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TITLE: Role of the XIAP/AIF Axis in the Development and Progression of Prostate Cancer

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT In the past year tremendous progress has been made towards completing the tasks outlined in the original Statement of Work. The physical association between XIAP and AIF was confirmed in living cells, and it was further determined that XIAP-mediated caspase inhibition was insensitive to AIF over expression. Furthermore, the caspase-inhibitory properties of XIAP were shown to be dispensable for interaction with AIF, and AIF was found to be a substrate of XIAP-mediated ubiquitination. Finally, AIF over expression was determined to result in significant increases in cellular reactive oxygen species levels, and XIAP protein levels were found to be greatly altered in tissues from Harlequin (AIF deficient) mice. These exciting findings not only confirm the physiological relevance of the association between XIAP and AIF, but also establish a functional link between XIAP and AIF. Importantly, that AIF fails to prevent XIAP-mediated caspase inhibition suggests that the pro-survival NADH-oxidase properties of AIF may play a significant role in tumor formation, despite any death stimulating properties of this protein. Taken together, these observations form the basis for determining the potential role these two proteins play in prostate cancer formation and progression.					
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Introduction

As the second most common form of cancer diagnosed among men in the western world, prostate cancer represents a significant healthcare threat, in part due to the lack of reliable biomarkers to predict disease stage and behavior. As part of my original research proposal, I described the identification of two well known regulators of programmed cell death, X-linked inhibitor of apoptosis (XIAP) and apoptosis inducing factor (AIF), as genes that are upregulated in a number of prostate tumors, and further presented data confirming a physical association between the XIAP and AIF proteins. Since these two genes represent both pro-survival (XIAP) and pro-death (AIF) regulators of the cell death cascade, I proposed to investigate the functional relevance of the physical association between XIAP and AIF, in terms of the described functions of both proteins, as well as their potential role in the pathogenesis of prostate cancer. This report summarizes the progress made in the past year towards completing the research proposal goals.

Body

I have made significant progress towards completing the tasks outlined in my original Statement of Work, as summarized below:

AIM 1. To determine the effects of AIF on XIAP function.

- a) **Characterization of the XIAP/AIF interaction under normal and apoptotic conditions.** The interaction of XIAP and AIF has been confirmed under normal cellular conditions through live cell imaging using Bi-molecular fluorescence complementation. I have determined that the $\Delta 102$ form of AIF (see below) strongly interacts with XIAP under non-apoptotic conditions (Figure 1). Evaluation of this interaction under apoptotic conditions is currently underway.
- b) **Assessment of the effects of AIF on the caspase inhibitory properties of XIAP.** I have determined that the caspase inhibitory properties of XIAP are unaffected by overexpression of either full-length, $\Delta 54$, or $\Delta 102$ AIF (Figure 2, see below for description of these AIF variants). This sub-aim is complete.
- c) **Assessment of the effects of AIF on the ability of XIAP to augment TGF- β , JNK, or NF- κ B signaling.** Experiments are in progress.
- d) **Evaluation of the effects of AIF on XIAP function in prostate tumor-derived cell lines.** Experiments are in progress.
- e) **Characterization of the effects of AIF on the copper metabolism regulatory properties of XIAP.** Experiments are in progress.

AIM 2. To determine the effects of XIAP on AIF function.

- a) **Assessment of the effects of XIAP on the death promoting properties of AIF.** I have determined that AIF overexpression does not induce apoptosis in multiple cell lines, including HeLa, Jurkat, and HEK293 (Figure 2). Further, AIF overexpression does not impair the survival of Jurkat cells overexpressing XIAP following treatment with apoptosis-inducing agents such as etoposide and agonistic anti-Fas. This sub-aim is complete.
- b) **Determination of the caspase-dependence of the interaction between XIAP and AIF.** I have determined that the caspase inhibitory properties of XIAP are dispensable for interaction with AIF, since the second BIR domain of XIAP (BIR2), which is incapable of inhibiting caspase activation, interacts with AIF to an extent comparable to that of wildtype XIAP or other BIR2-containing variants (Figure 3). This sub-aim is complete.

