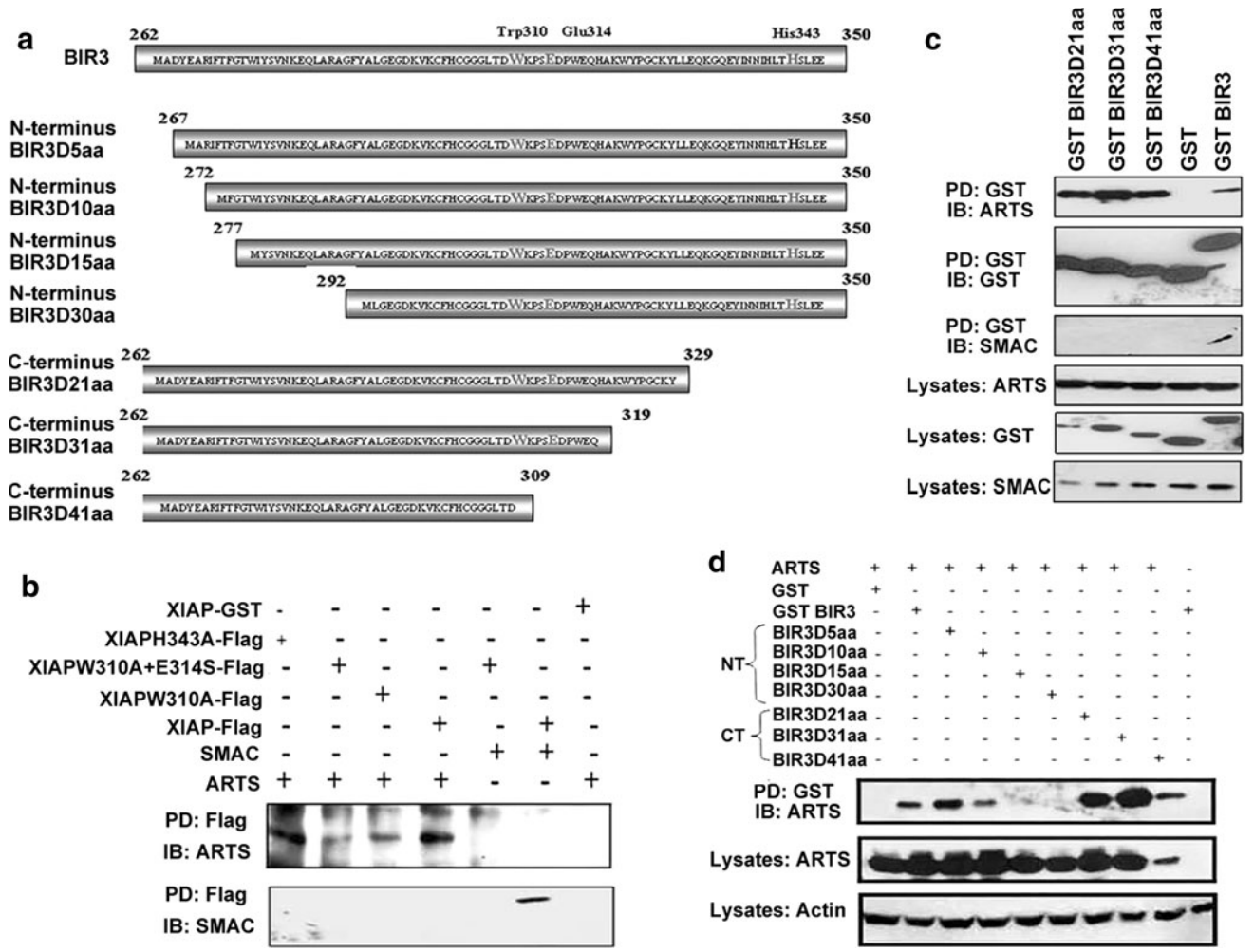

Fig. 5 ARTS binds to distinct sequences within XIAP-BIR3. **a** Schematic diagram of four N-terminal deletion constructs and three C-terminal deletion constructs of GST-BIR3 used in the assays described in this figure. **b** ARTS can bind to XIAP-BIR3 mutant constructs specifically mutated at the SMAC/Caspase 9 binding sites. COS-7 cells were co-transfected with 6myc-ARTS or SMAC together with XIAP-flag containing point mutations in W310, E314 or H343 amino acids. Control cells were transfected with 6myc-ARTS together with GST-XIAP. Cells were treated with 1.25 μM STS for 1 h. PD assays were carried out using anti-flag beads. Western blot analysis was performed using anti-ARTS anti-SMAC and anti-flag antibodies. The two amino acids (W310, E314) in BIR3 are essential for binding of both caspase 9 and SMAC [23, 53]. While ARTS could bind to these BIR3 mutants SMAC could not. **c** ARTS binds to different sequences than SMAC within XIAP-BIR3. COS-7 cells were transiently transfected with 6myc-ARTS together with mammalian GST constructs: GST-XIAP-BIR3, GST-XIAP-BIR3Δ21aa, GST-XIAP-BIR3Δ31aa, GST-XIAP-BIR3Δ41aa or GST empty vector. Cells were treated with 1.25 μM STS for 1 h. GST-PD assays were carried out, followed by Western blot analysis with anti-ARTS, anti-GST and anti-SMAC antibodies. Whole-cell lysates (input) showed that all the constructs were expressed