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ARTS and Siah Collaborate in a Pathway for XIAP Degradation

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SUMMARY

ARTS (Apoptosis-Related protein in the TGF-β Signaling pathway) is a mitochondrial protein that binds XIAP (X-linked Inhibitor of Apoptosis Protein) upon entering the cytosol, thus promoting cell death. Expression of ARTS is lost in some malignancies. Here we show that ARTS binds to XIAP at BIR1, a domain distinct from the caspase-binding sites. Furthermore, ARTS interacts with the E3 ligase Siah-1 (seven in absentia homolog 1) to induce ubiquitination and degradation of XIAP. Cells lacking either Siah or ARTS contain higher steady-state levels of XIAP. Thus, ARTS serves as an adapter to bridge Siah-1 to XIAP, targeting it for destruction.

INTRODUCTION

Inhibitor of Apoptosis Proteins (IAPs) play pivotal roles in oncogenesis by suppressing apoptosis induced by both intrinsic and extrinsic cell death pathways (Salvesen and Duckett, 2002). Most IAPs bind caspases, intracellular proteases responsible for apoptosis (Salvesen and Duckett, 2002; Eckelman et al., 2006). While binding caspases, only the XIAP protein has been shown to unequivocally inhibit the activity of caspases with physiologically relevant potency, owing to its ability to make contact with the caspases it targets at two sites rather than a single site as found thus far for other IAPs (Chai et al., 2001; Riedl et al, 2001; Shiozaki et al., 2003). Consequently, XIAP may have special importance among IAP family members as a suppressor of apoptosis.

All members of the human IAP family contain at least one baculoviral IAP repeat (BIR) domain (a distinctive zinc-binding protein fold). Several IAPs additionally contain a C-terminal RING domain that binds ubiquitin-conjugating enzymes (E2s), endowing them with E3 ubiquitin ligase activity (Srinivasula and Ashwell, 2008). Ubiquitin modifications that IAPs induce on their substrates vary, with K48-linked polyubiquitin chains targeting for proteasomal degradation (Li et al., 2002; Varfolomeev et al., 2007). In this regard, several

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IAPs appear to autoregulate their expression through self-ubiquitination, maintaining relatively low levels of these proteins in tissues.

Several endogenous antagonists of IAPs have been identified, including SMAC, HtrA2/Omi, ARTS, and XAF-1. SMAC and HtrA2/Omi are both targeted to mitochondria by an N-terminal leader sequence that is removed by proteolysis upon their import into these organelles (Shiozaki and Shi, 2004). SMAC and HtrA2/Omi are released from the inter-membrane space of mitochondria in response to apoptotic signals and bind via their cleaved N-termini to several IAPs. The interaction of SMAC and OMI with IAPs requires the N-terminal Ala of the processed mitochondrial proteins, which binds grooves on the surfaces of BIR domains, displacing caspases. Interestingly, SMAC binds with high affinity to XIAP, cIAP1, and cIAP2, but stimulates the E3 ligase activity only of cIAP1 and cIAP2 (Creagh et al. 2004), suggesting that XIAP ubiquitination may be regulated differently.

ARTS, a mitochondrial-localized protein, was shown to promote apoptosis by binding XIAP (Gottfried et al., 2004). Apoptotic stimuli cause the release of ARTS from mitochondria allowig its binding to XIAP, resulting in caspase activation and cell death (Gottfried et al., 2004; Larisch et al., 2000). ARTS expression is lost in some cancers. For example, ARTS expression is lost in > 70% of childhood of acute lymphoblastic leukemia (ALL) specimens during disease progression (Elhasid et al., 2004). ARTS is also implicated in the progression of acute myelogenous leukemia (AML), and leukemic cells lacking ARTS are resistant to apoptotic stimuli (Elhasid et al., 2004). Furthermore, the region encoding ARTS is deleted in a number of solid tumors (Kalikin et al., 2000). The loss of ARTS expression provides evidence that ARTS may function as a tumor suppressor (Yamamoto et al., 2004). We therefore investigated the mechanism by which ARTS antagonizes IAPs, in hopes that the resulting information might suggest strategies for restoring or mimicking ARTS activity, thus promoting apoptosis of cancer cells.

RESULTS

ARTS reduces XIAP protein levels

XIAP contains three BIR domains, where BIR2 binds downstream effector caspases (caspases-3 and -7) and BIR3 binds an upstream initiator caspase (caspase-9) (Reed, 2004). If ARTS promotes apoptosis in a manner analogous to other XIAP antagonists, SMAC and OMI/Htra2, then we anticipated it should interact with the BIR domains that bind caspases. However, examination of ARTS binding to various fragments of XIAP showed that it interacts predominantly with the BIR1 domain, not BIR2 or BIR3, and not the RING domain required for XIAP's E3 ligase activity (Figure 1A), as determined by co-immunoprecipitation (co-IP) experiments where epitope-tagged ARTS protein was co-expressed in HEK293T cells with XIAP fragments. HEK293T cells afford high levels of protein expression, such that even weak interactions might be detected, but binding was selective for BIR1.

Next, we performed *in vitro* protein binding studies by the GST-pull-down method, employing a panel of GST-fusion proteins containing a variety of fragments of XIAP and incubating them with lysates from HEK293T cells transfected with Myc-ARTS. As in the co-IP experiments, ARTS bound strongly to fragments of XIAP containing the BIR1 domain, including the isolated BIR1 domain (Figure 1B). We also detected a weaker interaction of ARTS with the isolated BIR3 domain of XIAP by GST pull-down (that was not observed by co-IP), 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) (Figure 1B). This observation suggests that ARTS may also bind the BIR3 domain of XIAP in a conformation-dependent manner, and is consistent with evidence

that some functions of IAPs may be controlled via intra-molecular interactions among domains found within these multifunctional proteins (Varfolomeev et al., 2007; Mace et al., 2008).

N-terminal peptides from the IAP antagonists SMAC and OMI bind BIR domains and displace caspases from XIAP (Shiozaki and Shi, 2004). When the SMAC tetrapeptide alanine-valine-proline-isoleucine (AVPI) (10 μM) was co-incubated with reaction mixtures containing ARTS and various fragment of XIAP, binding to full-length XIAP, the isolated BIR1 domain, or the isolated BIR3 domain was not inhibited, and in fact, binding was induced between ARTS and additional BIR3-containing fragments of XIAP (Figure 1B, bottom). In contrast, in separate reactions, addition of 10 μM of this peptide inhibited binding of recombinant full-length SMAC protein to full-length XIAP (Figure S1) and restored caspase activity in reactions inhibited by BIR2 or BIR3 domains of XIAP (Wang et al., 2004). We conclude from these experiments that (a) the mode of binding of ARTS to XIAP differs from other mitochondrial antagonists of IAPs such as SMAC, (b) interaction of ARTS with some regions of IAPs is likely to be conformation dependent, and (c) ARTS interacts with domains within XIAP that are not involved in caspase inhibition (e.g. BIR1).