- c) **Assessment of the effects of XIAP on the NADH-oxidase activity of AIF (Months 14-18).** I have constructed expression plasmids to express recombinant AIF protein, in order to evaluate NADH-oxidase activity in the absence and presence of XIAP.
- d) **Characterization of the ability of XIAP to ubiquitinate AIF.** I have determined that XIAP is capable of ubiquitinating the full-length, $\Delta 54$, and $\Delta 102$ forms of AIF (Figure 4). This sub-aim is complete.

In addition to the progress highlighted above, I have made further discoveries that relate to the biological functions of XIAP and AIF, which are supplemental to the tasks outlined in my proposal. First, I have determined that the amino-terminus of the mature form of AIF begins at amino acid residue 55 ($\Delta 54$ AIF, Figure 5), resulting in a significantly larger protein than what was first reported during the original cloning of AIF (Susin et al., 1999), which suggested that the mature amino-terminus begins at amino acid residue 103 ($\Delta 102$ AIF). This result has subsequently been confirmed by another group, which further showed evidence that only during the induction of apoptosis is AIF cleaved to the $\Delta 102$ form originally described.

Second, in light of the above observations and since the forms of AIF present in cells under normal and apoptotic conditions may interact differently with XIAP, I examined the cellular localization of both the $\Delta 54$ and $\Delta 102$ proteins, and determined that both are diffusely cytoplasmic (Figure 6).

Third, while investigating the effects of AIF on the formation of reactive oxygen species (ROS) in cells under both normal and apoptotic conditions, I determined that overexpression of AIF alone is sufficient to significantly increase the amount of ROS present in cells under normal conditions (Figure 7). Furthermore, upon treating cells with an apoptotic stimulus, there is a synergistic increase in ROS formation. Taken together these data suggest that increased AIF expression significantly increases steady state levels of cellular ROS, which is further augmented when cells receive an apoptotic stimulus.

Finally, in my original proposal I presented data that suggested that AIF protein levels were dramatically altered in tissues derived from XIAP-deficient mice. I have performed the reciprocal experiment to investigate if alterations are present in XIAP protein levels when tissues from the Harlequin (Hq) mouse, in which AIF proteins levels are reduced by 80% or more in all tissues, were examined. I have found that XIAP protein levels are significantly elevated in tissues derived from Hq animals when compared to wildtype controls (Figure 8).

Key Research Accomplishments

- XIAP was shown to preferentially interact with the $\Delta 102$ form of AIF in living cells
- It was shown that all forms of AIF (full-length, $\Delta 54$, and $\Delta 102$) fail to prevent XIAP-mediated caspase inhibition
- When overexpressed in a variety of cell lines, it was determined that all tested forms of AIF fail to induce cell death
- AIF was shown to be a substrate for XIAP-mediated ubiquitination
- The amino-terminus of mature AIF in healthy cells was shown to begin at amino acid 55
- Truncation AIF variants ($\Delta 54$, $\Delta 102$) were shown to be cytoplasmically localized in living cells
- AIF overexpression was determined to increase basal ROS levels, and synergistically increase ROS formation following exposure of cells to etoposide
- XIAP protein levels were determined to be elevated in tissues from the Hq strain of mouse.

Reportable Outcomes

The following abstract was presented at the 2005 Keystone Symposia on Cellular Senescence and Cell Death in the last year:

- **Wilkinson, J.C.**, Wilkinson, A.S., Scott, F.L., Csomos, R.A., Salvesen, G.S. and Duckett, C.S. Neutralization of Smac/DIABLO by IAPs: a caspase-independent mechanism for apoptotic inhibition.

Conclusions

Based on the progress so far, current conclusions include: 1) XIAP and AIF interact in living cells under non-apoptotic conditions, 2) AIF does not alter the caspase inhibitory properties of XIAP, 3) the caspase inhibitory properties of XIAP are not required for interaction with AIF, 4) AIF is a substrate of XIAP-mediated ubiquitination, 5) the mature amino-terminus of AIF begins at amino acid 55, 6) the $\Delta 54$ and $\Delta 102$ truncations of AIF are cytoplasmically localized, 7) AIF overexpression results in increased ROS formation that is further amplified during apoptosis, and 8) XIAP protein levels are significantly altered in tissues from Hq (AIF deficient) mice.

