An Exploration of Novel Physiological Roles for Smac/DIABLO

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Abstract

Second mitochondrial-derived activator of caspases (Smac), also known as direct IAP-binding protein with low pI (DIABLO) is known as a proapoptotic protein that inhibits the inhibitors of apoptosis family (IAPs). The members of the IAP family were characterized by their ability to inhibit proapoptotic caspases. More recently, the IAPs have also been shown to play major roles in regulating diverse signalling events within the cell. A physiological role for Smac remains elusive, but studies have focused primarily on its roles in regulating the IAPs in the context of apoptosis. In order to critically evaluate possible physiological roles for Smac, we sought to examine non-apoptotic signalling events regulated by the IAPs, including NF-κB induction, mitochondrial antiviral (MAVS) signalling, and necroptosis. While Smac knockdown revealed no obvious roles for Smac in context of MAVS signalling or NF-κB induction, we found that Smac contributes to necroptosis induction in L929 cells. While we hypothesized that Smac null mice would be protected from necroptosis induced by experimental colitis, we unexpectedly found that colitis was more severe in Smac null mice than in wild-type mice. Further investigation of how Smac is involved in the reaction to induced colitis might reveal a physiological role for Smac.

Résumé

"Second mitochondrial-derived activator of caspases" (Smac), aussi connu sous le nom de DIABLO pour "direct IAP-binding protein with low pI", est une protéine proapoptotique qui effectue son rôle en interagissant avec et inhibant l'action des membres de la famille des inhibiteurs d'apoptose (IAP).

Les membres de la famille des IAP ont été caractérisés par leur capacité d'inhiber l'action des caspases proapoptotiques prévenant ainsi l'apoptose cellulaire.

Les IAP sont aussi engagés dans diverses autres voies de signalisation, hormis leur rôle dans la régulation de l'apoptose. L'effet de Smac sur les IAP a été étudié dans le contexte de l'apoptose cellulaire et aucun autre rôle physiologique ne lui a été attribué jusqu'à nos jours. Afin d'évaluer diverses possibilités de rôles physiologiques pour Smac, nous désirions examiner les voies de signalisation non-apoptotiques régulées par les IAP, incluant l'induction de NF-κB, la signalisation antivirale mitochondriale (MAVS) ainsi que la nécroptose.

Nos résultats révèlent que la sous expression de Smac n'affecte pas l'induction de NF-κB et n'interfère pas avec la voie de signalisation engagée par les MAVS. Par contre Smac induit la mort des cellules L929 par nécroptose.

Pour examiner l'effet de Smac in vivo nous avons étudié son rôle dans un modèle de colite expérimentale. Nos résultats révèlent que les souris mutantes rendues nulles pour le gène Smac présentent une colite plus sévère

que les souris à phénotype sauvage.

Il serait ainsi nécessaire d'examiner de plus près la régulation de Smac dans le modèle de colite expérimentale pour essayer de mieux comprendre son rôle physiologique.

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List of Abbreviations

8-CPT-cAMP 8- (4- Chlorophenylthio)adenosine- 3', 5'- cyclic monophosphate
APAF1 Apoptotic protease activating factor 1
ARTS Apoptosis-related protein in the TGF-β signalling pathway
ATP Adenosine triphosphate
BAFF B-cell activating factor
BCS Bovine calf serum
BID BH3 interacting-domain death agonist
BIR Baculovirus repeat domain
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
CARD Caspase activation and recruitment domains
CD40L CD40 ligand
cIAP1 Cellular inhibitor of apoptosis 1
cIAP2 Cellular inhibitor of apoptosis 2
CREB cAMP response element-binding transcription factor
DExD/H DEAD box, DEAH, and the Ski families of proteins
DIAP1 Drosophila inhibitor of apoptosis 1
dmIKKε Drosophila IKK related kinase
DMEM Dulbecco's modified Eagle medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DSS Dextran Sodium Sulfate
ERK Extracellular signal-regulated kinase
FADD Fas-Associated protein with Death Domain
FasL Fas Ligand
FBS Fetal bovine serum
H&E Haematoxylin and Eosin
HEK293T Human embryonic kidney cell line
HBS Hepes-buffered saline
HCT116 Human colorectal carcinoma cell line
HT29 Human colon adenocarcinoma grade II cell line
HRP Horseradish peroxidase
IBM IAP-Binding Motif
IAP Inhibitors of apoptosis
IFN-β Interferon-beta
IFN-γ Interferon-gamma
IκB Inhibitor of kappa B
IKKε IκB kinase epsilon
IRSE Interferon-responsive sequence element
L929 Murine fibrosarcoma cells
LRR Leucine rich repeats
K48 Lysine-48 linked ubiquitination
K63 Lysine-63 linked ubiquitination

NAIP Neuronal apoptosis inhibitor protein
Nec-1 Necrostatin 1
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK NF-κB inducible kinase
NOD nucleotide-binding oligomerization domain
NS Non-specific
LUBAC Linear ubiquitin chain assembly complex
MAVS Mitochondrial antiviral signalling protein
MDA-MB-231 Human breast adenocarcinoma
MDA5 Melanoma Differentiation-Associated protein 5)
MEFs Mouse embryonic fibroblasts
MEKK2 Mitogen-activated protein kinase kinase kinase 2
ML-IAP Melanoma inhibitor of apoptosis
MLKL Mixed lineage kinase domain-like
MW Molecular weight
Omi Mammalian homolog of the bacterial high temperature requirement protein (also known as HTRA2)
PAMP Pathogen-associated molecular patterns
PBS phosphate-buffered saline
PGAM5 phosphoglycerate mutase family member
phospho phosphorylated
PRR pattern recognition receptor
RLR RIG-I-Like Receptors
RIG1 Retinoic acid inducible gene 1
RING Really interesting new gene
RIP1 Receptor interacting protein 1
RIP3 Receptor interacting protein 3
RNA Ribonucleic acid
ROS Reactive oxygen species
RT Reverse transcriptase
RT-PCR Reverse transcription polymerase chain reaction
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA Small interfering RNA
SM Smac mimetic
Smac second mitochondrial-derived activator of caspase/direct IAP-binding protein with low pI
Smac-β Smac beta
TAB transforming growth factor-β activated kinase
TBK1 Tank-binding kinase 1
TBS-T Tris buffered saline tween-20
TNFα Tumour necrosis factor
TS-IAP testis specific IAP
TRADD Tumour necrosis factor receptor type 1-associated DEATH domain protein
TRAF TNF receptor associated factor
TRAIL TNF-related apoptosis-inducing ligand

TWEAK TNF-related weak inducer of apoptosis
UBA ubiquitin-associated domain
WT Wild-type
XIAP X-linked inhibitor of apoptosis
zVAD-fmk carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Chapter One: Literature Review

Introduction

Programmed cell death is essential in development, the maintenance of homeostasis, and the elimination of disordered cells (Danial and Korsmeyer, 2004). Several forms of programmed cell death have now been described, the best characterized of these is apoptosis. The key effectors of cell death in apoptosis are caspases, members of a family of cysteine proteases (Thornberry and Lazebnik, 1998, Shi, 2002). Since tight regulation of programmed cell death is critical, the activation of caspases is controlled by many cellular factors. A family of proteins with a central role in the regulation of caspases is the inhibitor of apoptosis (IAP) protein family(Gyrd-Hansen and Meier, 2010). Through the characterization of the role of the IAPs in the regulation of apoptosis, proapoptotic factors that suppress the activity of the IAPs themselves have been also identified, including Smac (second mitochondria-derived activator of caspases), also known as Diablo (direct IAP binding protein with low PI) (Du et al., 2000, Verhagen et al., 2000).

Apoptosis

There are two well-characterized pathways involved in apoptotic signalling. The intrinsic pathway is triggered by cell stress and developmental cues. This stress triggers the release of death promoting factors like cytochrome C, and Smac from the mitochondria (Riedl and Shi, 2004). Cytochrome c binds

Apoptotic-protease-activating factor-1 (APAF1), which allows for activation and oligomerization with the initiator caspase-9 (Li et al., 1997). Caspase-9 can go on to activate effector caspases, caspase-3 and -7 to initiate apoptosis. The caspases are inhibited by members of the IAP family; Smac promotes apoptosis by inhibiting these inhibitors(Du et al., 2000). The extrinsic pathway is activated by binding of death inducing ligands like TNFα, TRAIL or FasL, to their respective receptors. This allows for the formation of ligand receptor complexes, recruitment of adaptor proteins, such as FADD, that in turn, recruit and allow for the activation of the initiator caspase-8. Caspase-8 can directly activate the effector caspase-3. Active caspase-8 can also cleave Bid to its activated truncated form, allowing for amplification of the death signal by allowing for release of Smac and cytochrome c from the mitochondria. Both the extrinsic and intrinsic pathways converge resulting in massive caspase activation(Riedl and Shi, 2004).

The IAPs

The IAPs are a family of proteins that are evolutionarily conserved from yeast to higher vertebrates. The first IAP was discovered in baculovirus, and was characterized by its ability to suppress virus-induced apoptosis in infected insect cells (Crook 1993). The IAPs are characterized by the presence of at least one Baculoviral IAP repeat (BIR) domain. Reports of high levels of IAP expression in a variety of neoplasms led to the idea that IAPs might contribute to the apoptosis resistance observed in many types of

cancer. Biochemical and structural studies to pursue this hypothesis revealed that BIR domains could bind to and inhibit the activity of the caspases, the key effectors in apoptosis.

Structure and Function of the IAP family members

Eight IAP family members have been described in humans, these are XIAP, cIAP1, cIAP2, NAIP, ML-IAP, Ts-IAP, survivin and apollon (Figure S1). These family members contain one to three BIR domains at their N-termini (Gyrd-Hansen and Meier, 2010). BIR domains, which are the defining structural characteristic of IAPs, are zinc-binding folds of approximately 70 amino acids that mediate protein-protein interactions(Srinivasula and Ashwell, 2008).

Individual BIR domains possess specific binding characteristics, but they can be approximately divided into two groups. Type I BIR domains possess a shallow pocket, while type II BIR domains posses a deep hydrophobic cleft. Through this deep hydrophobic cleft, type II BIR domains bind to conserved tetrapeptide sequences called IAP Binding Motifs (IBMs)(Gyrd-Hansen and Meier, 2010).

IAPs can contain additional functional regions. Some IAPs contain RING domains, which provides them with ubiquitin ligase activity, allowing them to mediate autoubiquitination or ubiquitination of target substrates. Ubiquitination can target proteins for destruction in the proteasome, but it can also act as post-translational modification that allows substrates to act as

scaffolds. Ub-associated (UBA) domains are also found in some IAPs, which allow them to associate with ubiquitinated proteins(Gyrd-Hansen and Meier, 2010).

CARD recruitment domains are present in cIAP1 and cIAP2. While CARD domains are typically involved in homotypic interactions with other CARD domain containing proteins, the function of the CARD domain in the IAPs was unknown until recently. Lopez, John et al. (2011) showed that the CARD domain of cIAP1 most likely does not play a role in the recruitment of substrates containing CARD domains, instead they showed it was important for regulation of cIAP1 ubiquitin ligase activity.

IAP inhibition of apoptosis

Early studies focused attention on the ability of the IAPs to inhibit apoptosis. In vitro and over-expression studies demonstrated that IAPs could inhibit apoptosis by inhibiting caspases. However, biochemical, structural and genetic evidence from further studies showed that this was only true for some of the IAP family members. Of the eight mammalian IAPs, XIAP is the only member of the IAP family that directly inhibits caspases.