in transfected cells. The GST–PD showed that ARTS was able to bind to all C-terminal deletions of BIR3 while SMAC could bind only to the full length BIR3 domain construct. **d** COS-7 cells were transfected with 6myc-ARTS together with various mammalian GST-XIAP-BIR3 C/N-terminus deletion constructs or GST empty vector. The C-terminus deletion constructs were: GST-XIAP-BIR3Δ21aa, GST-XIAP-BIR3Δ31aa and GST-XIAP-BIR3Δ41aa. The N-terminus deletion constructs were: GST-XIAP-BIR3Δ5aa, GST-XIAP-BIR3Δ10aa, GST-XIAP-BIR3Δ15aa and GST-XIAP-BIR3Δ30aa. Cells were treated with 1.25 μM STS for 1 h. GST–PD assays were carried out followed by Western blot analysis with anti-ARTS and anti-actin antibodies. Whole-cell lysates (input) showed that ARTS was expressed in the transfected cells. The GST-PD showed that ARTS could bind to all C-terminal BIR3 deletions, but could not bind to the BIR3 N-terminal deletion constructs: GST-XIAP-BIR3Δ15aa and GST-XIAP- BIR3Δ 30aa

Discussion

XIAP is considered to be the most potent inhibitor of caspases in vitro and elevated levels of this protein are found in human tumors [56–58]. On the other hand, because mice deficient for XIAP are viable and were initially reported to have no apparent apoptosis phenotypes [59], the physiological function of XIAP in situ has remained controversial. However, it was recently shown that loss of XIAP function causes elevated levels of