References

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. & Kroemer, G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446 (1999).

Appendix

Abstract presented at the 2005 Keystone Symposia on Cellular Senescence and Cell Death:

Neutralization of Smac/DIABLO by IAPs: a caspase-independent mechanism for apoptotic inhibition

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Numerous members of the IAP family can suppress apoptotic cell death in physiological settings. While certain IAPs directly inhibit caspases, the chief proteolytic effectors of apoptosis, the protective effects of other IAPs do not correlate well with their caspase inhibitory activities, suggesting the involvement of alternative cytoprotective abilities. To examine this issue, we have characterized the protective effects of an ancestral, baculoviral IAP (Op-IAP) in mammalian cells. We show that although Op-IAP potently inhibited Bax-mediated apoptosis in human cells, Op-IAP failed to directly inhibit mammalian caspases. However, Op-IAP efficiently bound the IAP antagonist Smac/Diablo, thereby preventing Smac/Diablo-mediated inhibition of cellular IAPs. While reduction of Smac/Diablo protein levels in the absence of Op-IAP prevented Bax-mediated apoptosis, overexpression of Smac/Diablo neutralized Op-IAP-mediated protection, and Op-IAP variants unable to bind Smac/Diablo failed to prevent apoptosis. Finally, Op-IAP catalyzed the ubiquitination of Smac/Diablo, an activity that contributed to Op-IAP-mediated inhibition of apoptosis. These data show that cytoprotective IAPs can inhibit apoptosis through the neutralization of IAP antagonists, rather than by directly inhibiting caspases.

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Meeting Code: J7 (Cellular Senescence and Cell Death)
Poster Session 3