The finding that ARTS prominently associates with the non-caspase-binding BIR1 domain of XIAP suggested its pro-apoptotic mechanism may involve more than competitive displacement of caspases from XIAP. In this regard, we noticed levels of XIAP protein were significantly reduced in cells in which ARTS was over-expressed by transfection (Figure 1C). A mutant of XIAP lacking the BIR1 domain, however, was resistant to ARTS-mediated reductions in expression (Figure 1D), with stable XIAPΔBIR1 protein levels achieved by transfection-mediated over-expression of ARTS (which causes the ARTS protein to accumulate in the cytosol was well as mitochondria). The BIR1 domain of XIAP has been reported to mediate its dimerization (Lu et al., 2007) However, binding of ARTS to XIAP does not appear to depend on BIR1-mediated XIAP dimerization, as a BIR1 mutant (V86A) reported to be defective in dimerization retained its ability to bind ARTS (Figure S1). In contrast to over-expression of ARTS, XIAP protein levels were elevated in cells in which endogenous ARTS expression was reduced using a short-hairpin RNA (shRNA) vector (Figure 1E). Analysis of XIAP mRNA levels did not reveal an effect of ARTS at the transcriptional level (not shown), suggesting that ARTS modulates the stability of the XIAP protein. Consistent with this hypothesis, experiments with the chemical inhibitor MG132 showed that ARTS-induced reductions in XIAP are dependent on the 26S proteasome (Figure S1). In contrast, a broad-spectrum inhibitor of caspases, benozyl-Valinyl-Alaninyl-Aspartyl-fluoromethylketone (zVAD-fmk) did not interfere with the ability of ARTS over-expression to induce reductions in XIAP protein.

We next investigated whether ARTS modulates ubiquitination of XIAP. When ARTS was over-expressed in the acute lymphocytic leukaemia (ALL) cell line MOLT4, in which endogenous ARTS gene expression is deficient (Figure S1), ubiquitination of XIAP protein was clearly induced, as demonstrated by experiments where XIAP was immunoprecipitated from cells and then analyzed by immunoblotting using anti-ubiquitin antibody (Figure 1F). Over-expression of ARTS in HEK293T cells also induced ubiquitination of XIAP (Figure 1G). Ubiquitination of XIAP was also induced in HEK293T cells following treatment with staurosporine (STS) (Figure 1G), a stimulus known to induce mitochondrial outer membrane permeability (MOMP) and release of ARTS from these organelles (Larisch et al., 2000), but not in cells in which ARTS was stably knocked down using a shRNA vector (Figure 1H).

ARTS binds the E3 ligase Siah-1

We considered that ARTS might activate the intrinsic E3 ligase activity of XIAP to induce its self-degradation. However, ARTS over-expression also accelerated the rate of

degradation of XIAP(H467A), an E3 ligase-defective mutant of XIAP in which the RING domain was disabled from binding E2s (Figure S2). While performing control experiments in which we compared the binding of ARTS to IAP family members with other RING-containing proteins by co-IP, we noticed that the E3 ligase Siah-1 associated with ARTS (Figure 2A). In contrast, ARTS did not bind to cIAP1 or cIAP2 (RING-containing IAP family members), nor did it bind Survivin or Bcl-X_L in these experiments. Interaction of endogenous ARTS with endogenous Siah-1 (Figure 2B) and endogenous XIAP with endogenous Siah-1 (Figure 2C) was also detected by co-IP, but only after treatment of cells with STS, suggesting that MOMP is required. [Note that we previously demonstrated that STS induces MOMP that is suppressed by Bcl-2/Bcl-X_L in the cell line used for these studies (Lin et al., 2004)].

These findings suggested that ARTS may operate as a molecular bridge that targets Siah-1 onto XIAP. To explore this hypothesis, we tested whether Siah-1 and XIAP form a complex as detected by co-IP and used shRNA gene silencing to ask whether ARTS is required for Siah-1 to associate with XIAP. STS induced formation of a complex containing both XIAP and Siah-1 when endogenous ARTS was present but not when ARTS expression was ablated by shRNA (Figure 2D).

To further evaluate the requirement of ARTS for joining XIAP and Siah-1, we prepared mutants of ARTS that fail to bind Siah-1, but retain the ability to bind XIAP. In this regard, the 3D-structure of Siah-1 has been determined (Polekhina et al., 2002), and mutagenesis studies have revealed that most of the known Siah substrates contain the consensus binding motif, RPVAxVxPxxR, which is essential for interaction with a crevice on the surface of Siah-family proteins (House et al., 2003). We compared the amino acid sequences of ARTS with other known Siah-binding partners in an attempt to locate a sequence in ARTS containing the consensus binding motif, finding a candidate Siah-binding motif at amino acids 37-48 (ASRPQVPEPRPQ) (Figure 3A).

Alanine substitution mutations were engineered within the 37-48 region to create four mutants: ASRAQAPAPAPQ R46A (MUT1), R46A, E44A (MUT2), R46A, E44A, V42A (MUT3); and R46A, E44A, V42A, P40A (MUT4). These mutant ARTS proteins were then expressed as Myc-tagged proteins in HEK293T cells and assessed for binding to FLAG-Siah-1 by co-IP assay (Figure 3B). ARTS MUT1 and MUT2 retained their ability to bind Siah-1, while ARTS MUT3 and MUT4 showed no interaction with Siah-1 (Figure 3B). We then compared these mutant versions of ARTS with the wild-type ARTS protein with respect to their ability to promote formation of Siah-1/XIAP complexes. In unstimulated cells where ARTS is associated with mitochondria, over-expression of wild-type (WT) but not MUT4 ARTS induced formation of a Siah-1/XIAP complex, as monitored by co-IP (Figure 3C). In STS-treated cells, where ARTS is released from mitochondria (Larisch et al., 2000), Siah-1 and XIAP formed a complex, which was increased in relative amounts by over-expression of WT ARTS, but completely abrogated by expression of MUT4 ARTS. We interpret this result as an indication that MUT4 ARTS competes with endogenous ARTS, thereby preventing recruitment of Siah-1 onto XIAP.

To further corroborate that ARTS is a Siah binding partner, we compared the ability of a synthetic ARTS peptide (ASRPQVPEPRPQ) to compete with epitopetagged-ARTS protein for binding to Siah-1 *in vitro*, using co-IP assays. For these experiments, full-length Siah-1 was expressed in HEK293T cells as a FLAG epitope-tagged protein together with Myc-tagged ARTS, then cell lysates were prepared to which the ARTS peptide was added. Incubation of cell lysates with the ARTS peptide blocked Myc-ARTS protein binding to Siah-1 in a concentration-dependent manner in vitro (Figure 3D, E). In contrast, addition of a negative control peptide to lysates did not disrupt ARTS binding to Siah (Figure 3D).

Additionally, we generated the same ARTS peptide with a HIV TAT sequence (HIV TAT-ASRPQVPEPRPQ), allowing for membrane penetration of the peptide into live cells. Treatment of cells with HIV TAT-ARTS peptide reduced interaction of Siah-1 with ARTS protein in a concentration-dependent manner (Figure 3D, F). In contrast, various HIV TAT control peptides did not disrupt ARTS/Siah-1 binding either when added to lysates or applied to cultured cells, including a control ARTS peptide representing an alanine-substitution mutant analogous to the engineered mutant ARTS protein (MUT4) that fails to bind Siah-1 (Figure 3D).