When overexpressed, XIAP inhibits caspase activation and apoptosis. Conversely, cells lacking XIAP are sensitized to apoptosis. Two regions of XIAP have been shown to be involved in the inhibition of caspases, one targeting effector caspases-3 and -7 while the other inhibits the initiator caspase-9. Caspase-3 and -7 are effector caspases that are activated by

cleavage that reveals a neo-amino terminus IAP binding motif (IBM). This IBM binds to the BIR2 domain of XIAP(Scott et al., 2005).

Inhibition occurs because the region that links the BIR1 and BIR2 domain of XIAP enters the catalytic pocket of the caspases, and prevents substrate entry. (Chai et al., 2001, Huang et al., 2001) Caspase-9 activation involves dimerization-induced conformational change to generate a functional catalytic pocket. XIAP inhibits this dimerization by binding to the homodimerization surface of caspase-9 through its BIR3 domain(Srinivasula et al., 2000). The role of the XIAP RING domain has not been firmly established, but might also be involved in caspase ubiquitination (Suzuki et al., 2001).

The closest homologs of XIAP, cIAP1 and cIAP2, are capable of binding caspases but this does not result in direct inhibition of their catalytic activity. Early studies indicated that cIAP1 and cIAP2 could bind and inhibit caspase-3 -7 and -9 like XIAP(Roy et al., 1997, Deveraux et al., 1998). They posses the IBM interacting groove that mediates caspase binding, but it was shown that they lack residues required for functional inhibition of caspases(Eckelman and Salvesen, 2006).

Despite inability to directly inhibit caspases like XIAP, there is strong evidence implicating cIAPs in apoptosis suppression, and they are overexpressed in some cancers. The anti-apoptotic activity of the cIAPs is facilitated by their RING domain; through ubiquitination of target substrates they promote apoptosis.

cIAP1 ubiquitinates caspase-3 and -7, targeting them for proteasomal degradation, and thus suppressing apoptosis(Choi et al., 2009). The cIAPs have also been shown to ubiquitinate RIP1, a kinase downstream of death receptor signalling(Bertrand et al., 2008). This promotes RIP1 to associate with downstream effectors in NF-κB survival signalling, rather than death signalling.

Inhibition of the Inhibitors by Smac

Smac is the best-studied inhibitor of the IAPs. Smac is a dimeric protein synthesized in the cytosol, and imported to the mitochondria. Its mitochondrial-targeting signal is cleaved to reveal an N-terminal tetrapeptide (AVPI) IAP binding motif (IBM). Smac is released back to the cytosol in response to mitochondrial outer membrane permeabilization, where it can go on to interfere with IAPs through its IBM. Smac has been shown to release inhibition of the apaf-1-caspase-9 apoptosome, and caspases 3 and 7 by XIAP (Srinivasula et al., 2000). Additionally, Smac has been shown to promote the autoubiquitination and subsequent proteasomal degradation of cIAP1 and cIAP2(Yang and Du, 2004).

The N terminal domain of Smac, with the residues AVPI has been shown to be necessary and sufficient for the removal of inhibition of caspase 9 by XIAP. Dimeric Smac interacts with the BIR3 and BIR2 domains of XIAP in a sequential manner. It first interacts with the BIR3 domain with one of its two N-terminal peptides. This first interaction allows for the second N-

terminal domain of Smac to interact with the BIR2 domain (Gao et al., 2007). By inhibiting both BIR2 and BIR3 of XIAP, dimeric Smac releases inhibition of caspase-3, -7 and 9 from XIAP suppression.

Cytosolic Smac

Smac possesses a mitochondrial targeting sequence at its N-terminus, which is cleaved in the mitochondria to reveal mature mitochondrial Smac. The mitochondrial targeting sequence is encoded by the first two exons. It has been shown that for human Smac, there is a splice variant that generate Smac lacking a mitochondrial targeting sequence, Smac-β, also known as Smac-S (Srinivasula et al., 2000). Smac-β is targeted to the cytosol, and in addition to lacking the mitochondrial targeting sequence, is missing the AVPI sequence that is responsible for the interaction of Smac with the BIR3 domain of XIAP. One group demonstrated that Smac-β was unable to enhance activation of caspase-9, and only displays 30% of the activity of wild type Smac with caspase 3 and 7 (Srinivasula et al., 2000), suggesting it might play a role in regulating their activity in vivo. While one group showed that Smac-β was still able to interact with the BIR2 domain of XIAP, another study found that Smac-β cannot interact with any IAPs within cells, but still retained proapoptotic activity (Roberts et al., 2001). This might indicate a role for Smac independent of IAP inhibition in apoptosis, but a mechanism for this has not been described.

Transcriptional Regulation of Smac

Regulation of Smac itself beyond its translocation from the cytosol to the mitochondria has not been extensively studied. Smac is deregulated in several types of cancer, including cervical cancer, sarcomas, lung cancer carcinomas and renal tumours (Yoo et al., 2003, Espinosa et al., 2004, Sekimura et al., 2004, Yan et al., 2004) One group found that a cAMP analog 8-CPT-cAMP, upregulates Smac transcription, by a CREB dependent mechanism (Martinez-Velazquez et al., 2007). Additionally, they found that apoptosis induced by cAMP requires Smac upregulation. Transcriptional regulation might be important for regulation of cytosolic Smac-β but this remains understudied.

Smac Mimetics

Smac mimetics are a class of compounds designed to function as IAP antagonists. These compounds mimic the neo-epitope of Smac that binds to IAP BIR domains. Smac mimetics are able to efficiently disrupt XIAP-caspase interactions. Additionally Smac mimetic treatment elicits auto-ubiquitination and degradation of the cIAPs (Varfolomeev et al., 2007, Sekine et al., 2008). By antagonizing XIAP, cIAP1 and cIAP2, Smac mimetic treatment increases apoptotic cell death in cancer cells. Smac mimetics are a promising therapeutic approach to promote cell death and arrest tumorigenesis in cancer and are being examined in clinical trials.

Physiological Role for Smac

Despite strong evidence in vitro of a role for Smac in apoptosis, Smac deficient mice do not exhibit an overt phenotype. Smac null mice are viable and mature normally (Okada et al., 2002). Additionally, mouse embryonic fibroblasts, lymphocytes and hepatocytes derived from Smac null mice exhibit normal responses to all apoptotic stimuli tested. The absence of an overt phenotype suggests functional redundancy between Smac and Omi (also known as HtrA2), a serine protease reported to bind to and proteolytically degrade IAPs. However, simultaneous deletion of these two genes does not lead to exacerbation of the Omi phenotype (Martins et al., 2004). A phenotype for Smac has only been shown in mice that also lack Caspase-3. Combined deletion of both caspase-3 and Smac results in perinatal lethality, and MEFs derived from these animals exhibit decreased effector caspase activity(Hui et al., 2011).

Non-apoptotic roles of the inhibitors of apoptosis

While IAPs are best known for their role in inhibiting apoptosis through caspase regulation, they have been shown to influence a multitude of other processes within the cell. The IAPs have emerged as regulators of mitochondrial antiviral signalling, NF-κB activation, and necroptosis. Roles for IAP regulators like Smac have not been examined in these contexts.

Mitochondrial Antiviral Signalling

Recently mitochondria have emerged as key players in the innate immune system, acting as signalling platforms in antiviral signalling responses(West et al., 2011) . IAPs have emerged as key elements in these mitochondrial antiviral signalling pathways**.**

An effective antiviral response involves the activation of many signalling pathways to facilitate the production of cytokines and other factors to inhibit viral replication and assembly. Following infection, cellular pattern recognition receptors (PRRs) sense pathogen associated molecular patterns (PAMPs). In the case of RNA virus infections, membrane bound Toll-like receptors and cytosolic receptors like retinoic acid-inducible gene-I (RIG1)-like receptors (RLRs) are the PRRs involved in sensing the PAMPs of viral RNA(Akira et al., 2006).

Mitochondria play an important role in signalling downstream of the cytosolic RLRs. The RLR family, composed of RIG1, MDA5 and LGP2, are present in both immune and non-immune cells. RIG1 and MDA5 posses CARD domains that are required for signalling, and DExD/H box domains to detect double stranded RNA in the cytoplasm. Mitochondrial antiviral signalling protein or MAVS (also known as IPS-1, VISA or CARDIF) was identified by four groups (Kawai et al., 2005, Meylan et al., 2005, Seth et al., 2005, Xu et al., 2005) as an essential adaptor for RLR signal transduction.

MAVS is localised on the outer mitochondrial membrane, and contains an N-terminal CARD, a proline rich region, and a transmembrane C-terminus.

MAVS has been shown to have prion-like activity; it forms detergent resistant aggregates upon activation, and these aggregates induce and propagate the antiviral response. The CARD domain of MAVS interacts with the CARD domains of RIG-1 and MDA5. The interaction of MAVS with these RLRs leads to activation of downstream NF-κB and IRF signalling pathways, resulting in the production of pro-inflammatory cytokines and IFN (West et al., 2011).

A study from Mao et al. (2010) demonstrated roles for both cIAP1 and cIAP2 in type1 IFN induction and cellular antiviral response. They showed viral infection resulted in the recruitment of cIAP1 and cIAP2 (cIAP1/2) to members of the TRAF protein family, TRAF3 and TRAF6, and the mitochondrial antiviral signalling protein MAVS. cIAP1/2 were shown to mediate the K48 and K63 ubiquitination of TRAF3 and TRAF6. No noticeable degradation of TRAF3/6 was observed following virus infection, so it was proposed that this ubiquitination might allow for interaction with downstream kinases essential for the antiviral response, like TBK1.

XIAP has also been implicated in antiviral signalling. The first hints of XIAP involvement in antiviral signalling come from drosophila studies (Kuranaga et al., 2006, Oshima et al., 2006) which have shown that Drosophila IKK-related kinase, a homolog of the noncanonical members of the IκB kinase family that regulate NF-κB activation or interferon 3 and 7 activation in mammals, regulates the turnover of DIAP1, the drosophila homolog of XIAP. A study from (Nakhaei et al., 2012) implicated XIAP in mammalian antiviral signalling. Following virus infection, they showed that

XIAP is phosphorylated by IKKε or TBK1, resulting in its K48 ubiquitination and degradation. Consequently, virus infected cells were sensitized to apoptosis.

IAPs in NF-κB signalling

NFκB transcription factors are important for the regulation of genes essential for inflammatory and immune responses, as well as processes of cell adhesion, differentiation, proliferation and apoptosis. IAPs have emerged as important regulators of NF-κB activation. Activation can occur through two signal transduction pathways that activate canonical and non-canonical NF-κB pathways, respectively. XIAP is also emerging as a regulator of NF-κB signalling.

cIAPs and the Canonical NFκB Pathway

The role of the IAPs in the canonical NF-KB signalling pathway downstream of the TNF receptor has been well described. cIAPs promote canonical NF-κB signalling, inducing the transcription of inflammatory and prosurvival genes. Under unstimulated conditions, the NF-κB transcription factor subunits are sequestered in the cytosol, inhibited IκBα and therefore unable to translocate to the nucleus.

As in the extrinsic pathway of apoptosis, the binding of TNFα to its receptor, TNFR1, leads to the recruitment of the adaptor protein TRADD, TRAF2, cIAP1/2 and RIP1 kinase to the receptor, forming "complex I"(Hsu et

al., 1995, Hsu et al., 1996, Shu et al., 1996). cIAP1/2 act as E3 ubiquitin ligases mediating K63 ubiquitination of RIP1(Bertrand et al., 2008) . K63 chains, in contrast to K48 chains that typically target proteins for proteasomal degradation, often act as scaffolds to promote signal transduction. The ubiquitin chains stimulate recruitment of the linear ubiquitin chain assembly complex (LUBAC) and the kinase complexes TAK1-TAB2-TAB3 (TGF-β related kinase, TAK1 binding protein 2 and 3) and IKKγ-IKKα-IKKβ (IκB kinase γ, α, β). LUBAC ubiquitinates IKKγ, promoting stabilization of the complex. TAK1 kinase phosphorylates of the IKK complex, activating them(Haas et al., 2009).