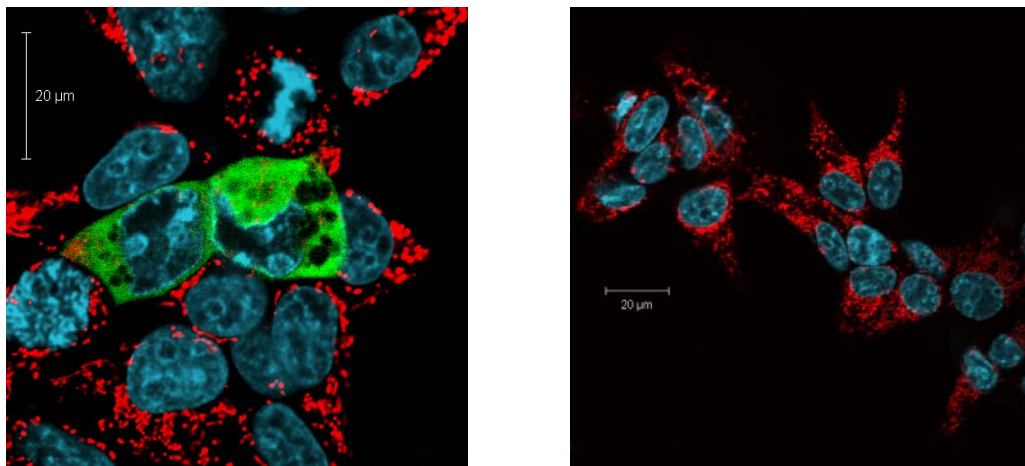


Figure 1. XIAP interacts preferentially with $\Delta 102$ -AIF. HEK293 cells were transiently transfected with an expression plasmid encoding XIAP fused amino-terminally with the amino terminal domain of YFP (YN-XIAP) along with expression plasmids encoding either $\Delta 102$ -AIF (left panel), $\Delta 54$ -AIF (right panel), or full-length AIF (data not shown) fused carboxy-terminally with the carboxy terminal domain of YFP ($\Delta 102$ -AIF-YC, $\Delta 54$ -AIF-YC, FL-AIF-YC). In this system, only cells in which an interaction occurs between XIAP and AIF will emit YFP fluorescence (shown as green in these images). Cells were co-stained with both Mitotracker Red (mitochondrial marker, red fluorescence) and Hoescht (nuclear marker, blue fluorescence), and fluorescence was observed using a Zeiss LSM 510 confocal microscope. Note the significant levels of green fluorescence, indicative of XIAP/AIF binding *in vivo*, evident only in the XIAP/ $\Delta 102$ -AIF sample (left panel). Both $\Delta 54$ -AIF-YC (right panel) and FL-AIF-YC (not shown) failed to produce fluorescence in combination with YN-XIAP. YN-XIAP/ $\Delta 102$ -AIF-YC signals were only detected in the cytoplasm, as confirmed by the absence of YFP signal localization with Mitotracker Red and Hoescht dyes. Data is representative of at least three experiments.

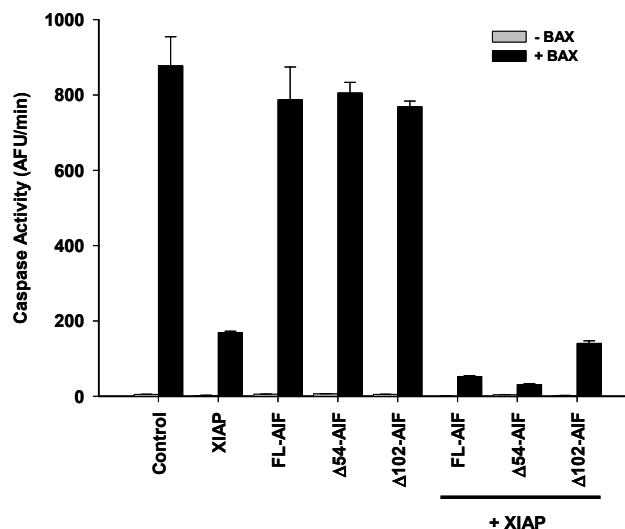


Figure 2. AIF does not abrogate XIAP-mediated caspase inhibition following Bax overexpression. HEK293 cells were transiently transfected with control, XIAP, or AIF (full-length AIF, $\Delta 54$ -AIF, $\Delta 102$ -AIF) expression plasmids in the absence (grey bars) or presence (black bars) of Bax. Additional samples in which AIF plasmids were co-transfected with XIAP were also prepared. Eighteen hours after transfection, cell lysates were prepared and tested for the presence of caspase-3 activity by incubation with the fluorogenic substrate DEVD-AFC. The averages $\pm$ S.D. of multiple independent measurements are shown, and data are representative of at least three experiments. Note the lack of basal caspase activation due to expression of the AIF variants (suggesting that AIF expression alone does not induce cell death), as well as the lack of an effect of any AIF variant upon XIAP-mediated caspase inhibition.