ARTS and Siah Collaborate to Reduce XIAP Protein Levels

We hypothesized that the interaction of Siah-1 with ARTS might provide a mechanism for recruiting the E3 ligase activity of Siah-1 to XIAP, thus accounting for the reductions in XIAP protein levels observed in cells over-expressing ARTS. Several experiments were designed to test this hypothesis. First, we determined the effects of over-expressing Siah-1 on ubiquitination of XIAP in cells transfected with control versus ARTS-targeting shRNA vector. Siah-1 over-expression increased ubiquitination of XIAP but not Bcl-X_L; (Figure S3) in control but not in ARTS knock-down cells (Figure 4A). Reconstituting ARTS expression in the knock-down cells by introducing an ARTS cDNA via an expression plasmid resulted in robust ubiquitination of XIAP (Figure 4A). Second, we assessed the effects on XIAP protein levels following exposure to a membrane-permeable ARTS peptide, which disrupts Siah-1 binding to ARTS. The ARTS competitive peptide increased XIAP but not cIAP1 protein levels in HEK293T cells (Figure 4B). Third, we used the ARTS MUT4 protein as a dominant-negative inhibitor, showing that expression of this mutant protein in cells results in an increase in XIAP protein levels, but not cIAP1 (Figure 4C). Fourth, we estimated the degradation rate of XIAP protein following over-expression of WT versus MUT4 ARTS in experiments where cells were transfected with a Myc-ARTS expression vector, then 1 day later, cycloheximide (CHX) was added to curtail protein synthesis. The rate of decline of XIAP protein levels following CHX exposure was faster in cells over-expressing ARTS compared to MUT4 ARTS (Figure 4D). Fifth, we utilized mouse embryonic fibroblasts (MEFs) lacking both Siah-1 and Siah-2 (Dickins et al., 2002) to explore the dependence on these E3 ligases for regulating levels of XIAP protein. Steady-state levels of XIAP protein were higher in Siah-1/Siah-2 double knock-out (DKO) cells (Figure 4E), consistent with a role for one or both of these E3 ligases in regulating XIAP stability.

Dominant-negative SiahΔRING interferes with XIAP ubiquitination and increases XIAP protein levels

Truncated Siah-1 protein lacking the RING domain needed for binding ubiquitin-conjugating enzymes (E2s) operates as a dominant-negative inhibitor of Siah-1-mediated protein degradation (Matsuzawa et al., 2002). We co-transfected HEK293T cells with epitope-tagged ARTS and either full-length Siah-1 or a RING-deficient mutant (SiahΔRING). Because XIAP is known to be cleaved by caspases, we also treated cells with the broad-spectrum caspase inhibitor, z-VAD-fmk to exclude a role for these proteases. ARTS expression with full-length Siah-1 caused a decline in XIAP protein levels, beginning at 12 hrs and becoming undetectable by 36 hrs (Figure 5A). In contrast, in cells co-expressing ARTS and Siah-1ΔRING, XIAP protein levels were sustained, thus demonstrating that the dominant-inhibitory mutant of Siah-1 blocks ARTS-mediated reductions in XIAP protein.

Next, the ubiquitination status of XIAP was examined in cells expressing ARTS with either full-length Siah-1 or ΔRING Siah-1. When HEK293T cells were transfected with plasmids encoding ARTS and Siah-1, extensive ubiquitination of XIAP was detected (Figure 5B) but not of control proteins such as Bcl-X_L (Figure S3) In contrast, when ARTS and

Siah-1ΔRING were co-expressed, little ubiquitination of XIAP occurred. Comparisons of full-length XIAP with XIAPΔRING mutant that fails to bind E2s showed that the intrinsic E3 ligase activity of XIAP is unlikely to be required for ARTS/Siah-1-mediated reductions in XIAP protein expression (Figure 5B).

To complement the Siah-1 dominant-negative studies, we also performed experiments in which Siah-1/Siah-2 double knock-out (DKO) cells were reconstituted by transfection with plasmids encoding Siah-1, Siah-2, or both. Over-expressing ARTS in cells reconstituted with Siah-1 showed clear evidence of XIAP ubiquitination, whereas Siah-2 reconstituted cells did not (Figure 5C), suggesting that Siah-1 plays a more prominent role in ARTS-mediated reductions in XIAP.

Cellular functions of XIAP are regulated by ARTS

The changes in XIAP ubiquitination and protein expression observed by experimental manipulation of ARTS cells were correlated with cellular functions of XIAP. Cells in which ARTS expression was reduced by stable transduction of ARTS-targeting shRNA vector were more resistant to cell death induced by STS, an apoptosis-inducing agents that induces MOMP (Lin et al., 2004), as measured by either ATP levels or by Annexin-V staining (Figure 6A, B). Clonogenic survival of STS-treated cells was also improved by ARTS knock-down (Figure 6C). Reconstitution of ARTS knock-down cells with ARTS expression plasmid completely restored apoptosis sensitivity, thus validating the results. Analogous observations were made with respect to XIAP-induced NF-κB, where we observed that: (a) higher levels of NF-κB activity were stimulated in cells with stable knock-down of ARTS compared to control cells; (b) over-expressing ARTS by transfection reduced NF-κB activity induced by XIAP (but not TNFα); and (c) an ARTS mutant lacking the C-terminal residues required for XIAP binding (ARTSΔC) failed to modulate XIAP-induced NF-κB activity, in contrast to over-expression of full-length ARTS (Figure S4).

To further confirm the importance of the interaction of ARTS with XIAP for regulating apoptosis, experiments were performed with the aforementioned XIAPΔBIR1 mutant that fails to bind ARTS but retains caspase-binding activity. *Xiap*$^{-/-}$ mouse embryo fibroblasts (MEFs) were reconstituted with XIAP or XIAPΔBIR1. XIAPΔBIR1 provided greater protection compared to full-length XIAP against cell death induced by STS (an inducer of MOMP that causes ARTS release from mitochondria) (Figure 6D). In contrast, full-length XIAP and XIAPΔBIR1 provided similar protection against cell death induced by TNFα/cycloheximide, which induces apoptosis via a mitochondria-independent mechanism in this cell line (Zhang et al., 2000). Apoptosis induced by over-expression of ARTS was also rescued by XIAPΔBIR1 but not by full-length XIAP (Figure 6D), thus correlating with our observation that XIAPΔBIR is resistant to ARTS-induced degradation (Figure 1C).

DISCUSSION

Here we describe a novel interaction of the E3 ligase Siah-1 with ARTS, a mitochondrial protein known to bind certain IAP family proteins and to promote apoptosis. Our data are consistent with a model in which ARTS serves as a molecular bridge between Siah-1 and XIAP, thus targeting the E3 ligase activity of Siah-1 onto XIAP. Consistent with this hypothesis: (1) Siah knock-out cells have reduced ubiquitination of XIAP and elevated levels of XIAP protein; (2) ARTS knock-out cells have elevated levels of XIAP protein and reduced ubiquitination of XIAP; (3) ARTS peptides that compete for Siah binding inhibit interaction of Siah-1 with ARTS and increase XIAP protein levels; (4) ubiquitination of XIAP and reductions in XIAP protein levels induced by over-expression of ARTS are blocked by co-expression of Siah-1ΔRING dominant-negative; and (5) ubiquitination of XIAP induced in cells by over-expression of Siah-1 requires ARTS.