Active IKK phosphorylates the inhibitor, IKBα, targeting it for K48-ubiquitination and proteasomal degradation, freeing the NF-κB dimer from inhibition(Li et al., 1999). NF-κB can then enter the nucleus to activate transcription of target genes.

cIAP1 and cIAP2 act to facilitate signalling in this pathway. As these proteins are highly similar, their function in facilitating the signalling in this pathway is thought to be redundant, as loss of either one does not impair activation . However when both cIAPs are lost, there is a significant loss of TNFα induced NF-κB induction.

cIAPs and Non-Canonical NF-κB activation

In opposition to their role in canonical NF-κB activation, IAPs impede signalling in the non-canonical pathway, preventing induction of cytokines

and proapoptotic genes. Activation of the non-canonical NF-κB signalling pathway occurs primarily downstream of TNF family ligands CD40L, BAFF, and TWEAK (Gyrd-Hansen and Meier, 2010). When these ligands bind to their respective receptors they initiate signalling that results in the accumulation of NIK kinase in the cytosol. NIK can then go on to phosphorylate and activate IKKα, which phosphorylates the NF-κB subunit p100. P100 is ubiquitinated and processed by the proteasome into its mature form p52. P52 can then dimerise with NF-κB family member RelB to translocate into the nucleus and activate transcription of cytokines and pro-apoptotic genes (Hayden and Ghosh, 2008).

In resting cells, activation of the non-canonical pathway is suppressed by cIAP1 and cIAP2 mediated degradation of NIK, preventing its accumulation in the cytosol. Under unstimulated conditions, cIAP1 and cIAP2 act in a complex with TRAF2 and TRAF3(Vallabhapurapu et al., 2008, Zarnegar et al., 2008) . While TRAF2 and TRAF3 possess RING domains, it is cIAP1 and cIAP2 that are responsible for the ubiquitin ligase activate in this complex. TRAF2 and TRAF3 act as adaptor proteins that permit the cIAP-mediated K48-ubiqutination of NIK, targeting it for proteasomal degradation. When receptors are stimulated, activated TNF family receptors recruit the cIAP1/2-TRAF2/3 complex, and components of the complex are degraded by the proteasome. No longer able to target NIK for degradation, NIK accumulates in the cytosol and activates non-canonical NF-kB (Vallabhapurapu et al., 2008, Zarnegar et al., 2008).

XIAP and NF-κB

The involvement of XIAP in regulation of NF-κB signalling pathways was first indicated by several studies in which overexpression of XIAP led to increased NF-κB reporter activity(Hofer-Warbinek et al., 2000, Birkey Reffey et al., 2001, Levkau et al., 2001). Recently, XIAP-deficient cells were found to have an impairment of canonical NF-KB activation by TNFα(Winsauer et al., 2008). When examining the kinetics of NF-κB activation by TNFα in Xiap-deficient cells they found a marked impairment, compared to control cells, of nuclear RelA (p65) in a second wave of activation that was found to occur around 60 minutes after stimulation. They found that XIAP functions in the canonical pathway to control a second-wave of nuclear translocation of NF-κB , leading to prolonged activation, through interacting with the kinase MEKK2.

Necroptosis

Recently it has been shown that cIAP1 plays a protective role against necroptosis (Vanlangenakker et al., 2011). Additionally Smac mimetics have been shown to sensitize some cell lines to necroptosis, and to exacerbate necroptosis induction by death-inducing ligands (He et al., 2009) .

Signalling in Necroptosis.

Necroptosis, also known as programmed necrosis, is morphologically necrotic cell death and involves signalling that is caspase-independent. In necroptosis, as in the extrinsic pathway of apoptosis, stimulation of the TNF receptor leads to receptor trimerization and recruitment of a complex that includes FADD and RIP1. Under caspase-deficient conditions, RIP1 associates with RIP3, and this is dependent on the kinase activity of RIP1(He et al., 2009). The association of RIP1 and RIP3 leads to downstream events that involve increased energy metabolism and the generation of reactive oxygen species (ROS), ultimately resulting in morphologically necrotic cell death.

Two components in the necroptosis pathway downstream of RIP1 and RIP3, mixed lineage kinase domain-like protein (MLKL) and phosphoglycerate mutase 5 (PGAM5), have recently been identified (Sun et al., 2012, Wang et al., 2012, Zhao et al., 2012). MLKL is a kinase dead protein that is recruited to RIP1 and RIP3, and is phosphorylated by RIP3. Since it's a kinase dead protein, it was postulated by Sun et al. (2012) that it might facilitate interaction with downstream effectors. PGAM5 is a mitochondrial phosphatase normally localized on the outer mitochondrial membrane. PGAM5 was found in complex with RIP1, RIP3, and MLKL after necroptosis induction, and that it is phosphorylated by RIP3, resulting in increased PGAM5 phosphatase activity(Wang et al., 2012). Additionally, they found that PGAM5 recruits and activates the GTPase that controls mitochondrial fission,

dynamin-related protein 1 (DRP1), and that cells undergoing necroptosis undergo mitochondrial fission. It is not yet clear how mitochondrial fission leads to cell death by necroptosis. Additional downstream components and parallel pathways downstream of RIP1 and RIP3 need to be elucidated and require further study.

Necroptosis is a physiologically relevant cell death mechanism. In a mouse model of stroke, where the middle cerebral artery is transiently occluded, production of cytokines like TNFα are increased, and cell death results in infarct. A drug called necrostatin-1 can inhibit the size of the infarct(Degterev et al., 2005); necrostatin-1 is specifically designed to inhibit caspase-independent necrotic cell death, and in fact is an allosteric inhibitor of RIP1 kinase activity(Degterev et al., 2008). Necroptosis has also been shown to play a role in models of myocardial infarction and in *in vitro* studies of excitotoxicity (Xu et al., 2007, Kung et al., 2011).

Necroptosis is also implicated in the elimination of viral infections (Cho et al., 2009). Some viruses encode caspase inhibitors to evade apoptosis. Vaccinia, the cowpox virus, encodes a caspase inhibitor called SP12. When TNFα encounters a vaccinia-infected cell, it cannot activate apoptosis, but instead the cell undergoes necroptosis. In this way, necroptosis acts as a backup mechanism to eliminate virally infected cells if the apoptotic pathway is blocked. Animals that are lacking RIP3, essential for necroptosis, have high viral titres and succumb to infection(Cho et al., 2009). Necroptosis also acts to release damage associated molecular pattern

molecules, which together with the viral antigens act to activate the immune system, resulting in the elimination of the viral factory.

Necrostatin works to inhibit necroptosis through inhibition of RIP1 kinase activity (Degterev et al., 2008). RIP1 is a member of the RIP family of serine/threonine kinases, involved in innate and adaptive immunity. It is a key player in prosurvival NF-κB signalling, death receptor mediated apoptosis, and necroptosis. RIP1 is made up of a kinase domain, and intermediate domain, a RIP homotypic interaction motif, and a C terminal death domain(Galluzzi et al., 2009). RIP1 interacts with adaptor proteins at death receptors through its death domain. When RIP1 is K63 polyubiquitinated, it can act as a scaffold to allow for the assembly of TAK1-TAB2/3 complexes, as well as for the IKK complex, ultimately leading to activation of NF-κB and survival. When RIP1 is not ubiquitinated, it can no longer act as a scaffold for NF-κB activation. After death receptor stimulation, non-ubiquitinated RIP1 is found in complex with caspase-8, which goes on to cleave RIP1 and initiates apoptosis (Bertrand et al., 2008).

Under caspase-inhibited or deficient conditions, non-ubiquitinated RIP1 is found in complex with FADD and RIP3(Cho et al., 2009, Galluzzi et al., 2009, He et al., 2009). RIP1 interacts with RIP3 through the RIP-homotypic-interaction motifs. Interdependent phosphorylation of these two kinases is essential for downstream signalling leading to necroptosis. Importantly, the kinase activity of RIP1 is necessary for necroptosis, but dispensable in NF-κB signalling and apoptosis. Necrostatin-1 acts as an allosteric inhibitor that

specifically blocks the kinase activity of RIP1, and consequently inhibits necroptosis.

The IAPs and Necroptosis

The ubiquitination state of RIP1 is regulated by the cellular inhibitors of apoptosis proteins(Bertrand et al., 2008). cIAP1 and cIAP2 target RIP1 for K63 polyubiquitination, allowing it to act in the NF-κB pathway. Under caspase-deficient conditions, when TNFα binds its receptor, it recruits a complex that includes the adaptor FADD, cIAP1/2 and RIP1(He et al., 2009). When the cIAPs are downregulated or eliminated by Smac mimetic treatment, non-ubiquitinated RIP1 accumulates. While RIP1 can go on to activate apoptosis signalling, when caspases are inhibited RIP1 is able to go on in complex with FADD to interact with RIP3. Interdependent phosphorylation of these two kinases, leads to downstream effects including increased energy metabolism, oxidative phosphorylation and reactive oxygen species, ultimately causes necrosis(Cho et al., 2009, He et al., 2009).

While evidence of IAP regulation in many signalling contexts beyond apoptotic cell death, what is missing from the literature is a re-evaluation of the roles IAP inhibitors, including Smac. Many studies have utilized Smac mimetics as tools to dissect the functions of the IAPs in these pathways, but they failed to consider a role for endogenous Smac in these processes.

Chapter 2: Rationale, Methods and Results

Goals of this study

Smac is a highly conserved protein with an elusive physiological role. While overexpression studies indicate its importance in apoptosis, Smac knockout mice have no apoptotic deficits. The evidence that Smac is able to bind, inhibit, and promote the degradation of IAP proteins is very strong. Lately, the IAPs have emerged as regulators of many processes beyond apoptosis.

In this study we sought to:

1. Determine if Smac inhibits IAP function in non-apoptotic contexts
2. Examine *Smac -/-* mice for deficit in identified signalling contexts to reveal a non-redundant physiological role for Smac.

To this end, we decided to examine the roles of Smac in regulation the IAPs in the context of mitochondrial antiviral signalling, canonical NF-κB activation, and necroptotic cell death.

Experimental Procedures

Materials

siRNA directed against human and mouse Smac and corresponding control RNAs were all Stealth siRNAs purchased from Invitrogen Canada Inc. siRNA targeting two distinct regions of the mRNA were chosen and typically used in parallel experiment together with matched controls.

Cell cultures and transfections

Cell lines: All cell lines were maintained at 37°C and 5% CO2. 293T cells were maintained in DMEM (Hyclone) supplemented with 10% BCS, 2mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin. HCT116 and HT29 cells were in McCoy's Media (Wisent) supplemented with 10% FBS, 2mM L-glutamine and 100 Units/ml penicillin and 100 μg/ml streptomycin. MDA-MB-231 and MEFs were maintained in DMEM supplemented with 10% FBS and , 2mM L-glutamine,100 Units/ml penicillin and 100 μg/ml streptomycin.

Lipofectamine transfection: For knockdown experiments, cells were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturers protocol.

Calcium phosphate transfection of HEK293T cells: 10^6 HEK293T cells were

seeded on 10 cm plates on the day before transfection. For the transfection mix, 5µg of DNA was diluted in 250µl TE, to which 250µl of CaCl2 was added. 500µl of 2X HBS was added dropwise, and the mixture was incubated for 20 minutes at room temperature. 18 hours following transfection, the media was replaced, and cells were harvested 48 hours after transfection.