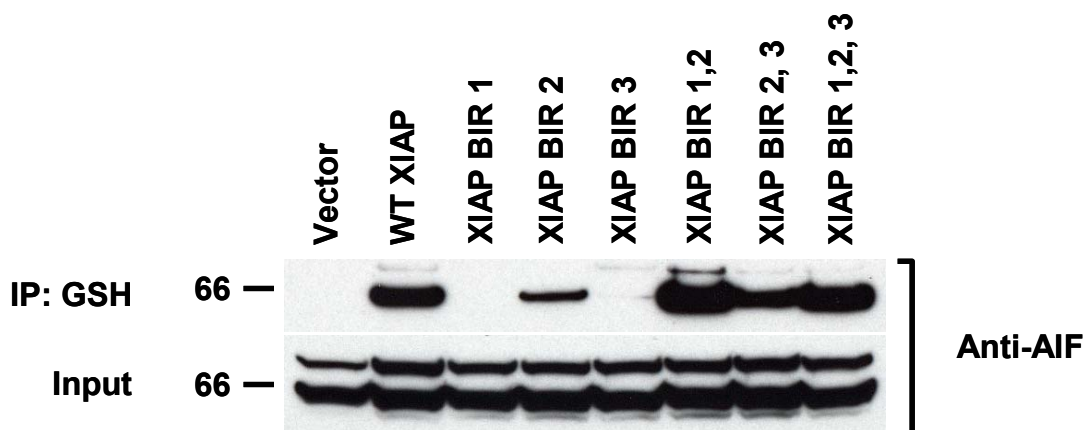


Figure 3. XIAP BIR2 is sufficient for binding to AIF. HEK293 cells were transiently transfected with plasmids expressing various XIAP domains in fusion with GST, along with a plasmid expressing full-length AIF in fusion with a carboxy-terminal FLAG epitope. Cells were lysed, and XIAP proteins were precipitated with glutathione beads. The presence of AIF in precipitated complexes was determined by immunoblotting with anti-FLAG (top). Equivalent AIF expression was determined by immunoblotting input samples with anti-FLAG (bottom). Note that only those XIAP variants that contain BIR2 precipitate AIF, suggesting that BIR2 is necessary and sufficient for interaction with AIF.

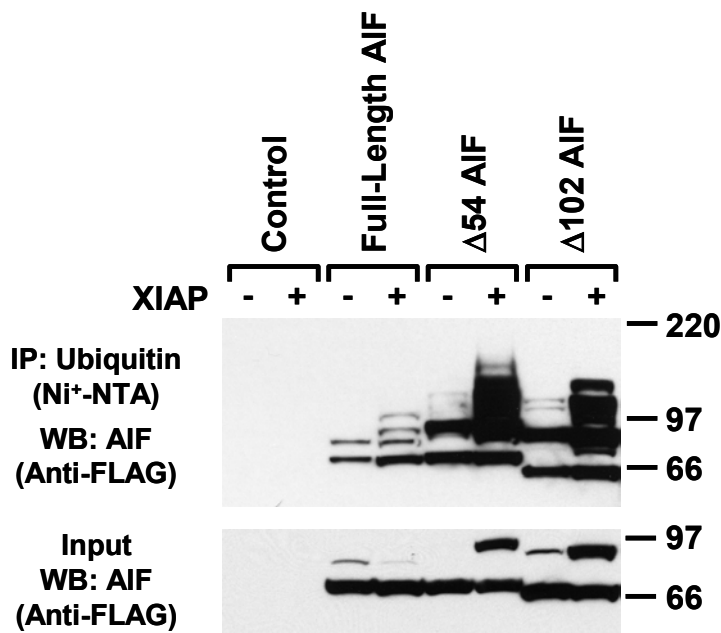


Figure 4. AIF is a substrate of XIAP-mediated ubiquitination. HEK293 cells were transiently transfected with His-tagged ubiquitin and either control or C-terminal FLAG-tagged AIF variants (full-length, Δ54-AIF, Δ102-AIF) in the absence and presence of XIAP. Ubiquitinated material was then precipitated using Ni²⁺-NTA beads, and the presence of FLAG-tagged proteins (AIF) in precipitated complexes (IP) was detected by immunoblot analysis (WB). Equivalent protein expression for all AIF variants was confirmed by immunoblot analysis of input samples using anti-FLAG. Note the increase in ubiquitinated AIF material when XIAP is co-expressed.