The ARTS protein contains a canonical Siah-binding motif. Mutagenesis of residues within this motif abrogated binding to Siah-1 and synthetic peptides corresponding to this motif blocked binding of Siah-1 to ARTS *in vitro* and in cells. While ARTS binds Siah-1, levels of ARTS proteins do not appear to be reduced, suggesting that ARTS functions not as a substrate of the E3 ligase activity of Siah but as an adapter that bridges Siah-1 to substrates such as XIAP. The ability of Siah-1 to bind such adapters or bridging proteins without necessarily promoting their degradation has been observed previously for proteins such as SIP, BAG-1, and Vav (Reed and Ely, 2002).

While XIAP possesses an E2-binding RING domain and is capable of auto-ubiquitination, we observed that an intact RING domain of XIAP is unnecessary for ubiquitination and XIAP degradation induced by ARTS/Siah-1. Thus, while we cannot exclude the possibility that ARTS stimulates the E3 ligase activity of XIAP against itself in some contexts, we do not believe this is required. Also, if ARTS induced self-ubiquitination of XIAP, then we would not have expected to observe effects of various experimental manipulations of Siah-1 on XIAP ubiquitination and XIAP protein degradation. In contrast, cells in which either ARTS or Siah expression was genetically ablated show elevated steady-state levels of XIAP. The auto-ubiquitination sites of the XIAP were previously localized to Lys 311, Lys322, and Lys328 on the BIR3 domain (Shin et al., 2003). Mutating these sites reduces XIAP ubiquitination but has little effect on the ability of ectopically expressed XIAP to rescue cells from apoptosis (Shin et al., 2003), suggesting that auto-ubiquitination of XIAP may have a minimal effect on XIAP protein levels. We also observed that these self-ubiquitination site mutants of XIAP remain sensitive to ARTS-induced ubiquitination and degradation (Figure S5), further arguing that ARTS/Siah-1 utilizes alternative sites in the XIAP protein to mediate ubiquitination. XIAP-deficient cells reconstituted with these auto-ubiquitination site mutants of XIAP were also equally sensitive to cytotoxicity of ARTS over-expression as wild-type XIAP (not shown), suggesting ablation of these sites does not render XIAP resistant to ARTS (unlike deletion of the ARTS-binding BIR1 domain).

In our experiments, ARTS bound selectively to XIAP among IAP family members tested, which included XIAP, cIAP1, cAP2, and Survivin. Our domain mapping experiments demonstrated that ARTS binds to BIR1 of XIAP reproducibly, including the isolated BIR1 domain and several different fragments of XIAP that contained the BIR1 domain, but not to XIAP lacking BIR1 (ΔBIR1) (Figure S5). However, ARTS also shows some tendency to bind the isolated BIR3 domain, but not to some fragments of XIAP that contained BIR3 along with other domains, unless SMAC peptide was added. In this regard, domains within the XIAP protein have been postulated to undergo intra-molecular interactions, controlling conformational states of XIAP that regulate its E3 ligase activity (Eckelman et al., 2006; Schile et al., 2008). Thus, the BIR3 domain in XIAP may become accessible to ARTS only in some conformational states. It remains to be determined whether ARTS interacts with other members of the IAP family in a conformationally restricted manner. Binding of ARTS to XIAP does not appear to depend on BIR1-mediated XIAP dimerization, as a BIR1 mutant (V86A) reported to be defective in dimerization retained its ability to bind ARTS. Also of note, various studies have provided evidence that the mode of ARTS binding to XIAP is unrelated to the mechanism used by endogenous antagonist SMAC, with ARTS depending on its C-terminus (versus SMAC requiring its proteolytically processed N-terminus) (Gottfried et al., 2004). Further arguing for a different mode of IAP antagonism, we observed binding of ARTS to XIAP even in the presence of SMAC N-terminal peptides that displace caspases from the BIR2 and BIR3 domains of XIAP.

ARTS protein is reported to be predominantly localized to the interior of mitochondria, becoming released following stimulation with apoptosis-inducing agents that trigger outer mitochondrial outer membrane permeabilization (MOMP) (Larisch et al., 2000). However,

we observed changes in XIAP protein levels even in normal culture conditions when (a) ARTS expression was knocked-down by shRNA vectors; (b) HIV TAT-ARTS competitive peptide was introduced into cells; and (c) Siah-1 expression was ablated by homologous gene recombination. These observations thus suggest that even in normal circumstances, ARTS and Siah-1 contribute to homeostasis of XIAP protein levels. However, when MOMP was induced in cultured cells that contain endogenous ARTS, greatly increased ubiquitination of XIAP was induced in a Siah-dependent manner. Thus, circumstances known to induce ARTS release from mitochondria clearly induce a burst of ubiquitination of XIAP. Conceivably, ARTS plays both a baseline, tonic role in maintaining levels of XIAP, while also allowing for rapid degradation of XIAP following MOMP. Experimental over-expression of ARTS causes XIAP ubiquitination and degradation because ARTS protein is produced in excess amounts where its import into mitochondria is saturated, resulting in accumulation of ARTS in the cytosol. It remains to be clarified whether ARTS similarly targets XIAP for Siah-1-dependent ubiquitination and degradation while passing through the cytosol in route to the mitochondrial import machinery versus other possible explanations.

In summary, we have identified a novel function for ARTS as an adapter for the E3 ligase Siah-1, showing that collaboration of ARTS and Siah-1 controls XIAP protein levels. These findings provide insights into the putative tumor suppressor role of ARTS as pertains to apoptosis regulation, and also suggest strategies for cytoprotective therapies where chemical mimics of ARTS peptides that disrupt binding to Siah-1 might be employed to elevate endogenous XIAP levels for purposes of preserving neurons or other types of vulnerable cells. Given the recent implication of XIAP in signaling by NLR family proteins, our findings may additionally have implications for innate immunity (Bertrand et al., 2009; Krieg et al., 2009).

EXPERIMENTAL PROCEDURES

Additional methodological details are provided as supplemental information.

Cell Culture and Transfections

Cells were grown in either Dulbecco's modified Eagle's medium (HEK293T, MEF) or RPMI (MOLT4) media supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. HEK293T and MEF cell transfections were performed using Lipofectamine2000 reagent (Invitrogen) in Opti-MEM-reduced serum medium (Invitrogen). The Lipofectamine transfection mix was replaced with fresh medium after 4 hrs. MOLT4 cells were electroporated using the Amaxa Biosystems (Amaxa, Gaithersburg, MD) Cell Line Nucleofector Kit L, utilizing the reagents and following the recommendations of the manufacturer (program C-005).