MEF generation

Fibroblasts were prepared from E12. 5 wild-type mouse embryos. Embryos were dissected in cold PBS. Heads, membranes and internal organs were removed and the remaining tissue was washed and then dissociated with trypsin. Embryos were further dissociated using a razor blade, and incubated for 20 minutes at 37°C. The trypsinization was stopped by adding DMEM medium supplemented with FBS, and the obtained solution was filtered using 40µM cell strainer to remove any clumps. The cells were plated on 10cm plates.

Luciferase Reporter Assay

1 x 10^6 293Tcells were seeded in 10cm plates and tranfsected 16h later with siRNA targeting Smac/DIABLO or non-specific siRNA by lipofectamine 2000 (Invitrogen). The next day, 1 x 10^3 cells were split into 96 well plates. These cells were transfected with luciferase reporter plasmids and mammalian expression plasmids, also using lipofectamine 2000. To normalize transfection efficiency, 100ng of Renilla luciferase plasmid was added to each transfection mix. Approximately 24h after transfection, Lucifer's assays were

performed using the dual-glo luciferase assay kit (Promega). Firefly luciferase activities were normalized based on Renilla luciferase activities.

Western Blot Analyses

Equivalent sample volumes were loaded onto 8%, 10% or 12% SDS-PAGE gels to resolve proteins. Following transfer, nitrocellulose membranes (GE Water & Process Technologies, Trevose, PA) were blocked at room temperature for 1 hour. Phospho-specific primary antibodies were blocked using 2% BSA diluted in TBS-T (20 mM Tris, pH 7. 6, 140 mM NaCl, 0. 1% (v/v) Tween-20) to reduce non-specific binding; all other antibodies were blocked with 5% dry skim milk powder diluted in TBS-T. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Membranes were washed in TBS-T and incubated with HRP-conjugated secondary antibodies diluted in 5% milk/TBS-T for 60-90 minutes at room temperature. Blots were washed again in TBS-T. Immunoreactive bands were detected using enhanced chemiluminescence (PerkinElmer Life Sciences; Emeryville, CA). The following antibodies were used : mouse anti-actin (clone C4; Fisher), mouse anti-Smac (BD Biosciences), rabbit anti-Smac (Calbiochem), rabbit IkB α (Santa Cruz), mouse anti-XIAP (BD Biosciences), goat anti-cIAP1 (R&D), goat anti-cIAP2 (R&D) and rabbit anti-cIAP1/2 (a gift from Dr. Robert Korneluk, University of Ottawa).

Cell Viability Assays

$2x10^4$ cells per well were seeded in opaque 96-well plates. The next day cells were treated as indicated with various concentrations of TNF (R&D systems, 20μM zVAD-fmk (R&D systems), 10μM Nec-1 (Tocris) and 20nm Smac mimetic AEG730 for 24h. Cell viability was determined by measuring the intraceullar levels of ATP using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturers instructions. Viability was graphed relative to control cultures. Luminescence was detected by microplate reader.

Mitochondrial Fractionation

L929 cells were grown to 90% confluency on 150mm plates. Following indicated treatments, cells were washed with PBS and scraped into eppendorf tubes. Cells were spun at 1000g for 5 minutes and resuspended in mitobuffer (20mM Hepes pH 7. 4, 220mM mannitol, 68mM sucrose, 76 mM KCl, 4mMKAc, 2mM $MgCl_2$ 0. 5mM EGTA, with protease inhibitor tablet). Cells were lysed using a 25-gauge syringe. The lysate was spun down at 1000g for 5 minutes and transferred to a fresh tube, and spun at 10000g for 10 minutes. The supernatant (cytosolic fraction) was transferred to a new tube, and the pellet (mitochondria) was resuspended in mitobuffer, and re-pelleted. The mitochondrial fraction was resuspended in mitobuffer + 10% glycerol.

DSS Colitis

For the acute colitis experiment, C57BL/6 and Smac-deficient mice, were given 4% Dextran Sodium Sulphate (MW 36,000-50,000; MP Biomedicals) in drinking water for 6 days (days 0–5) and then placed on regular water for 3 days (days 6–8). For sustained colitis, mice were given 3% DSS in drinking water for 8 days. Mice were monitored daily for body weight loss.

Histopathological Analysis and Immunohistochemistry Colons were fixed in 10% formalin and embedded in paraffin. Serial 5-mm- thick sections were cut onto glass slides and stained with haematoxylin and eosin (H&E). Sections were assessed for loss of crypts, an indicator of colitis.

Statistical Analysis Statistical differences were calculated GraphPad Prism software. Two-way ANOVA or unpaired, two-tailed Student's T-tests were used as indicated.

Results

Smac in mitochondrial antiviral signalling

IAPs have emerged as regulators of the immune response to RNA viruses. After RNA viruses are detected by cytosolic receptors, RIG-1 activates MAVS on the mitochondria, causing it to aggregate. MAVS recruits a variety of proteins, including TRAF3, TRAF6, cIAP1 and cIAP2. cIAP1 and cIAP2 mediate ubiquitination of TRAF3 and 6, which is believed to allow for their interaction with downstream kinases essential for propagating the antiviral response. Reduction of cIAP1 or cIAP2 by knocking down these proteins or by Smac mimetic treatment inhibits the antiviral response. XIAP has also been implicated in signalling downstream of MAVS.

Given the roles for both cIAP1 and cIAP2 in propagating the antiviral response, we sought to evaluate the potential role for their endogenous inhibitor, Smac. We hypothesized that Smac inhibition of cIAP1 and cIAP2, by promoting their autoubiqutination and proteasomal degradation, would attenuate the antiviral response.

Antiviral signalling can be elicited by overexpression of constitutively active RIG1 (ΔRIG1), or overexpression of the MAVS protein in 293T cells. Activation of signalling can be easily detected by co-transfection with IFN-β and IRSE promoter reporter constructs. Mao et al. (2010) showed that overexpression of MAVS or ΔRIG1 activates reporter activity, and that knockdown of cIAP1 and cIAP2, alone or in combination, inhibits activation of these promoters. They had similar results when using Smac mimetic,

which depletes the cells of cIAP1 and cIAP2. We hypothesized that knocking down Smac would have the opposite effect of cIAP1 or cIAP2 knockdown, because inhibition of cIAP1 and CIAP2 would be released. With cIAP1 and cIAP2 free from inhibition, we predicted that there would be increased activation of both IFN-β and IRSE reporter activity.

Consistently, we found that overexpression of ΔRIG1, or MAVS led to activation of both IFN-β (Figure 1A) and IRSE reporter activity (Figure 1B). Additionally, Smac mimetic treatment, which inhibits cIAP1 and cIAP2, significantly inhibited activation of IFN-β and IRSE reporter activity. Smac knockdown did not enhance activation of either reporter when ΔRIG1, or MAVS was overexpressed. In fact, in opposition to our hypothesis, Smac knockdown decreased activation of the IFN-β reporter when MAVS was overexpressed (Figure 1A). This suggests Smac might promote activation of IFN-β through an alternative pathway downstream of MAVS. However, no significant change in IFN-β activity downstream of ΔRIG1 was observed, and we saw no significant changes in IRSE reporter activity downstream of ΔRIG1 or MAVS, and I concluded that Smac does not play a major role regulating mitochondrial antiviral signalling.

Smac in Canonical NF-κB Signalling

In the canonical NF-κB pathway, the cellular IAPs promote NF-κB activation. NF-κB goes on to induce transcription of genes involved in inflammation and proliferation. Additionally, XIAP has been shown to play a role in promoting

a second wave of NF-κB activation. Given the role for these IAPs in the canonical NF-κB signalling pathway, we sought to uncover a role for their endogenous inhibitor, Smac, in the process.

Activation of canonical NF-κB requires phosphorylation and proteasomal degradation of IkBα, an NF-κB inhibitor. Degradation of IkBα frees the NF-κB dimer from inhibition, allowing it to translocate to the nucleus where it will then activate transcription of target genes. Activation of canonical NF-κB can be elicited by TNFα treatment. To determine if Smac inhibits canonical NF-κB activation, we performed a TNFα time course in two different cell lines, 231s (Figure 2A) and MEFs (Figure 2B) where cells were treated with non-specific or Smac siRNA. We expected that Smac knockdown would free the cIAPs and XIAP from inhibition and therefore enhance and prolong NF-κB activation. Degradation of IkBα was assessed by western blot, and observed after 15 minutes of TNFα stimulation in both cell lines. No considerable change in the time course of IkBα degradation was observed when Smac was reduced by knockdown. I concluded that Smac does not play a major role in the regulation of canonical NFκB signalling.

Smac in Necroptosis Signalling

Smac promotes necroptosis in L929 cells

Members of the IAP family are involved in the inhibition of not only apoptosis, but also a newly described form of programmed cell death, necroptosis(He et al., 2009, Vanlangenakker et al., 2011). In light of this, we

sought to test if there is a role for endogenous Smac in the regulation of necroptosis. The L929 cell line has been used to study necroptosis. These cells express RIP3 and are very sensitive to TNFα induced cell death.

As a first approach, we confirmed that L929 cells undergo cell death after 24h treatment with TNFα, and that cell death occurs even when caspases are inhibited with the pan-caspase inhibitor zVAD-fmk (Figure S2). A functional definition of necroptosis is cell death that can be inhibited with nec-1, a specific inhibitor of RIP1 kinase activity; TNFα induced cell death in L929 cells is strongly inhibited by nec-1 treatment.

Smac mimetics have been shown to enhance both apoptosis and necroptosis. We confirmed that the Smac mimetic AEG730, in addition to enhancing apoptosis, as in the HCT116 cell line (Figure S3), also enhances necroptosis in L929 cells. HT29 cells, like L929 cells, have been used in studies of necroptosis. These cells have been shown to be sensitized to necroptosis by Smac mimetic treatment. We confirmed that treatment of HT29 cells with AEG730 sensitizes this cell line to necroptosis, while AEG730 alone does not affect cell viability (Figure 2B).

To address whether endogenous Smac might play a role in necroptosis, L929 cells were transfected with siRNA targeting Smac 48 hours prior to necroptosis induction. Cells with Smac knockdown were resistant to necroptosis induced by TNFα compared to control cells. This result indicates Smac contributes to necroptosis signalling in L929 cells.

Smac is present in the cytosol of L929 cells

Smac is normally sequestered in the mitochondria but released during apoptosis. In order to determine how Smac might be acting in necroptosis, we first sought to address the localization of Smac in L929 cells. One possibility is that necroptosis signalling downstream of the TNF receptor leads to Smac release from the mitochondria as in apoptosis. In order to determine if there is flux of Smac from the mitochondria during necroptosis, mitochondrial and cytosolic fractions of L929 cells were examined in the presence and absence of necroptosis-inducing treatments. L929 cells were treated with vehicle, or with TNFα and zVAD-fmk, for 2h, to induce necroptosis. Additionally, cells were also treated with TNFα and zVAD-fmk in the presence of Nec-1 to inhibit necroptosis signalling. When mitochondrial and cytosolic fractions from L929 cells were examined for Smac, it was clear that Smac was present in both fractions. Surprisingly, it was evident that there was no flux of Smac from the mitochondria as a consequence of treatment, rather, it seems that in L929 cells Smac is constitutively present in the cytosol.