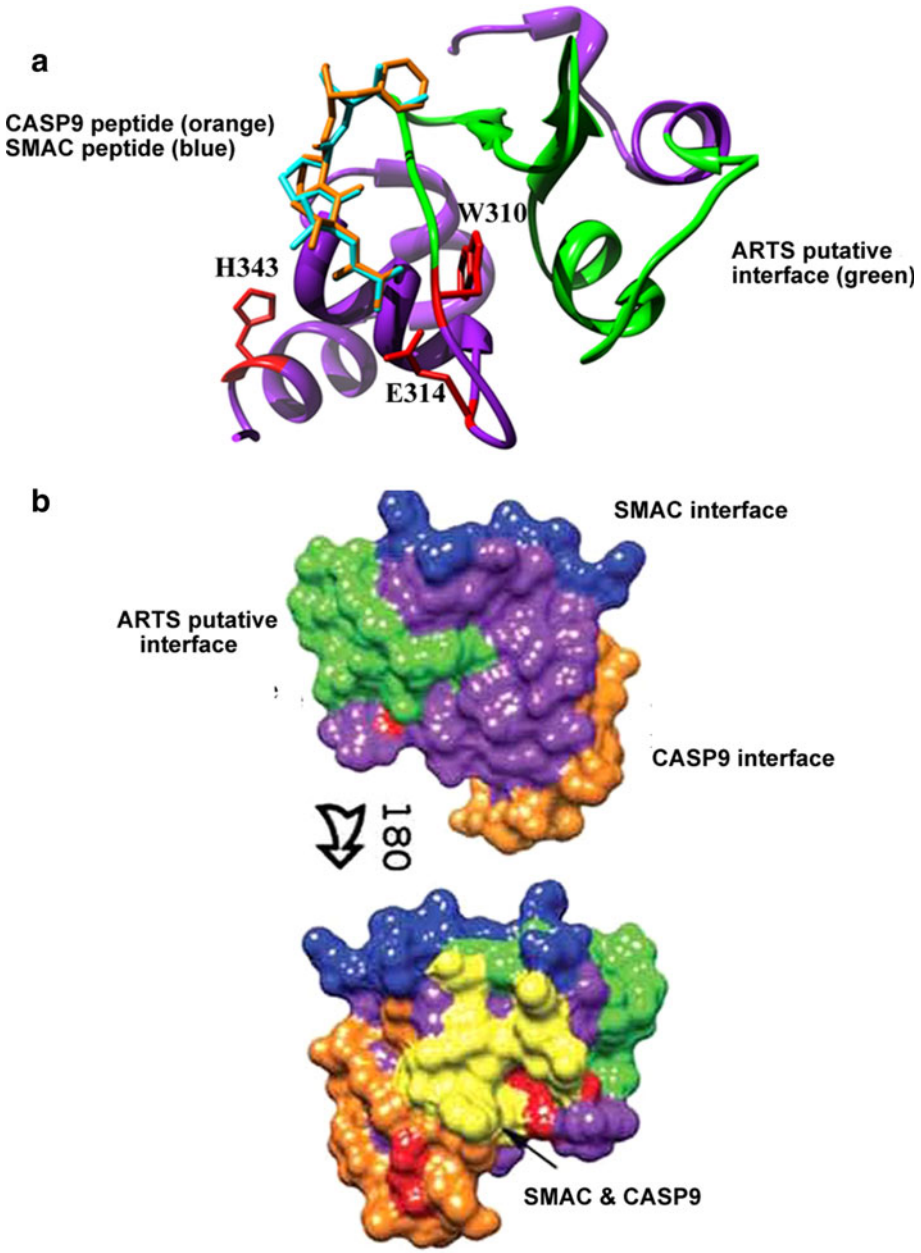

Fig. 6 Computational model of XIAP-BIR3 structure exhibiting the Caspase 9 (CASP9), SMAC and putative ARTS interfaces. The structure was extracted from the BIR3-CASP9 complex (Protein Data Bank PDB, code 1nw9). **a** Schematic model of the structural interaction of XIAP BIR3 domain with CASP9, SMAC and ARTS. The *green* aria within BIR3 domain of XIAP represents the aria which ARTS binds to SMAC and CASP 9 peptides are represented in *blue* and *orange*, respectively. **b** Filled in model of CASP9, SMAC and ARTS interactions. In both panels, the residues forming the BIR3-CASP9 interface are colored *orange*, BIR3-SMAC interface residues are colored *blue* and the residues forming the putative BIR3-ARTS interface region (aa 272-292 in BIR3) are colored in *green*. Residues binding both CASP9 and SMAC are colored *yellow*. BIR3 residues which are not a part of any interface are colored in *purple*. Molecular images were produced using the [75] and edited with GIMP (Graphic Manipulation Program, http://www.gimp.org) (Color figure online)

caspase 3 enzyme and sensitizes certain primary cells towards apoptosis [16]. In addition, XIAP-mutant mice are protected against Eμ-Myc-driven lymphoma due to increased apoptosis of pre-malignant lymphocytes. Significantly, the ability of XIAP to inhibit caspases and apoptosis was dependent on the E3-ligase activity of XIAP [16]. Conversely, loss of ARTS function has been implicated in human hematopoietic malignancies and ARTS/*Sept4* null mice develop spontaneous tumors and show accelerated tumor development in an Eμ-Myc background [50, 60]. The tumor phenotypes of ARTS/*Sept4* null mice are suppressed by inactivation of XIAP, providing strong evidence that the functions of ARTS are mediated through its action as an XIAP antagonist in vivo [50]. Interestingly, genetic inactivation of SMAC and Omi/HtrA2 has failed to reveal any physiological requirement of these IAP-binding proteins in apoptosis, IAP regulation, or tumor suppression [61–63]. One possible explanation for this lack of mutant phenotypes is the potential functional redundancy of different IAP antagonists in the mouse.