Immunoprecipitations and immunoblotting

Cells were lysed at 24 hrs post-transfection in ice-cold immunoprecipitation buffer [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, and a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN)]. Lysis with immunoprecipitation buffer was complemented with sonication (5 pulses) on ice. Lysates were cleared of cell debris by centrifugation at 16,000 × *g* at 4°C for 30 min. The supernatant was saved and aliquots corresponding to 20 μg and 250 μg total protein were used for SDS-PAGE/immunoblotting and immunoprecipitations, respectively. IPs were preformed using anti-Flag or anti-Myc antibodies conjugated to Sepharose beads (Sigma, Saint Louis, MO) (Santa Cruz, Santa Cruz, CA) and with gentle overnight agitation. Beads were washed 3 times in lysis buffer, resuspended in 2X Laemmli buffer, and boiled for 5 min to release bound proteins. Proteins were analyzed by SDS-PAGE and immunoblotting after transfer to polyvinylidene

difluoride membranes (Osmonics, Inc., Minnetonka, MN). Antibodies used include anti-XIAP, anti-cIAP1, anti-cIAP2, anti-His, and anti-GFP; and anti-ubiquitin (Cell Signaling Technologies Danvers, MA); anti-ARTS, anti-β-actin, and anti-FLAG M2 (Sigma); anti-HA and anti-Myc (Roche); and anti-Siah1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). An enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL) was used for detection.

GST-Pull Down

HEK293T cells were transfected with Myc-ARTS and lysed 24 hrs later in ice cold buffer [0.2% NP40, 1 mM EDTA, 135 mM NaCl, 20 mM Tris, 10 % Glycerol,1 mM DTT, 10 mM NaF, 20 μM Leupeptin, 2 mM PMSF, 2 mM ortho-vanadate, protease inhibitor mixture (Roche)]. Lysates were cleared of cell debris by centrifugation at 16,000 × *g* at 4°C for 30 min. GST-tagged proteins were incubated with cell lysates overnight at 4°C with rotation. Beads were washed 3 times in lysis buffer, resuspended in 2X Laemmli buffer, and boiled for 5 min to release bound proteins. Proteins were analyzed by SDS-PAGE and immunoblotting after transfer to polyvinylidene difluoride membranes (Osmonics, Inc., Minnetonka, MN). Antibodies used include anti-Myc and -GST.

Apoptosis Assays

For Annexin-V assays, HEK293T cells (1×10^6 cells) treated with 2.5 μM staurosporine or TNFα (Invitrogen) at 20 ng/mL for 24 hrs, with or without pre-treatment for 2 hrs with 10 μg/mL cyclohexamide. Cells were double-stained with fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide (PI) using a kit according to the manufacturer's instructions (BioVision, Mountain View, CA). The percentage of apoptotic (annexin-V positive + PI negative) cells was determined by flow cytometric analysis (Becton Dickinson, San Jose, CA).

Clonogenic Assays

Wild-type, stable scrambled shRNA, and stable ARTS shRNA HEK293T cells were transfected with empty plasmids or ARTS. At 24 hrs post-transfection, cells were treated with (STS 2.5 μM). Cells were then split and 300 cells of each group were seeded in 6-cm dishes and allowed to grow for 10 days. Colonies greater then 50 cells were then counted.

HIGHLIGHTS

- ARTS binds to XIAP at BIR1, a domain distinct from the caspase-binding sites.
- The E3 ligase Siah-1 interacts with ARTS.
- ARTS Siah-1 complex induces ubiquitination and degradation of XIAP.
- ARTS clears XIAP protein from cells, allowing apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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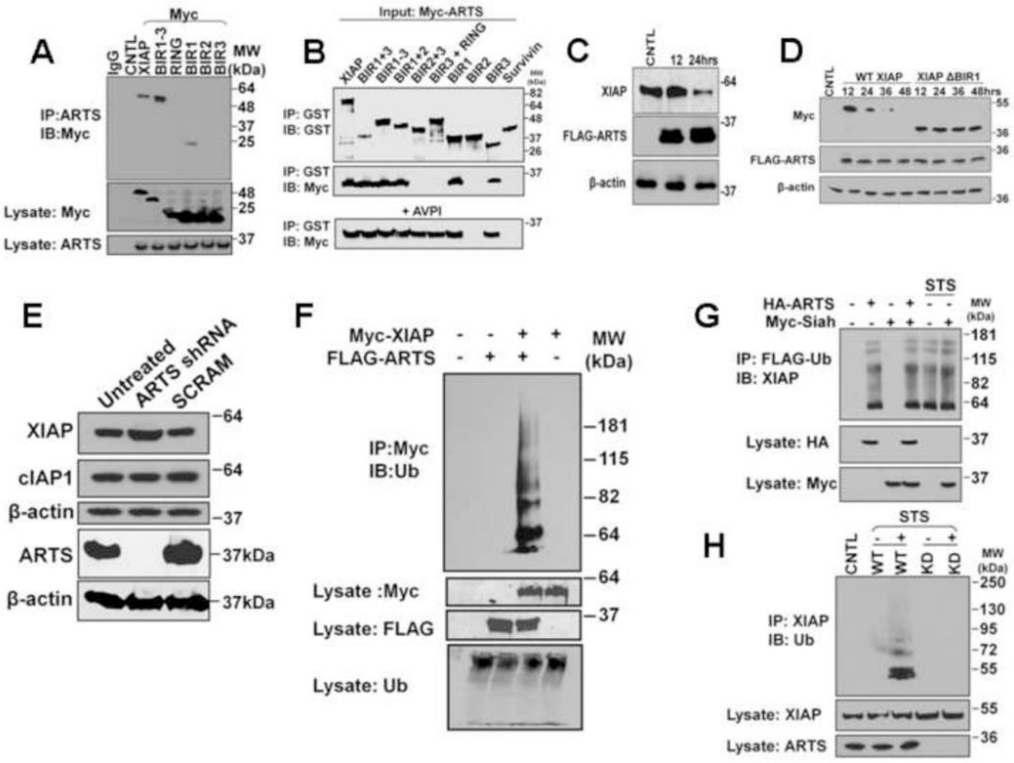

Figure 1. ARTS binds XIAP and reduces XIAP protein levels

A. ARTS binds to XIAP at the BIR1 region. The plasmids encoding the indicated Myc-tagged XIAP protein fragments or empty vector were transfected into HEK293T cells with or without plasmid encoding FLAG-ARTS. At 24 hrs post-transfection, cell lysates were normalized for protein content and ARTS was immunoprecipitated (IP'ed) using control IgG (first lane) or mouse monoclonal anti-FLAG antibodies. IPs and lysates were analyzed by immunoblotting using anti-Myc or -FLAG antibodies. **B.** SMAC mimetic, AVPI, promotes ARTS binding to BIR3. GST fusion proteins of varying domains of XIAP were produced in bacteria. IPs were performed using anti-GST in the presence (bottom) or absence (middle) of 10 μM AVPI. Immune-complexes and GST fusion proteins were analyzed by immunoblotting using anti-GST or anti-Myc antibodies. **C.** ARTS reduces XIAP protein levels. HEK293T cells were transiently transfected with FLAG-ARTS and endogenous levels of XIAP were analyzed at the indicated times by immunoblotting using lysates normalized for total protein content. Blots were also probed with anti-FLAG and β-actin antibodies. **D.** Non-ARTS-binding mutant XIAP is resistant to ARTS-mediated degradation. HEK293T cells were transfected with FLAG-ARTS and either Myc-XIAP or Myc-XIAPΔBIR1. Cell lysates were analyzed by immunoblotting with anti-myc, anti-FLAG, and anti-actin antibodies. **E.** ARTS reduction elevates XIAP protein levels without changing cIAP1. Lentiviral shRNA targeting ARTS was stably integrated into HEK293T cells to knockdown ARTS protein levels. Lysates from untreated, ARTS shRNA, and scrambled ARTS (SCRAM) shRNA HEK293T cells were analyzed by immunoblotting using the indicated antibodies. **F.** ARTS induces ubiquitination of XIAP. MOLT4 cells were electroporated with empty plasmid, FLAG-ARTS, and/or Myc-XIAP. IPs were performed with anti-Myc. The immune-complexes (IPs) and lysates were analyzed by immunoblotting and probed with anti-Ub, -Myc, or -FLAG antibodies. **G.** Ubiquitination of endogenous XIAP is induced by ARTS. HEK293T cells were transiently transfected with plasmid encoding FLAG-ubiquitin (Ub) and either HA-ARTS or empty vector, then cultured with or without 1.5 μM STS for 4 hrs. Cell lysates were subjected to IP using anti-FLAG. IPs and