***Smac -/-* mice are not resistant to DSS induced colitis, but have a more severe phenotype**

As noted above, Smac null mice have no overt phenotype; this might be due to the fact that Smac mice have been examined for apoptotic phenotypes, rather than necroptotic ones. To determine the necessity of

Smac for necroptosis in vivo, the Dextran Sodium Sulphate (DSS) -induced colitis model was used.

Feeding mice DSS is a model of experimental intestinal inflammation, inducing colitis. DSS colitis is characterized by bloody diarrhea, ulcerations and inflammatory infiltration of the colon(Wirtz et al., 2007). Necroptotic cell death contributes to this phenotype; administration of necrostatin-1 is protective against DSS-induced injury. Indeed, DSS colitis was recently used to demonstrate that caspase-8 regulates TNF induced epithelial necroptosis in the colon(Gunther et al., 2011). Interestingly, this study also found a potential role for necroptosis in the pathogenesis of Chron's disease. They found high levels of RIP3 in human paneth cells, and increased necroptosis in the terminal ileum of patients with Chron's disease.

We predicted that *Smac -/-* mice would have a less severe DSS induced colitis compared to wild type mice, demonstrating a physiological role for Smac. To assess this, wild type and Smac -/- mice were subjected to sustained (Figure 5) and acute (Figure 6) DSS colitis. The animals were assessed by measuring body weight daily over the course of treatment. Histopathology of the colon was examined; the length of damage along the colon, resulting in loss of basal crypts, was measured and compared to the total length of the colon. No significant changes were found when comparing histology. Colitis can cause shrinking of the colons, no significant differences between wild-type and *Smac -/-* mice were observed. However, after sustained DSS treatment of 8 days, *Smac -/-* mice had lost more weight than

wild-type mice. This was even more evident after acute DSS treatment, *Smac* -/- mice had lost significantly more weight by day 5 and this difference was maintained until the end of the experiment at day 8. Weight loss after DSS colitis occurs because damage to the intestinal walls reduces the ability of the animal to absorb nutrients, and because diarrhea causes food to move faster through the body, allowing less time for food to be absorbed. *Smac* -/- mice lost more weight, indicating that they were more susceptible to the effects of DSS induced colitis. While this was the opposite of what we predicted from the in vitro data, the differences in weight loss here were substantial, and the mechanisms responsible for this requires further investigation.

Chapter 3: Discussion and Future Directions

Functional Redundancy of IAP inhibitors

While Smac may play a role in inhibiting the IAPs in many signalling contexts, the lack of an overt phenotype might be explained by functional redundancies between Smac and other IAP inhibitors. Known IAP inhibitors include Omi, ARTS, and IKKε.

Omi is a serine protease that, like Smac, is released from the intermembrane space of the mitochondria during apoptosis. Omi possesses an IBM allowing it to interact with XIAP, cIAP1 and cIAP2, cleave these IAPs and relieve caspase inhibition(Srinivasula et al., 2003, Yang et al., 2003). While overexpression of Omi leads to apoptosis, mice lacking Omi like *Smac-/-* mice, do not exhibit reduced rates of cell death. Unlike *Smac-/-* mice, these animals have an overt phenotype; these mice have a neurodegenerative disorder with a Parkinsonian phenotype due to a loss of striatal neurons (Martins, Morrison et al. 2004). Simultaneous deletion of both Smac and Omi does not alter the phenotype of these mice, indicating that Omi mediated cleavage of other proteins, and not specifically the IAPs is what results in striatal neurodegeneration.

ARTS is a septin that binds to XIAP and promotes its proteasomal degradation(Gottfried et al., 2004) . Unlike Smac and Omi, ARTS does not have an IBM, but binds to the BIR3 domain via unique sequences(Reingewertz et al., 2011). Translocation of ARTS from the mitochondria to the cytosol during apoptosis has been shown to occur ahead

of the mitochondrial outer membrane permeabilization, before release of cytochrome C and Smac (Edison et al., 2012) . Interestingly, deletion of *Sept4*, the gene encoding ARTS, in mice, results in elevated levels of XIAP protein, increased numbers of hematopoietic stem and progenitor cells and increased resistance to cell death in these cells. These mice also exhibited spontaneous development of hematopoietic malignancies(Garcia-Fernandez et al., 2010). Defects in more differentiated cells were not observed, suggesting that the role of XIAP is non-redundant at the progenitor stage but not later on. ARTS does not lead to cIAP1 degradation, so its role in inhibiting the IAPs in roles beyond apoptosis would be limited to the functions of XIAP.

Recently, the kinase IKKε has been implicated in XIAP inhibition. In drosophila, dmIKKε kinase phosphorylates the drosophila homolog of XIAP, DIAP1, activating its autoubiquitination and degradation (Kuo et al., 2006, Kuranaga et al., 2006). This has been shown to occur in mammalian cell lines, IKKε phosphorylates XIAP and causes its proteasomal destruction(Nakhaei et al., 2012). Because IKKε is a cytosolic protein, it is a good candidate for mediating inhibition of the IAPs in non-apoptotic signalling contexts.

Smac and MAVS signalling

When we examined Smac in the context of mitochondrial antiviral signalling we did not see striking effects. While Smac mimetic treatment, which inhibits the IAPs, led to decreased antiviral signalling, downregulating Smac with siRNA did not show the increased activation of IFN-β and IRSE we predicted.

It is possible that this is due to functional redundancy between other IAP inhibitors like Omi, ARTS or IKKε.

Smac did effect IFN-β signalling downstream of MAVS overexpression, but instead of increasing activity of the reporter, as would be expected if Smac was inhibiting cIAP1 and cIAP2 in the pathway, reporter activity was decreased. One possibility is that Smac impacts signalling downstream of MAVS in an IAP independent manner. One group, looking for interactors of TBK1, identified Smac as one of many components in a TBK1 complex (Goncalves et al., 2011). TBK1 is an important regulator of interferon production, and is activated downstream of MAVS. While the relationship between TBK1 and Smac was not the focus of this study, it is possible that Smac interaction with TBK1 is important for its activation, and this would explain why Smac downregulation might decrease IFNβ interaction. Further exploration of the relationship between TBK1 and Smac might reveal a novel IAP-independent role for Smac.

Smac and NF-κB signalling

We examined Smac in the context of canonical NF-κB signalling. TNFα treatment led to degradation of IkBα in both MDA-MB-231 cells and MEFs. Smac downregulation did not alter the course of this degradation in either cell line. Phosphorylation of p65 was also examined and no significant differences were seen when Smac was downregulated (data not shown). The

lack of phenotype might be due to functional redundancy between Smac and the other IAP inhibitors, like ARTS or Omi.

Loss of XIAP leads to a second wave of NF-κB activation. The second wave of this activation involves IkBβ, rather than IkBα (Winsauer et al., 2008). An effect of Smac might be more evident if degradation IkBβ is examined; Smac might be involved in promoting a second wave of activation by inhibiting XIAP.

We only examined Smac in the context of canonical NF-κB signalling. It is possible that a role for Smac might be elucidated in the context of non-canonical Nf-κB signalling. This could be accomplished by examining TNFα treated control and Smac-depleted cells for changes in NIK protein levels.

Smac and Necroptosis

We examined the possibility that Smac plays a role in necroptosis and found that Smac knockdown in L929 cells was protective against necroptosis (Figure 3). Interestingly, we found that Smac is constitutively in the cytosol of L929 cells. Many studies have examined the localization of Smac, and have detailed the kinetics of Smac release from the mitochondria after mitochondrial outer membrane permeabilzation during apoptosis (Springs et al., 2002, Munoz-Pinedo et al., 2006, Bhola et al., 2009). These studies examined overexpressed, fluorescent-tagged Smac, rather than endogenous Smac, and thus might not represent what is really happening within the cell. One possibility is that constitutive Smac flux might occur in L929 cells, as

previous studies have been limited to HeLa cells (Springs et al., 2002, Munoz-Pinedo et al., 2006, Bhola et al., 2009). Another possibility that is that an alternatively spliced variant of Smac, like Smac-β, that lacks a mitochondrial targeting signal, accounts for the Smac we observe in the cytosol in L929 cells. Smac-β has been indentified in human cell lines but a mouse Smac-β has not been described. We found that Smac-β is expressed in HT29 cells, a human cell line that also undergoes necroptosis (Figure S4). We also performed mitochondrial fractionation in this cell line to examine the localization of Smac protein but had variable results (data not shown).

Addressing how Smac-β could contribute to necroptosis would be an important next step. This could be accomplished by using overexpression constructs of cytosolic Smac with a mutated AVPI. While we were to effectively overexpress Smac constructs in HEK293T cells (Figure S5), our attempts to overexpress Smac in L929 cells using transfection techniques or tiviral transduction were unsuccessful (data not shown). Creation of stable L929 cells overexpressing these constructs might be a useful approach to address the role of Smac-β in future experiments.

There are many unknowns in the necroptosis signalling pathway. The effectors that link RIP1 and RIP3 phosphorylation to downstream increased energy metabolism and reactive oxygen species are only beginning to be identified. Two substrates of RIP3 phosphorylation essential for necroptosis, MLKL and PGAM5, were just recently identified (Sun et al., 2012, Wang et al., 2012, Zhao et al., 2012). Increased phosphorylation of ERK has been reported

to occur downstream of TNFα induced necroptosis in L929 cells (Vanlangenakker et al., 2011). We confirmed that phospho-ERK levels in L929 cells were increased after two hours of necroptosis induction. However, we did not see a significant change when Smac was downregulated by siRNA (data not shown). This indicates ERK phosphorylation is upstream of Smac activity.

We also examined the effect of Smac knockdown in HT29 cells, and had variable results. An added layer of complexity in the context of the HT29 cells is that these cells do not undergo programmed necroptosis except when these cells are treated with Smac mimetic. While they express Smac-β, we do not know if this transcript is actually translated.

We used DSS colitis as an in vivo model of necroptosis. We expected that *Smac -/-* would be protected from necroptosis induce by DSS but found the opposite to be true. These mice lost more weight than wild-type mice after acute and sustained colitis. It is possible that Smac might be protective against colitis injury due to an IAP independent mechanism. Further investigation of Smac in colitis is necessary.

In two different situations downregulation of Smac led to an outcome that was the opposite of our hypothesis. Smac knockdown, like IAP downregulation by Smac mimetic treatment, led to a decrease in IFN-β reporter activity when MAVS was overexpressed. *Smac -/-* mice had an increased susceptibility to DSS colitis compared to wild-type controls. One possibility is that Smac might be required to enhance to IAP function in these

contexts rather than inhibit them. Smac inhibits the cIAPs by activating their autoubiqutination, leading to their K48 ubiquitination and proteasomal destruction(Yang and Du, 2004). If Smac promotes not just the autoubiqutination of the cIAPs leading to their proteasomal degradation, but also promotes their ubiquitin ligase activity that leads to K63 ubiquitination of RIP1 and other substrates, Smac could facilitate cIAP function. Surprisingly, when Smac constructs were overexpressed in HEK293T cells, we did not significant reduction of cIAP1 or 2 (Figure S5). It might be interesting to examine the ubiquitin ligase activity of the cIAPs after Smac overexpression, as well as the ubiquitination state of cIAP substrates using K48 and K63 specific antibodies.

Is there more to Smac than AVPI?

While the tetrapeptide neo-epitope of Smac is responsible for its interaction with BIR domains of the IAPs, and necessary and sufficient for inhibiting these proteins, the length of the Smac protein is highly conserved throughout phylogeny. Importantly, Smac-β, which lacks the AVPI sequence, has been shown to sensitize cell to apoptosis, suggesting that Smac could function in IAP-independent contexts.