In this study, we show that the mechanism by which ARTS binds to XIAP is distinct from any other known pro-apoptotic protein. First, the unique C-terminus of ARTS that has been implicated in XIAP-binding shows no detectable sequence similarity with other IAP-antagonist, including IBM-containing proteins (such as Reaper,

SMAC, Omi/HtrA2), and XAF1 [64]. Thus, ARTS contains a novel IAP-binding motif. Second, the cellular localization of ARTS is different than both IAP-antagonists SMAC and Omi/HtrA2. While ARTS is localized at the mitochondria outer membrane (MOM) [49], SMAC and Omi/HtrA2 are localized at the intermembrane space of the mitochondria [46, 65, 66]. The cellular localization of these proteins is highly relevant to their function. We have found that translocation of ARTS from mitochondria to the cytosol and its binding to XIAP precedes the release of SMAC and is required for it [49]. Thirdly, in this study we show that although both ARTS and SMAC bind to the BIR3 domain of XIAP, ARTS-binding to BIR3 involves different sequences than those bound by SMAC (Fig. 5) [24, 42]. Further structural analysis using X-ray crystallography will be useful in providing more extensive information regarding the binding site of ARTS within BIR3. Finally, SMAC selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP [67]. Moreover, SMAC-based IAP antagonists have the ability to induce degradation of cIAPs, but not XIAP [68, 69]. In contrast, ARTS appears to promote apoptosis through specifically reducing XIAP levels via UPS-mediated degradation (Fig. 2bI, 2bII).

Collectively, our results suggest that ARTS and SMAC act in distinct ways to target different IAP family members via different mechanisms and kinetics, and that XIAP is a major target for ARTS during caspase activation and tumor suppression. In addition, we show that treatment of cells with the proteasome inhibitor MG132 blocked ARTS-induced reduction of XIAP levels, indicating that ARTS promotes degradation of XIAP via the UPS (Fig. 3c). XIAP is an E3-ubiquitin ligase and its E3-ligase activity is important for both self-conjugation and caspase regulation [16, 70, 71]. The observed ARTS-induced UPS-mediated degradation of XIAP may occur through auto-ubiquitination of XIAP. The possibility that decreased levels of XIAP leading to caspase activation occurs through autoubiquitination, was raised in several reports [32, 34, 68, 71, 72]. Alternatively, degradation of XIAP could be promoted by activation through another E3-ligase. A recent study has shown that ARTS collaborates with the E3-ligase Siah1 to degrade XIAP [73]. Garrison et al. found that ARTS acts as an essential adaptor between XIAP and Siah-1 allowing XIAP degradation by interacting exclusively with the BIR1 domain of XIAP [73]. These findings raise the possibility that the distinct binding affinities between ARTS to XIAP-BIR1 versus XIAP-BIR3 may reflect different functional outcomes induced by ARTS. We speculate that the binding of ARTS to BIR1 allows the recruitment of Siah1, which acts as an E3-ligase of XIAP, while the binding of ARTS to BIR3 specifically enables the auto-ubiquitination of XIAP followed by its degradation via the UPS. Another possibility is that binding of ARTS to BIR3 may also induce conformational changes that could result in caspase 9 de-repression and activation.

Consistent with our results, Garrison et al. also report interaction of ARTS with the isolated BIR3 domain of XIAP when using GST pull-down assays, but not with other BIR3-containing fragments that included adjacent domains located on either the N- or C-terminal sides of BIR3 (XIAP BIR2+BIR3 and XIAP BIR3+UBA+RING). Thus, ARTS may bind the BIR3 domain of XIAP in a conformation-dependent manner. This is consistent with evidence that some functions of IAPs may be controlled via intramolecular interactions among domains found within these multifunctional proteins [68, 73, 74].

In this study, we also show that MEFs from ARTS/*Sept4* null mice exhibit elevated levels of XIAP (Fig. 3d). This confirms the physiological role of ARTS as a XIAP-antagonist in vivo. Furthermore, it suggests that ARTS can regulate steady-state levels of XIAP under normal, non apoptotic conditions. Consistent with these results, elevated levels of XIAP were found in several cell types from ARTS/*Sept4* null mice under non-apoptotic conditions [50, 51]. There are at least two possible explanations for this phenomenon; One, that some ARTS-XIAP complexes may form all the time at the MOM and hence affect steady-state levels of XIAP. Alternatively, there may be a constant (low) "flux" of ARTS from the MOM to the cytosol. Such a dynamic flux would be insufficient to inhibit XIAP strongly enough to induce apoptosis, but it would explain the observed increase of steady-state XIAP protein level. Based on our results we suggest that the interaction between ARTS and XIAP may also occur in living cells, but is strongly increased under apoptotic conditions allowing de-repression of caspases, and apoptosis. In summary, our results define ARTS as a unique IAP-antagonist that induces apoptosis by a mechanism distinct from all other known IAP-antagonists.

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