cell lysates were analyzed by immunoblotting using anti-XIAP (*top*), anti-HA (*middle*), and anti-Myc (*bottom*) antibodies. **H.** Ubiquitination of XIAP requires ARTS. HEK293T (WT) or HEK293T stable shARTS (KD) cells were treated with or without 1.5 μM STS. Cell lysates were subjected to IP using anti-XIAP. IPs and cell lysates were probed by immunoblotting using anti-Ub (*top*), anti-XIAP (*middle*), or anti-ARTS (*bottom*) antibodies.

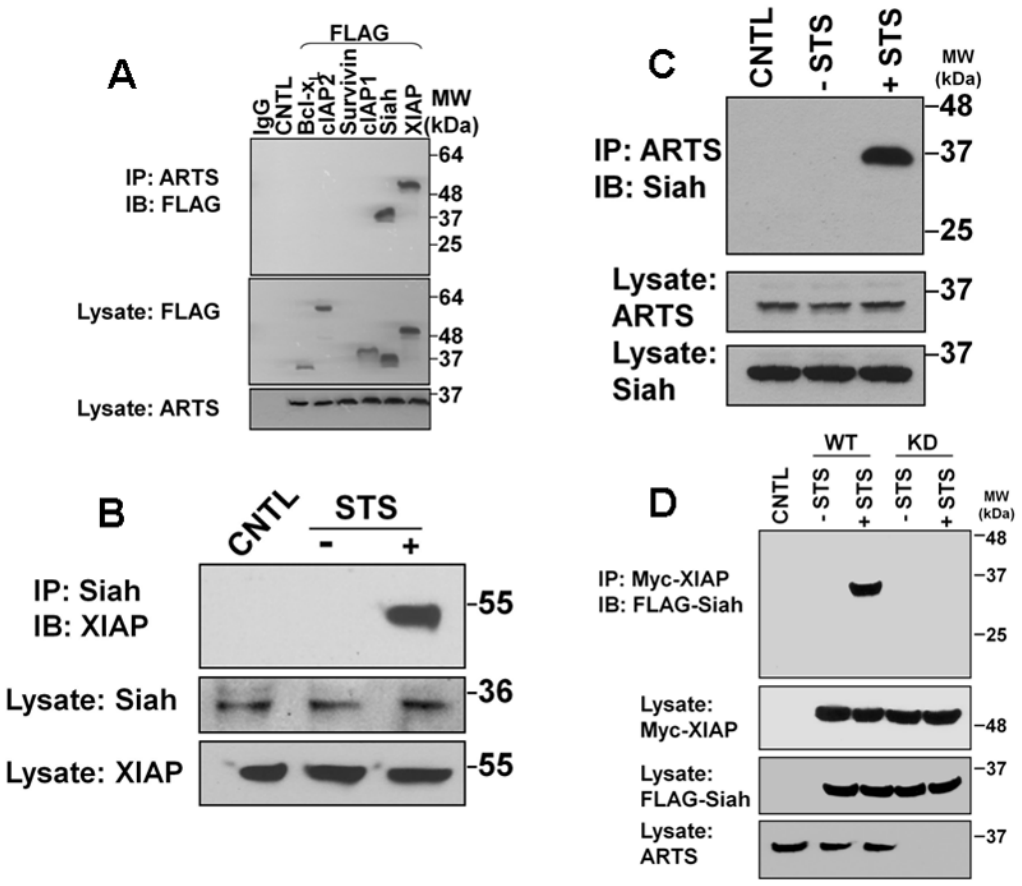

Figure 2. ARTS binds the E3 ligase Siah-1

A. ARTS binds to Siah. HEK293T cells were co-transfected with plasmids encoding Myc-ARTS and FLAG-Bcl-X_L, FLAG-Survivin, FLAG-cIAP1, FLAG-cIAP2, FLAG-Siah-1, FLAG-XIAP, or empty vector as indicated. After 24 hrs, cell lysates were normalized for protein content and ARTS was IP'ed using control IgG (first lane) or anti-Myc antibody. Immune-complexes (IP) and lysates were analyzed by immunoblotting and probed with the indicated antibodies. **B.** The ARTS:Siah interaction is MOMP-dependent. HEK293T cells were treated with or without 1.5 μM STS for 4 hrs. Cells were lysed and IPs were performed using control IgG or mouse monoclonal ARTS-antibodies. IPs and lysates were analyzed by immunoblotting and probed with anti-Siah or anti-ARTS antibodies. **C.** Endogenous XIAP and Siah interact. HEK293T cells were treated with or without 1.5 μM STS for 4 hrs hrs. Cell lysates were subjected to IP using anti-Siah antibody. IPs and cell lysates were analyzed by immunoblotting using anti-XIAP (top, bottom) and anti-Siah (middle) antibodies. **D.** XIAP binds Siah-1 in an ARTS-dependent manner. HEK293T control (CNTL), scrambled ARTS (SCRAM) shRNA, and ARTS shRNA (knock-down, KD) cells were transfected with plasmids encoding Myc-XIAP and FLAG-Siah and cultured with or without STS for 4 hrs. Cell lysates were normalized for protein content and IP'ed with anti-Myc. IPs and lysates were analyzed by immunoblotting using anti-FLAG, -Myc, or -ARTS antibodies.