There are three possibilities for the importance of the rest of the protein. First, it may be important for Smac homodimerization, which has been shown to be essential for antagonizing the BIR2 domain of XIAP. Another possibility is that Smac can interact with proteins outside of the IAP

family through alternative residues. Smac might also have non-apoptotic roles inside the mitochondria.

Conclusion

In this study we sought to examine non-apoptotic signalling events regulated by the IAPs, including NF-κB induction, mitochondrial antiviral (MAVS) signalling, and necroptosis. We found revealed no obvious roles for Smac in context of MAVS signalling or NF-κB induction, but found that Smac contributes to necroptosis induction in L929 cells. While we hypothesized that Smac null mice would be protected from necroptosis induced by experimental colitis, we unexpectedly found that colitis was more severe in *Smac -/-* mice than in wild-type mice. Further investigation of how Smac is involved in the reaction to induced colitis is required to elucidate the mechanism of its action in this setting. Additionally, examination of IAP-independent roles for Smac might reveal further roles for Smac.

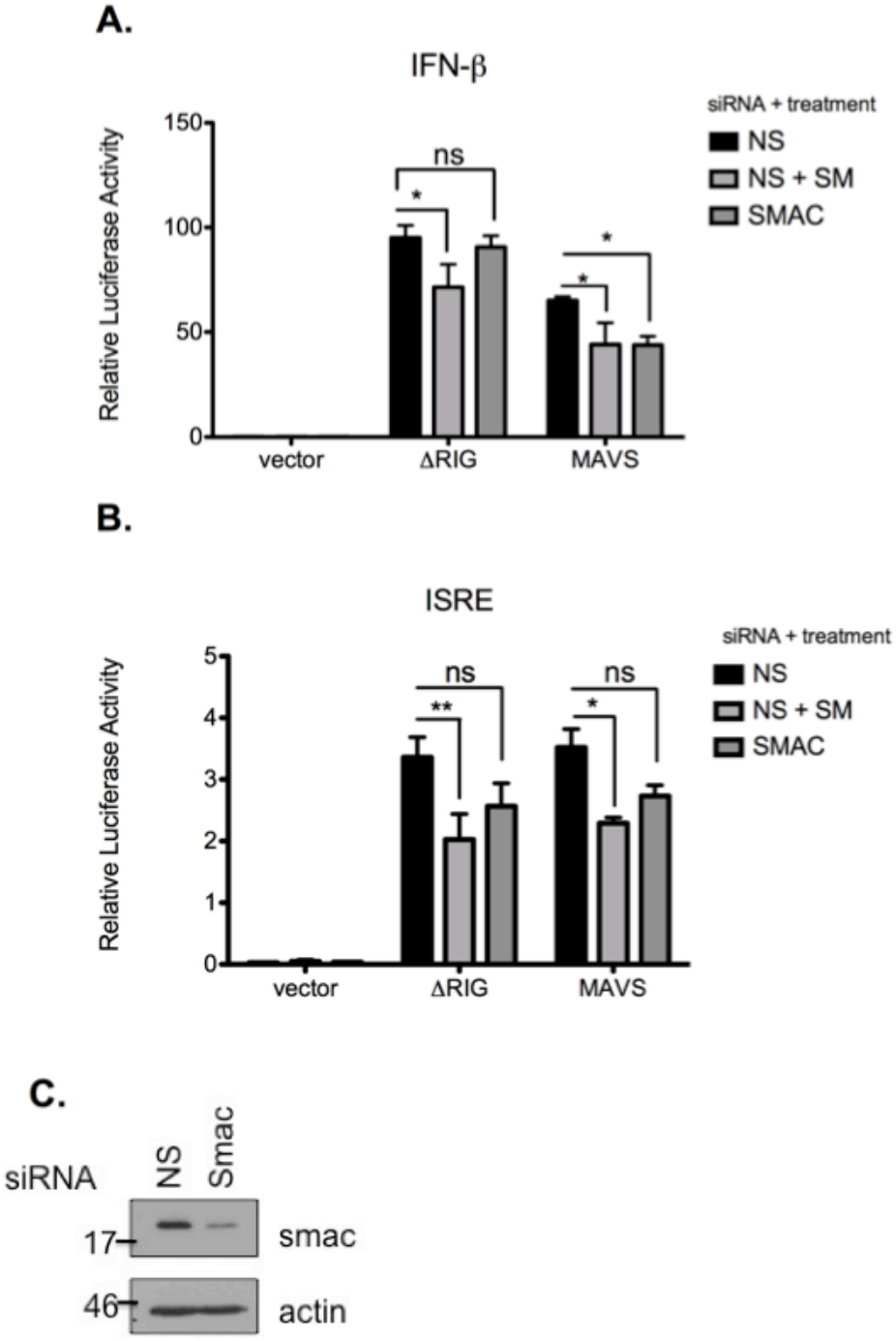


Figure 1. Smac mimetic treatment downregulates virus-induced signalling downstream of RIG1 and MAVS, but loss of endogenous Smac

does not enhance signalling. The effects of Smac mimetic (SM) and Smac knockdown on the activation of the IFN-β promoter (A) and IRSE (B) mediated by components of virus triggering signalling pathways were examined by reporter assays. HEK293T cells were transfected with non-specific or Smac siRNA. 24h later, the cells were split into a 96 well plate (1 x 10^3 per well). These cells were then further transfected with luciferase reporter plasmids and mammalian expression plasmids and also treated with Smac mimetic or vehicle. Approximately 24h later luciferase assays were performed. Renilla luciferase was used to normalize transfection efficiency. Efficiency of Smac knockdown is shown in (C) by western blot. "***" indicates p<0. 001, "**" indicates p<0. 01, and "*" indicates p<0. 05, analyzed by two-way ANOVA. Error bars represent SEM. Data shown is one representative result from two independent experiments.

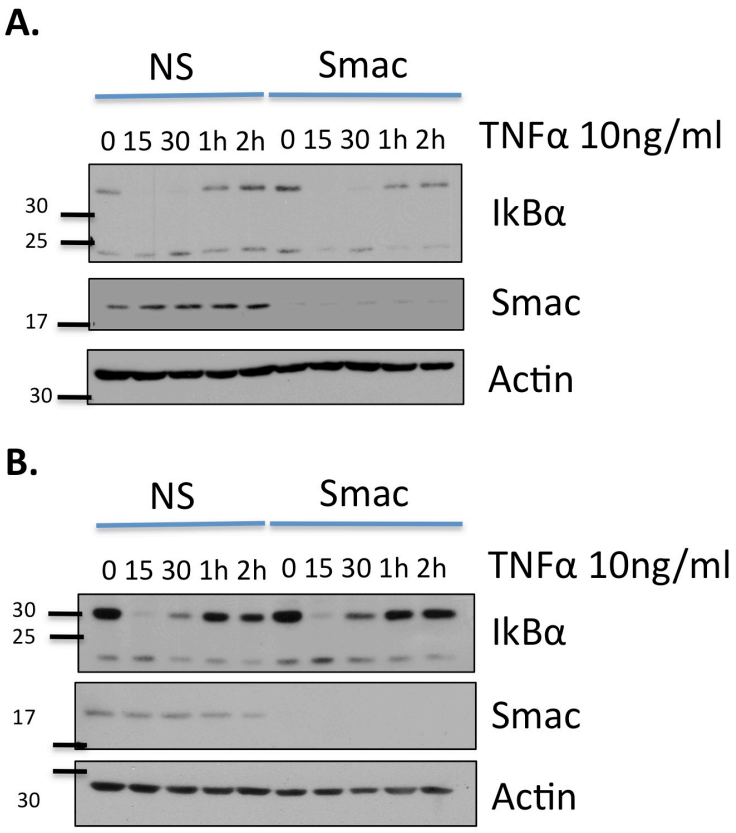


Figure 2. Smac depletion does not affect activation of canonical NF-κB signalling. 231 cells (A) and MEFs (B) were transfected with nonspecific (NS) or Smac siRNA. 48h later they were stimulated with TNFα for specified amounts of time. IKBα degradation was assessed by western blot and is shown in the top panel of (A) and (B) Efficiency of Smac knockdown is shown in the middle panels. One representative result from two independent experiments is shown.

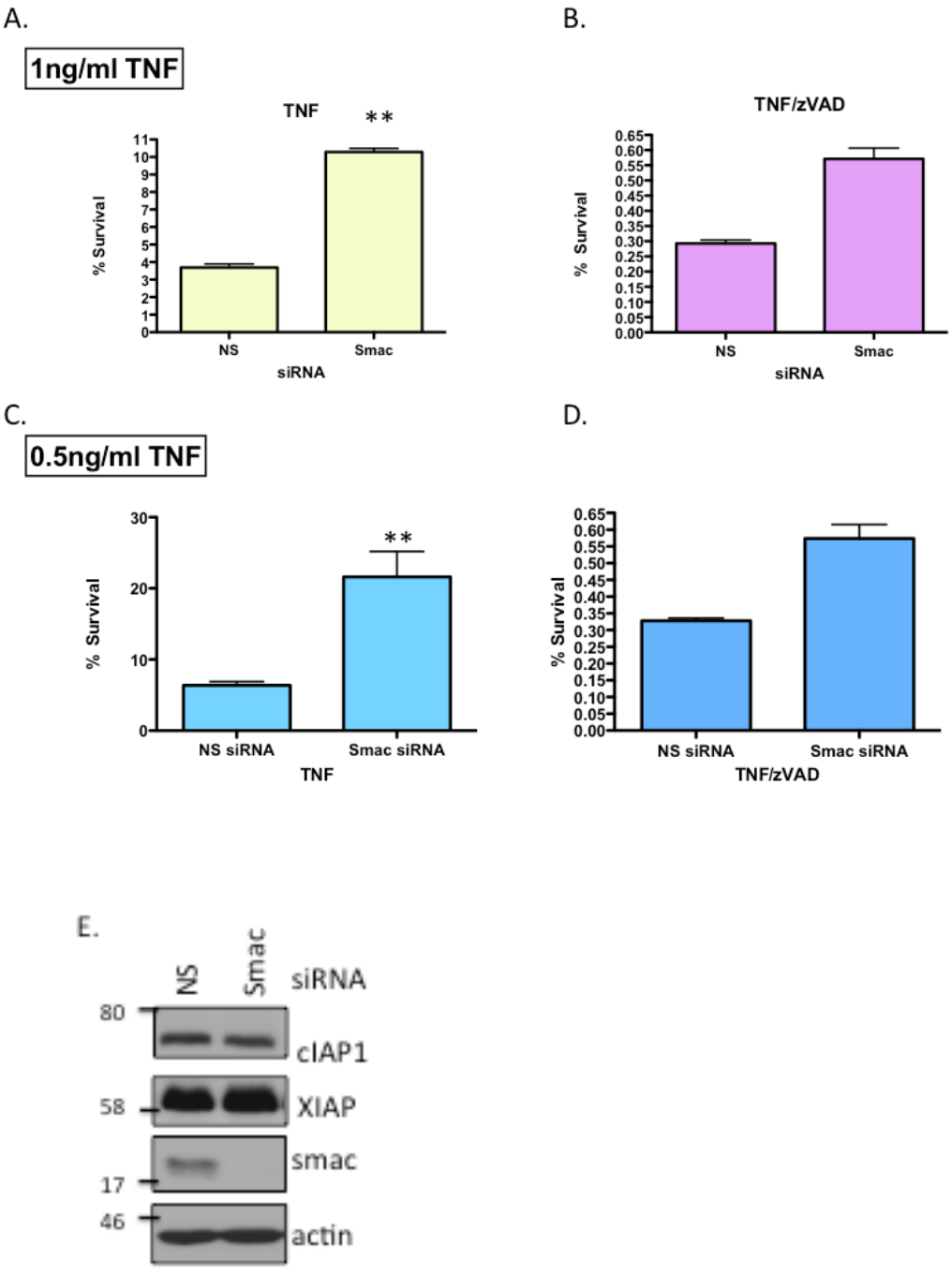


Figure 3. Smac knockdown reduces necroptosis in L929 cells.