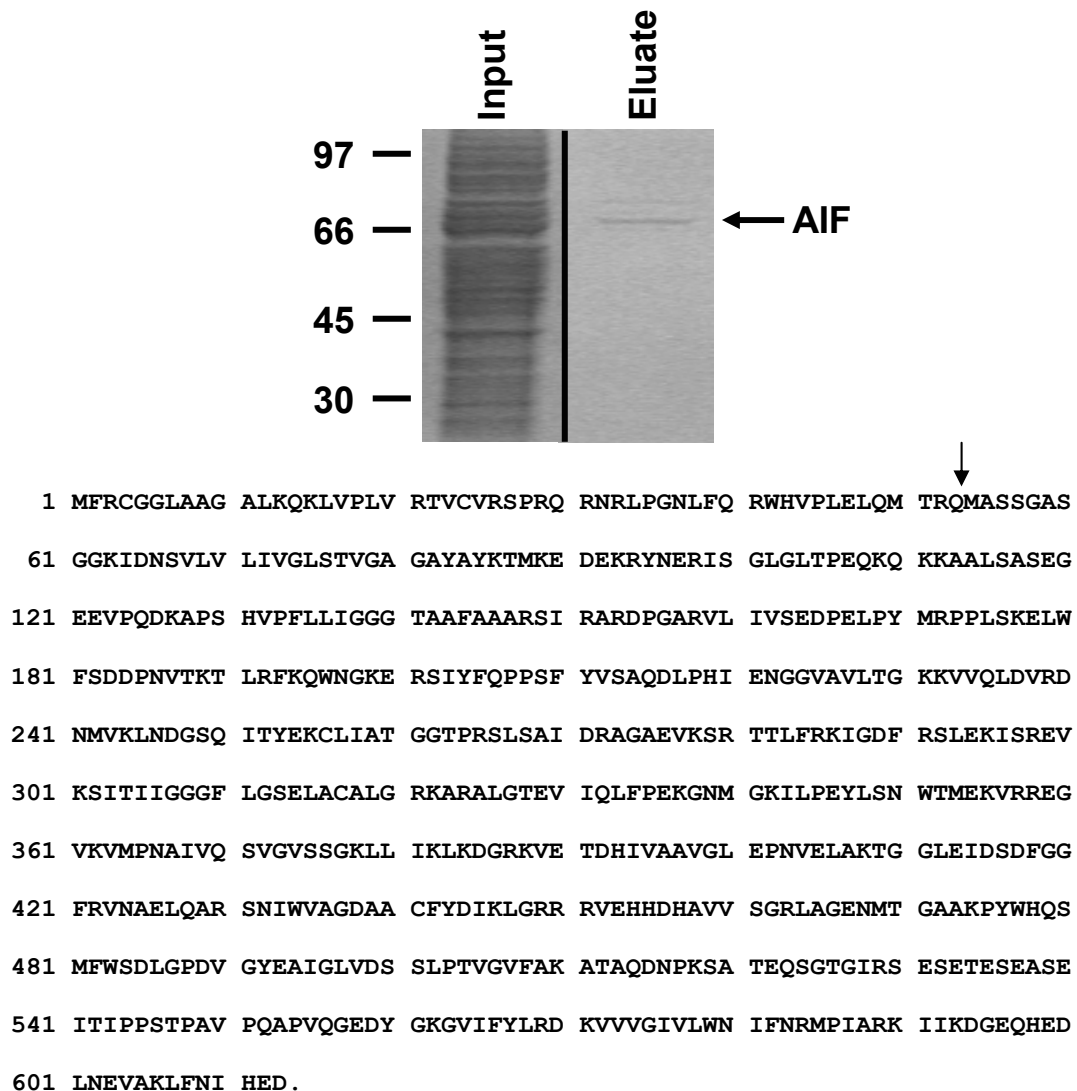


Figure 5. The amino-terminus of mature AIF begins at amino acid 55. Top panel: HEK293 cells were transiently transfected with an expression plasmid encoding full-length AIF in fusion with a carboxy-terminal tandem affinity purification (TAP) tag. A cellular lysate was prepared (Input), and purified AIF-TAP (eluate) was subjected to Edmund degradation in order to determine the mature amino terminus. Bottom panel: the primary amino acid sequence of human AIF is displayed. The arrow indicates the cleavage site generating the mature amino-terminus, as determined from Edmund degradation of purified AIF-TAP.