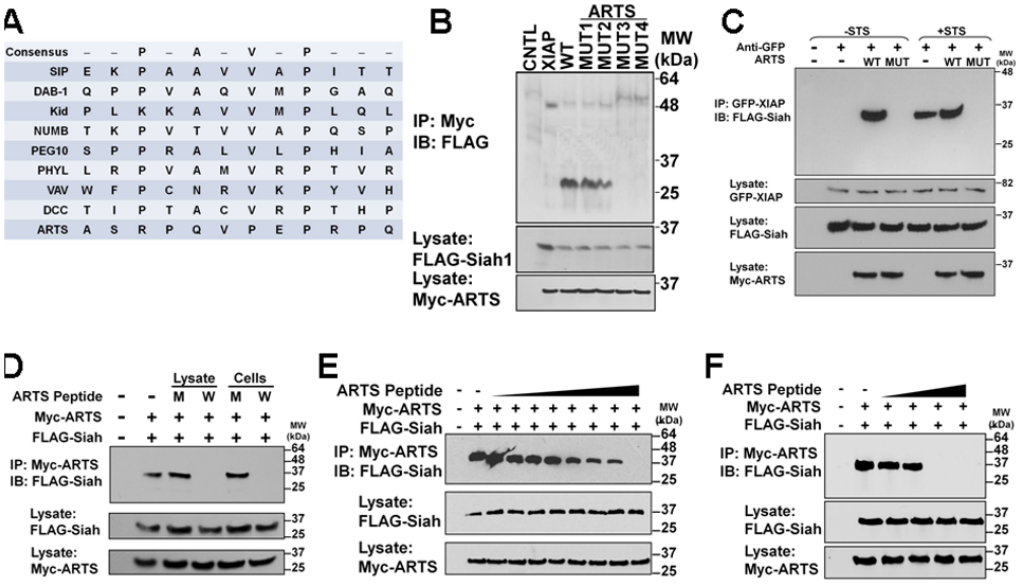

Figure 3. Elucidation of Siah-1 binding site on ARTS

A. ARTS contains the consensus binding motif of Siah-interacting proteins. Sequence alignment of proteins known to interact with Siah-1 via the RPVAxVxPxxR motif. **B.** The conserved Siah-binding motif (RPVAxVxPxxR) is responsible for binding ARTS. Specific residues of the ARTS protein were modified by site-specific mutagenesis (MUT1-4, converted to alanine). HEK293T cells were transfected with the FLAG-Siah and either Myc-tagged XIAP, or various forms of Myc-ARTS. IPs were performed using IgG control (CNTL) or anti-Myc antibody, then analyzed by immunoblotting. **C.** The XIAP:Siah interaction is blocked by ARTS dominant-negative mutant. HEK293T cells were transfected with GFP-XIAP and FLAG-Siah-1, and either empty plasmid (−), Myc-ARTS WT, or MUT4. After 24 hrs, cells were cultured with (+) or without (−) STS for 4 hrs followed by cell lysis and IP with anti-GFP antibody (+) or IgG control (−). IPs and lysates were analyzed by immunoblotting. **D.** ARTS peptide disrupts ARTS:Siah interaction. HEK293T cells were transfected with plasmids encoding Myc-ARTS and FLAG-Siah or empty plasmid (−). In some cases, cells were cultured for 24 hr with wild-type (W) or mutant (M) (R46A, E46A, V42A, E40A) HIV TAT-ARTS peptide (“cells”) prior to lysis or alternatively the ARTS peptides were added to cell lysates (“lysates”). Lysates were subjected to IP with anti-Myc and analyzed by immunoblotting. **E, F.** Co-IP of Myc ARTS and FLAG-Siah-1 was performed as described above. Prior to IP, lysates were incubated with various concentrations of ARTS peptide (ASRPQVPEPRPQ) **(E)**, or the peptide (HIV TAT-ASRPQVPEPRPQ) was added to cultures of cells for 6 hrs **(F)**.

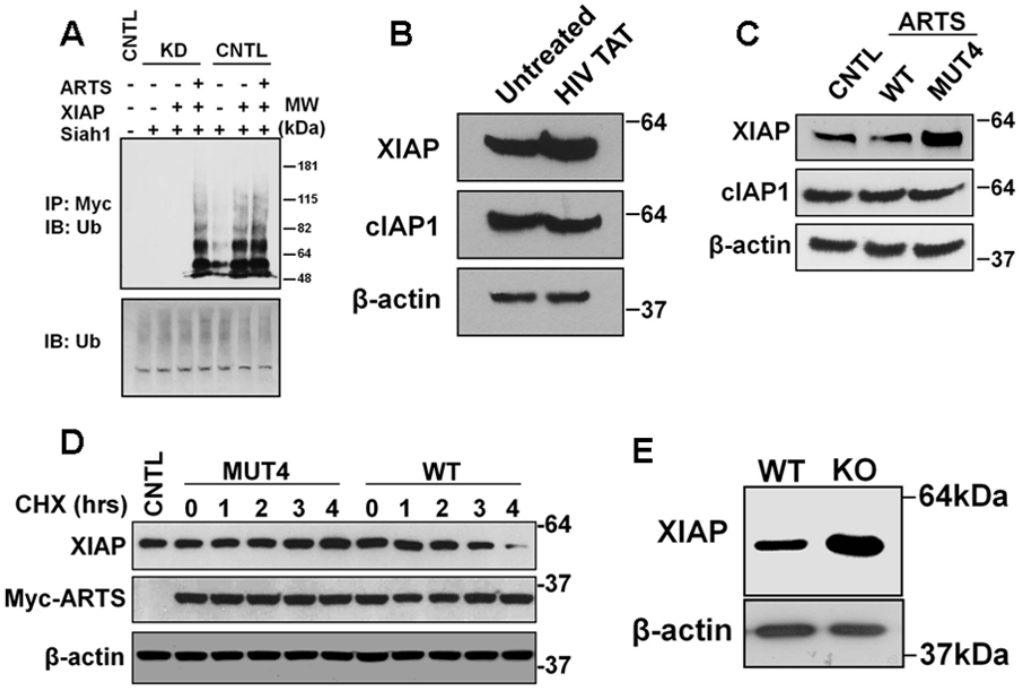

Figure 4. ARTS and Siah collaborate to reduce XIAP protein levels

A. ARTS is required for ubiquitination of XIAP. HEK293T cells stably containing ARTS-targeting shRNA (KD) or scrambled control (CNTL) vectors were transfected with Myc-XIAP, HA-ARTS, FLAG-Siah, or various combinations. (*Note that the shRNA vector targets the 3′ untranslated region of endogenous ARTS mRNA whereas the HA-ARTS-encoding plasmid contains a cDNA encompassing only the open reading frame*). After 24 hrs, cell lysates were normalized for protein content and IP'ed using control IgG (CNTL) (first lane) or mouse monoclonal anti-Myc antibodies. IPs (*top*) and lysates (*bottom*) were analyzed by immunoblotting with anti-ubiquitin. **B.** ARTS peptide increases XIAP protein levels. HEK293T cell were cultured with or without the ARTS HIV TAT peptide (HIV TAT-ASRPQVPEPRPQ) for 6 hrs. Cells were lysed and analyzed by immunoblotting with the indicated antibodies. **C.** Mutant ARTS protein increases XIAP protein levels. HEK293T cells were transfected with empty vector, wild-type (WT) ARTS, or ARTS (MUT4). After 24 hrs, cells were lysed and analyzed by immunoblotting with the indicated antibodies. **D.** ARTS reduces the half-life of XIAP. HEK293T cells were transfected with Myc-ARTS or empty plasmid (CNTL) then cultured 1 day later with 120 μg/ml cycloheximide for various times. Cell lysates were analyzed by immunoblotting. **E.** Cells lacking Siah have increased endogenous XIAP protein. Lysates from wild-type (WT) MEFs and Siah1/2$^{-/-}$ MEFs (KO) were analyzed by immunoblotting using anti-XIAP (*top*) and anti-β-actin (*bottom*) antibodies.