L929 cells were transfected with control or Smac siRNAs. 48h post-transfection, cells were treated with vehicle and TNFα (A) and (C) or TNFα and zVAD-fmk (B) and (D). Two doses of TNFα were used, 1ng/ml in (A) and (B) and 0. 5ng/ml in (C) and (D). 24h after treatment, cell viability was determined by measuring ATP levels using the Cell-titer-glo kit. The knockdown efficiency of cell lysates was determined by western blot analysis of Smac and actin levels. IAP levels are also shown(E). Error bars represent SEM. ** = $p<0.01$ by two-way ANOVA. One representative result of three independent experiments is shown.

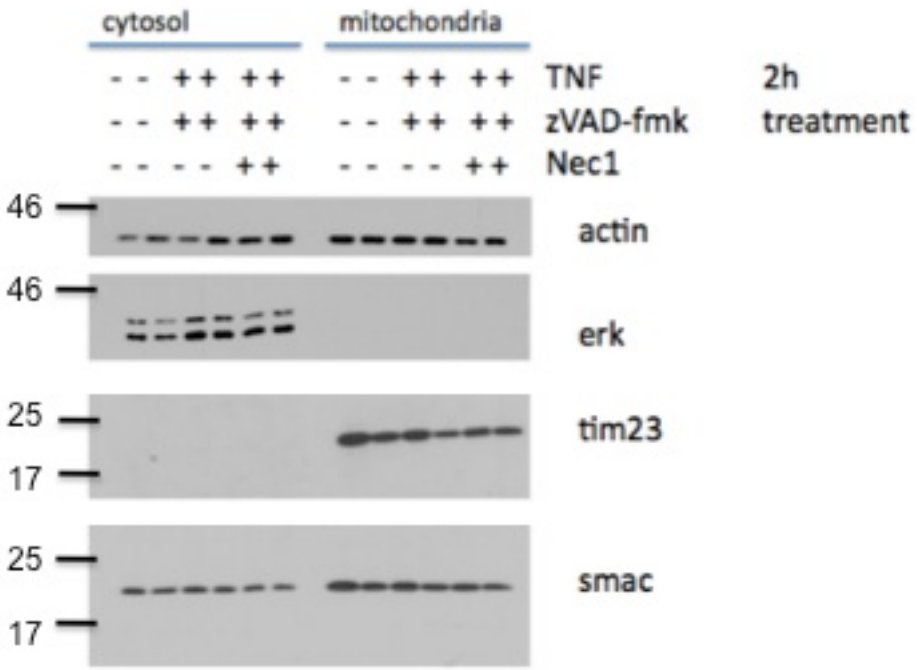


Figure 4 Smac is present in cytosolic fractions form L929 cells. L929 cells were treated with vehicle, TNF alpha and ZVAD, or TNF alpha and ZVAD with Necrostatin for 2 hours. After treatment, mitochondrial and cytosolic fractions were isolated and analysed by Western Blot. Tim23 served as a mitochondrial marker, and ERK was used as a cytosolic marker. One representative result of two independent experiments is shown.

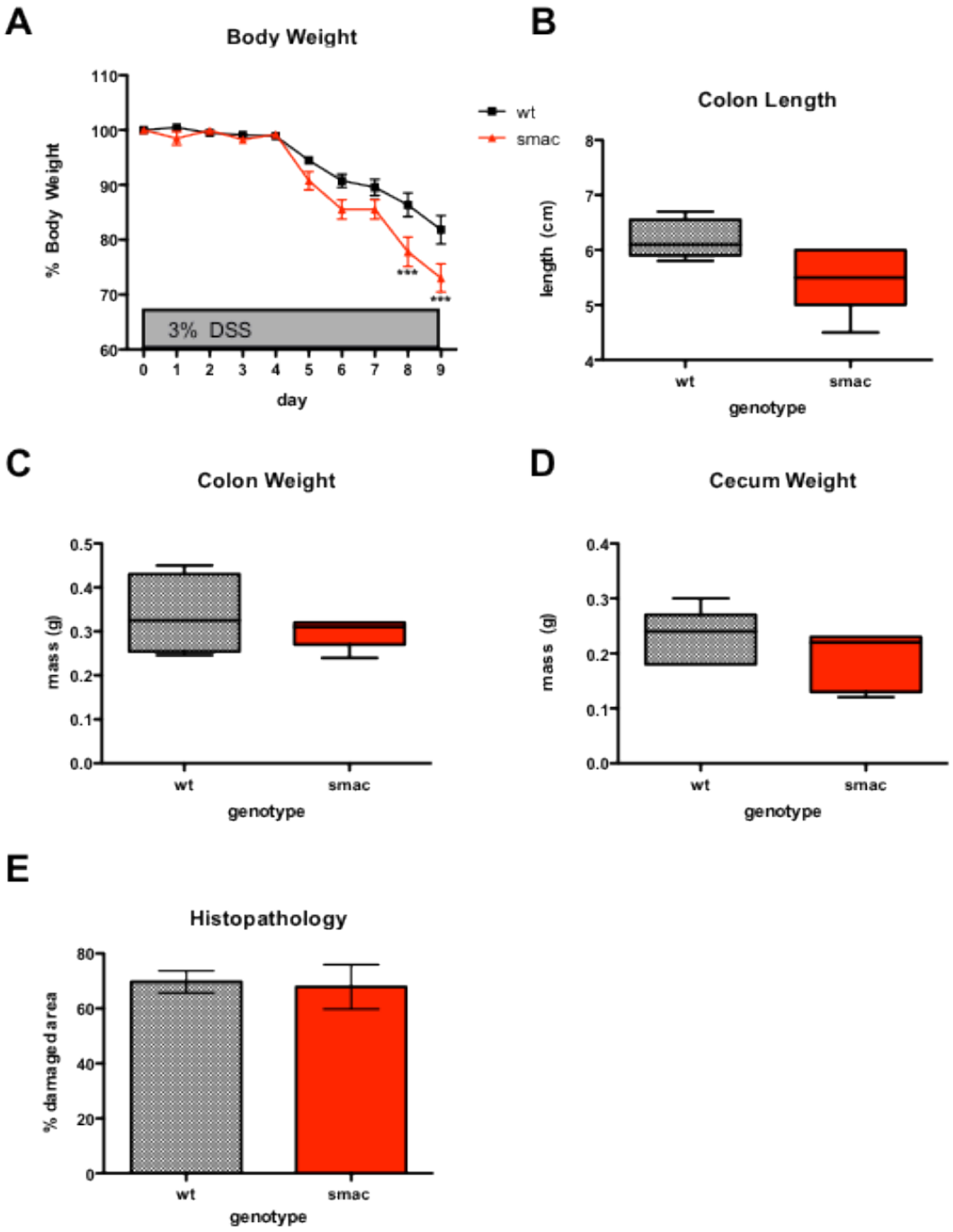


Figure 5 Smac null mice lose more weight than wild-type after sustained experimental colitis. Wild-type (n=5) and *Smac* -/- (n=5) mice were

treated with drinking water containing 3% DSS for 8 days. Mice were measured daily (A). Colon length (B), weight (C) and cecum weight (D) were assessed. Colons were fixed, embedded and stained with haematoxylin and eosin. Loss of crypts was used as an indicator of damage (E). Error bars represent SEM. "***" indicates $p<0.005$. Body weight was analyzed by two-way ANOVA, other measures were analyzed by Student's T-tests.

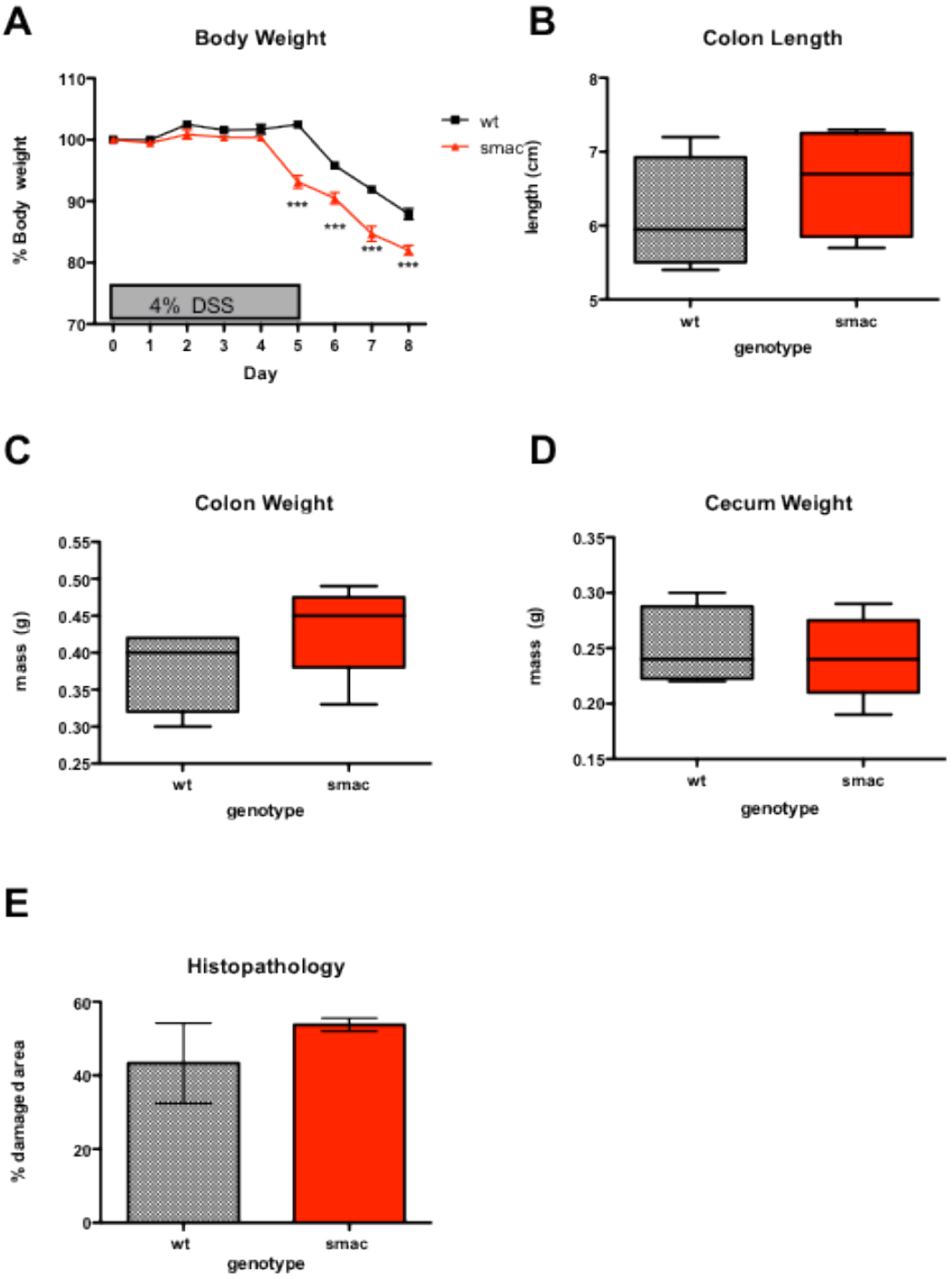


Figure 6. Smac null mice lose more weight than wild-type after acute experimental colitis. . Wild-type (n=4) and *Smac* -/- (n=5) mice were treated with drinking water containing 4% DSS for 5 days followed by 3 days

of regular drinking water. Mice were measured daily (A). Colon length (B), weight (C) and cecum weight (D) were assessed. Colons were fixed, embedded and stained with haematoxylin and eosin. Loss of crypts was used as an indicator of damage (E). Error bars represent SEM. "***" indicates $p<0.005$. Body weight was analyzed by two-way ANOVA, other measures were analyzed by Student's T-tests.