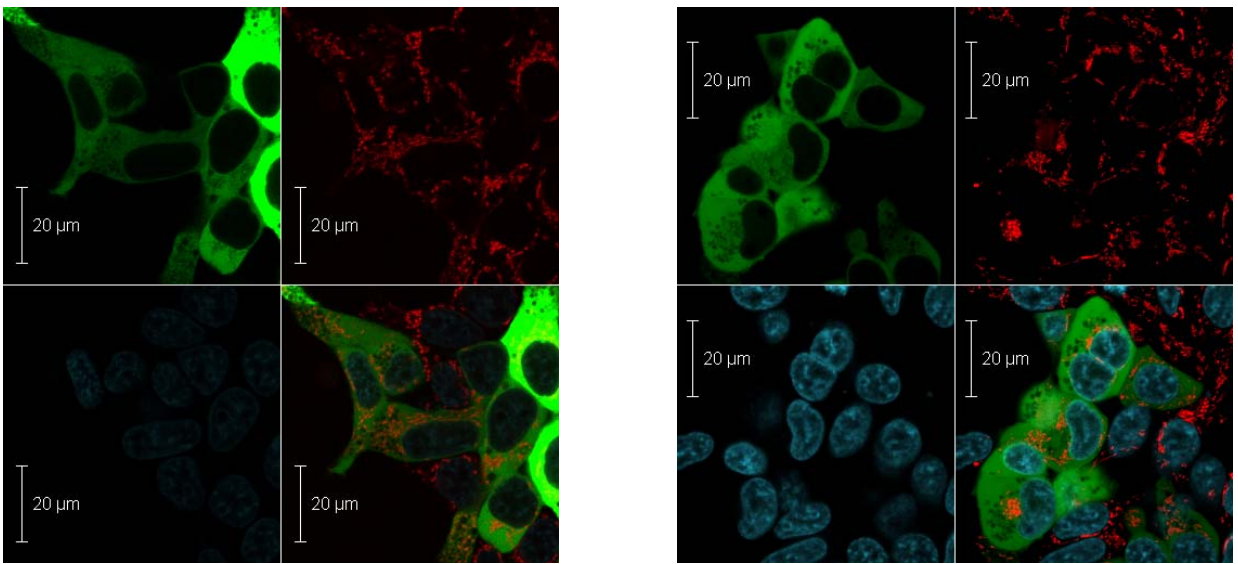


Figure 6. AIF truncation variants are cytoplasmically localized. HEK293 cells were transiently transfected with expression plasmids encoding either $\Delta 54$ -AIF (left panel) or $\Delta 102$ -AIF (right panel) in fusion with YFP (green fluorescence in these images) at the carboxy terminus. Cells were co-stained with both Mitotracker Red (mitochondrial marker, red fluorescence) and Hoescht (nuclear marker, blue fluorescence), and fluorescence was observed using a Zeiss LSM 510 confocal microscope. Note the cytoplasmic localization of both proteins, as evidenced by the lack of signal co-localization with either Mitotracker Red or Hoescht dyes.