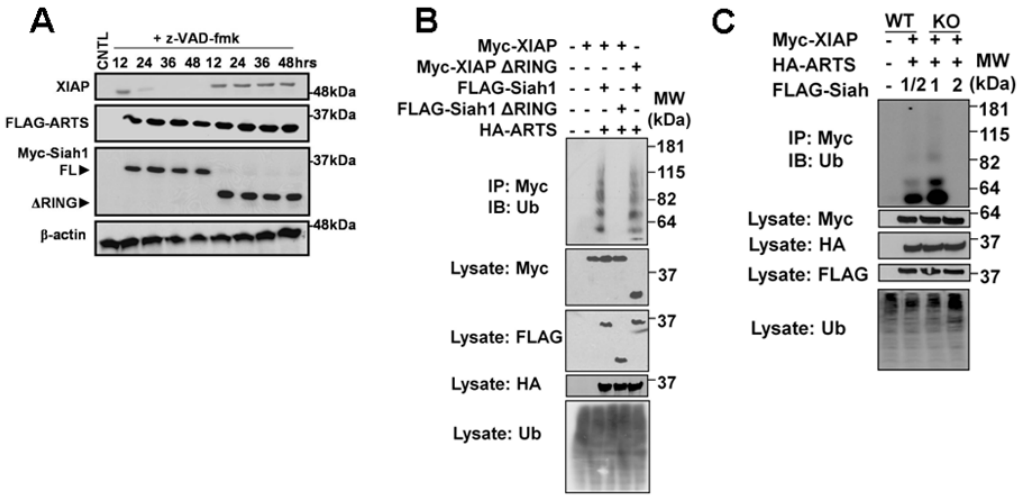

Figure 5. Dominant-negative Siah alters XIAP protein levels

A. Siah-1ΔRING dominant-negative blocks ARTS-induced decline in XIAP protein levels. HEK293T cells were transfected with plasmids encoding FLAG-ARTS versus empty plasmid (CNTL) together with plasmids encoding full-length (FL) Myc-Siah-1, or Myc-Siah ΔRING in the presence of the pan-caspase inhibitor z-VAD-fmk (20 μM). At various times, cell lysates were analyzed by immunoblotting. **B.** RING domain of Siah-1 but not RING of XIAP is required for ARTS-induced ubiquitination of XIAP. HEK293T cells were transfected with Myc-XIAP, Myc-XIAPΔRING, HA-ARTS, FLAG-Siah-1, and/or FLAG-SiahΔRING. After 24 hrs, cell lysates were normalized for protein content and IPs were performed using mouse monoclonal anti-Myc antibody. IPs and lysates were analyzed by immunoblotting. **C.** Siah-1 is required for ARTS-induced ubiquitination of XIAP. Wild-type (WT) or *Siah1/2* −/− double knockout (KO) MEFs were transiently transfected with Myc-XIAP, HA-ARTS, and/or plasmids encoding FLAG-Siah-1, FLAG-Siah-2, or both. Lysates were IP'ed using anti-Myc. IPs and lysates were analyzed by immunoblotting.

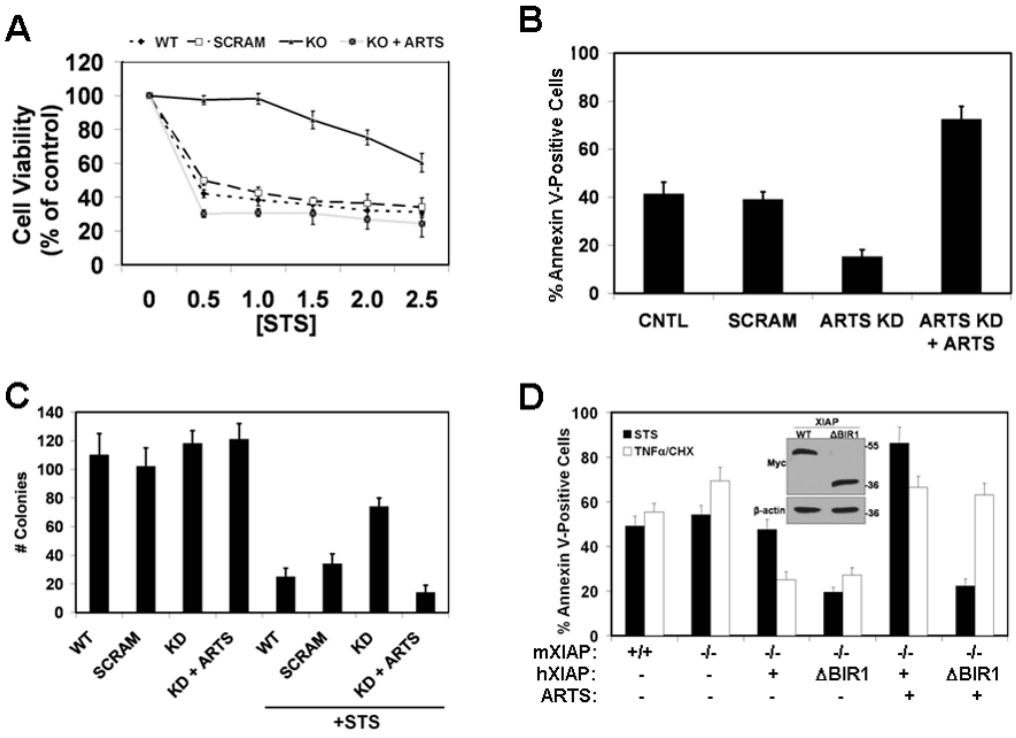

Figure 6. ARTS regulates cellular functions of XIAP

A. ARTS sensitizes cells to STS-induced cell death. Control untransfected (*diamonds*), stable scrambled shRNA (*white squares*), and stable ARTS shRNA (*triangles*) HEK293T cells were transfected with empty plasmids or ARTS-encoding plasmid (*black squares*). After 24 hrs, cells were treated with (STS 0–2.5 μM) for 24 hrs, then cell viability was assessed by measuring ATP levels, expressing data as % control relative to no STS (mean ±SD; n = 3). **B.** Knockdown of ARTS protects cells against STS-induced apoptosis. Control untransfected, stable scrambled shRNA, and stable ARTS shRNA HEK293T cells were transfected with empty plasmids or a plasmid encoding ARTS. After 24 hrs, cells were treated with (STS 2.5 μM) for 24 hrs, then cell death was assessed by Annexin-V staining, expressing data as percentage Annexin V-positive cells (mean±SD; n = 3). **C.** Knockdown of ARTS increases clonogenic survival. Wild-type, stable scrambled shRNA, and stable ARTS shRNA HEK293T cells were transfected with empty plasmids or ARTS. After 24 hrs, cells were treated with 2.5 μM STS for 1 day. Cells were then washed and 300 cells of each group were seeded into 6-cm dishes and allowed to grow for 10 days. Colonies greater then 50 cells were then counted (mean±SD; n = 3). **D.** Murine (m) *Xiap*+/+ or *Xiap* −/− MEF cells were transiently transfected with various plasmids encoding full-length human (h) XIAP or XIAPDBIR1 deletion mutant. After 24 hrs, cells were cultured with either 2.5 μM STS or 20 ng/ml TNFα (10 μg/ml CHX) for an additional 24 hrs, then Annexin-V staining was performed (% positive cells; mean±SD; n =3).