Supplemental Figures

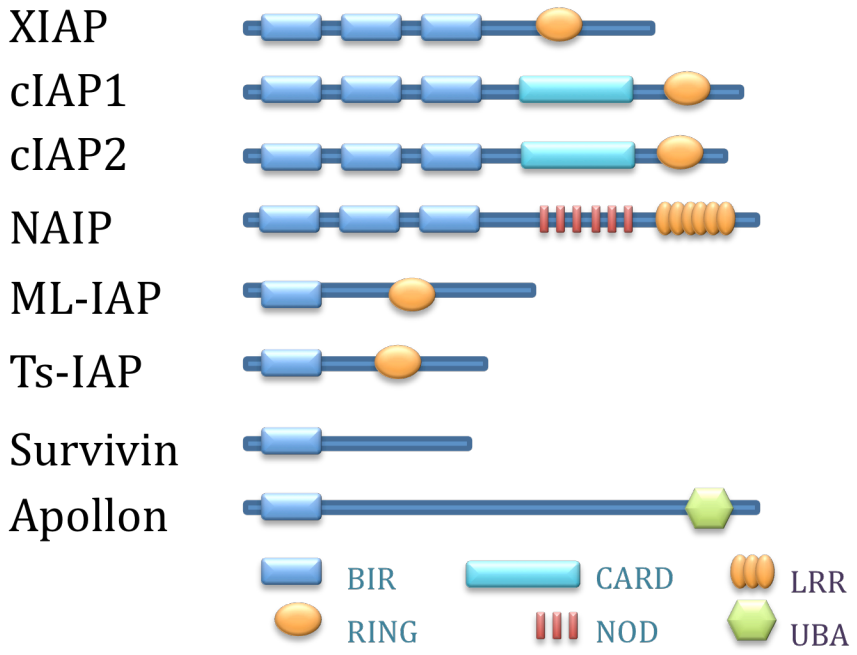


Figure S1. Schematic Representation of human IAP family proteins.

Adapted from (Srinivasula and Ashwell, 2008)

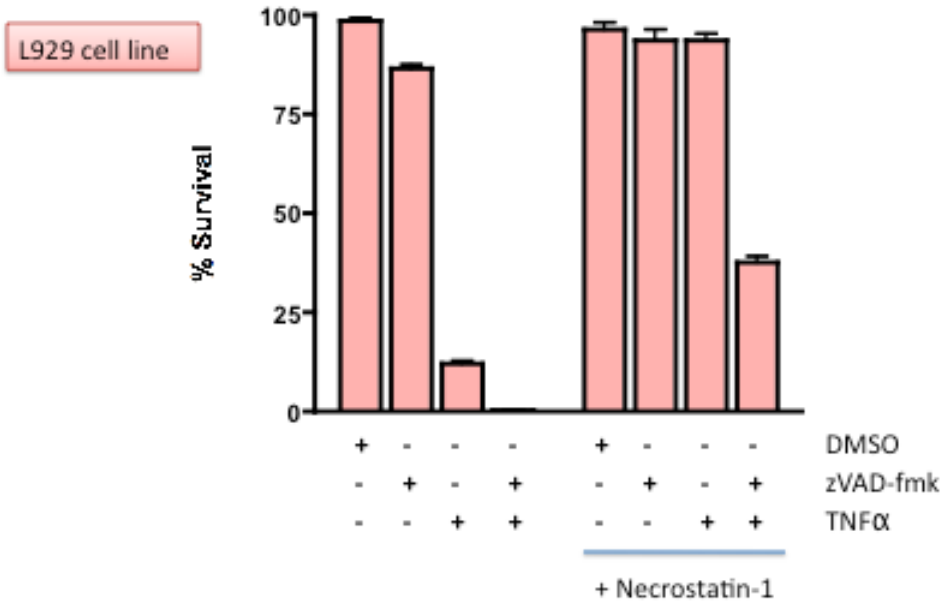
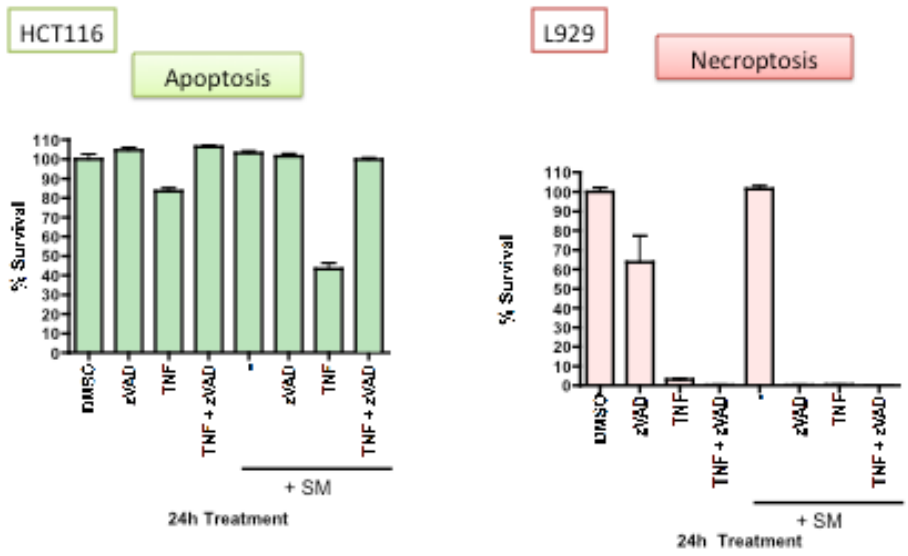


Figure S2. TNFα induces necroptotic cell death in L929 cells. Cells were treated with vehicle (0. 1% DMSO), TNFα, zVAD-fmk and Necrostatin-1 as indicated. 24h after treatment, cell viability was determined by measuring ATP levels using the Cell-titer-glo kit. Viability was graphed comparing luminescence to control cultures.



HT29

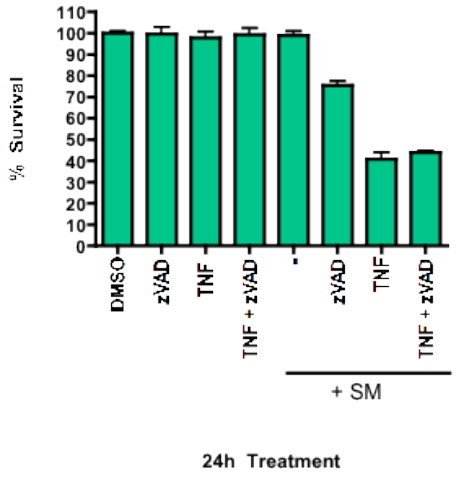


Figure S3. Smac mimetic sensitizes cells to apoptotic and necropotic cell death. Cells were treated with 20μM zVAD-fmk, 20ng/ml TNFα, and or 20nm of the Smac Mimetic (SM) AEG730 and assessed for cell viability by measuring ATP levels with the Cell-Titer Glo kit after 24h of treatment. AEG730 treatment alone does not result in a loss of cell viability in HCT116, L929 or HT29 cells. HCT116 cells were sensitized to apoptosis after SM

treatment death while HT29 cells were sensitized to necroptosis induced by TNFα after SM treatment. Necroptosis in L929 cells was exacerbated with TNFα treatment.

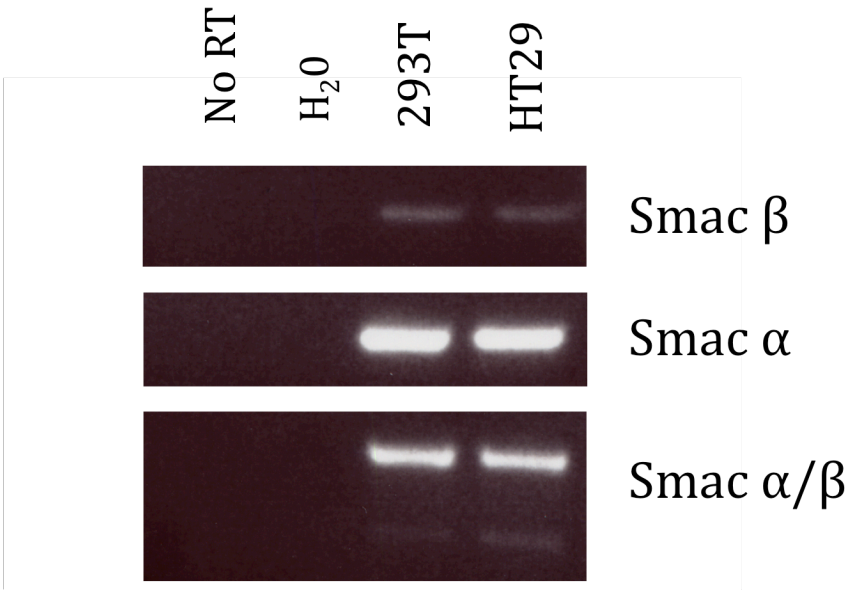


Figure S4. HT29 cells express Smac β. RNA was isolated from HEK293T and HT29 cells. RT-PCR was performed using the same 3' reverse primers for Smac-α and Smac-β but distinct 5' primers were used to detect each individually. Smac α and β were detected together using 5' primers to detect both.

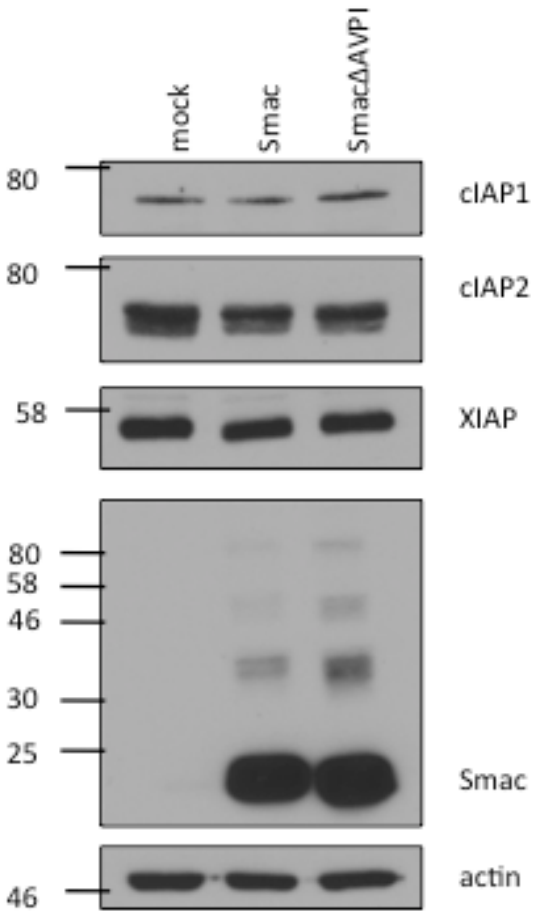


Figure S5. Overexpression of cytosolic Smac in HEK293T cells does not lead to a significant reduction in IAP levels. HEK293T were mock transfected, transfected with cytosolic Smac or transfected Smac with a deletion of the IAP binding domain (SmacΔAVPI). Cells were lysed 48h after transfection and analyzed by Western Blot.

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