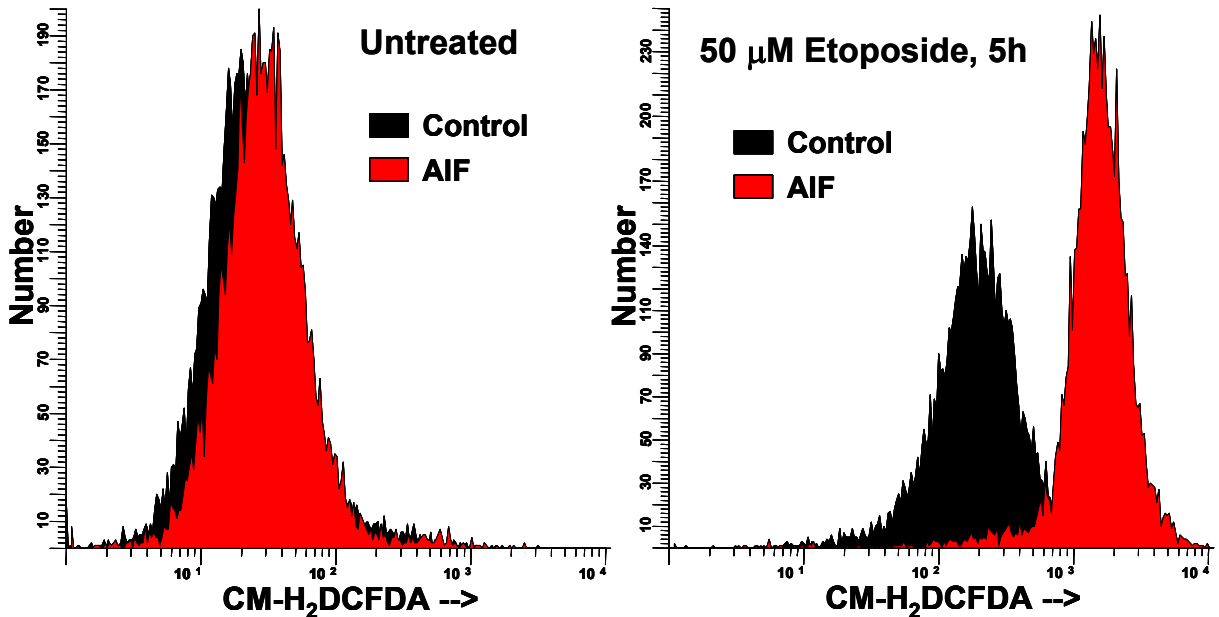


Figure 7. ROS formation in Jurkat cells overexpressing AIF. Jurkat cells were transiently transfected with either control (black) or full-length AIF (red) expression plasmids. Forty eight hours after transfection, cells were left untreated (left panel) or treated with etoposide (right panel) for 5 h. Following treatment, cells were harvested and stained with the ROS indicator CM- H_2 DCFDA, and ROS levels were quantified by flow cytometry. Note the increase in ROS due to AIF overexpression under both basal (left panel) and stressed (right panel) conditions.

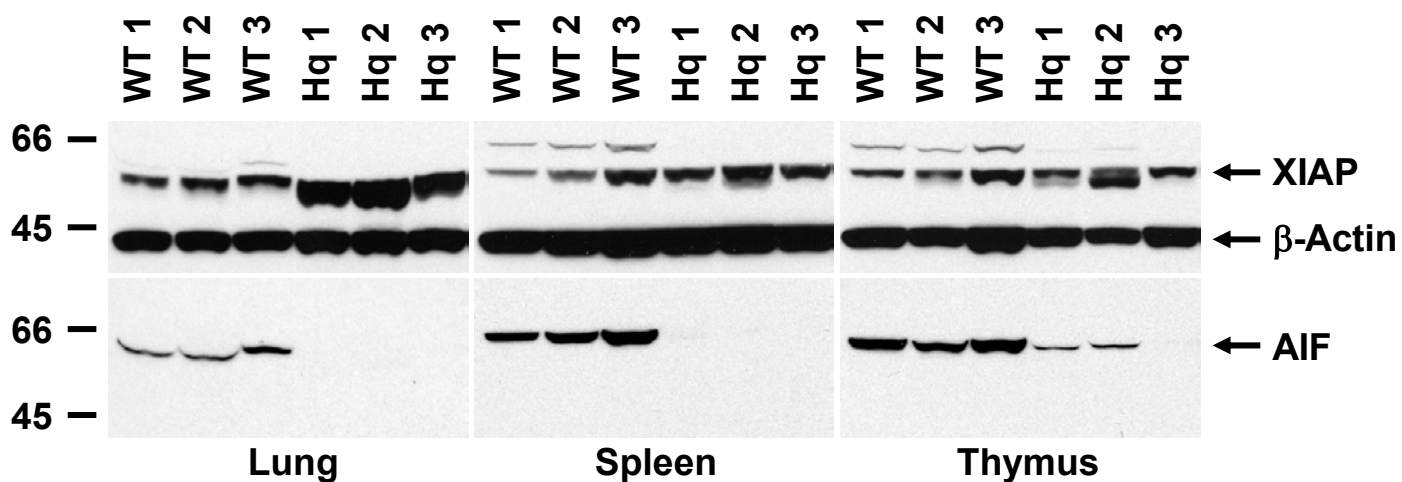


Figure 8. XIAP protein expression is tissues derived from Hq mice. Lung, spleen, and thymus were isolated from three Harlequin (Hq) and three corresponding wildtype (WT) littermate control mice. Protein extracts were prepared and immunoblotted for the presence of XIAP (top panel) and AIF (bottom panel). As a loading control, the top panel was also immunoblotted for the presence of β -actin. Note either the absence or reduction of AIF protein in all Hq tissues, as well as the increase in XIAP protein in Hq tissues, especially lung.