# REGULATION OF INTRACELLULAR SIGNALING BY THE INHIBITOR OF APOPTOSIS (IAP) PROTEINS

by

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## DEDICATION

This dissertation is dedicated to my family and friends, who have shaped me, for better or worse, into the man I am today.

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## PREFACE

At the time of the submission of this dissertation, the data presented in Chapter II have been submitted to Oncogene for publication and are currently in press with the citation listed below.

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## ABSTRACT

Members of the TNF receptor superfamily, many of which are important for immune and inflammatory responses, have been shown to utilize ubiquitination for signal transduction. Two proteins, cellular inhibitor of apoptosis proteins 1 and 2 (c-IAP1/2), play central roles in the signaling cascades from these receptors through their ubiquitin ligase activity. In certain pathways, c-IAP1/2 actively propagate receptor-mediated signaling through the construction of ubiquitin scaffolds within the receptor signaling complex, while in other situations, c-IAP1/2 constitutively target substrate proteins for ubiquitination and degradation, inhibiting signal transduction. Activation of these signaling pathways is dependent on the degradation of c-IAP1/2, a process that is associated with stimulation of specific members of the TNF receptor superfamily. While the biological properties of c-IAP1/2 are context-dependent, the exact roles and outcomes of c-IAP1/2 activity in these different circumstances are unclear. In the work presented in this dissertation, these diverse roles of c-IAP1/2 in signaling were further explored, characterizing the signaling pathways in which they participate, investigating their regulation, and identifying the downstream consequences of their activity. Using multiple methods to study the function of c-IAP1/2, including activation of TNF receptor superfamily members and treatment with synthetic compounds that target and degrade c-IAP1/2, novel aspects of c-IAP1/2 activity in signaling were characterized. Specifically, the regulation of canonical and non-canonical NF-kappa B activation by c-IAP1/2 was investigated, finding that the consequences of c-IAP1/2 activity were cell type-specific.

Furthermore, important regulatory crosstalk between the NF-kappa B signaling pathways was identified in cells that activated both canonical and non-canonical NF-kappa B. Additionally, novel roles for c-IAP1/2 were identified using transcriptome analysis and a technique called Bru-seq, and these roles included the regulation of ribosomal gene expression and protein synthesis. These results may have important clinical implications, since the IAPs are actively being studied as potential therapeutic targets. Collectively, these findings expand our understanding of ubiquitin-dependent signaling and, more specifically, provide crucial insight into the multifaceted functions of c-IAP1 and c-IAP2 in key intracellular signaling pathways.

# CHAPTER I

## Introduction

### The Inhibitor of Apoptosis Protein Family

Control of cellular fate is vital for developing and maintaining a properly functioning immune system. Proper regulation of cell death is necessary for producing functional immune cell repertoires, eliminating infected cells, and contraction of the effector cell populations following termination of the immune response (Cohen and Duke, 1992). Additionally, evasion of cell death is a known characteristic of cancer and has been the focus of numerous therapeutic strategies (Hanahan and Weinberg, 2011). The study of cell death and its regulation led to the discovery and characterization of a family of factors called the inhibitor of apoptosis (IAP) proteins, and over the last few decades, the IAP protein family has been found to play major roles in a multitude of cellular processes. The first IAP was discovered in 1993 through a gene complementation assay (Crook et al., 1993) and was found to prevent baculovirus-induced cell death in insect cells. Furthermore, sequence analysis showed that the *iap* gene encoded for a zinc finger-like motif, dubbed a baculovirus IAP repeat (BIR) domain (Clem and Miller, 1994), and homology studies identified *iap* genes in the genomes of multiple species, including yeast, nematodes, fruit flies, and humans, indicating that these genes were highly evolutionarily conserved (Uren et al., 1999; Fraser et al., 1999; Hay et al., 1995;

Rothe et al., 1995; Uren et al., 1996; Roy et al., 1995; Liston et al., 1996; Duckett et al., 1996).

The mammalian IAP family consists of eight members, all of which share the family-defining BIR domain (Fig. 1.1), and while every IAP member possesses at least one BIR domain, many members contain multiple BIR domains. Three of the best characterized IAPs are x-linked IAP (XIAP), cellular IAP 1 (c-IAP1), and cellular IAP 2 (c-IAP2), each of which possess three BIR domains (Fig. 1.1) that are responsible for protein-protein interactions between the IAPs and other factors, such as caspases (Scott et al., 2005; Wilkinson et al., 2008). In addition to the BIR domains, XIAP and the c-IAPs contain a carboxy-terminal really interesting new gene (RING) domain, which confers E3 ubiquitin ligase activity and plays an important role in the functional activities of these proteins (Fig. 1.1). The c-IAPs also contain two additional domains: a caspase-associated recruitment domain (CARD) and an ubiquitin-associated (UBA) domain (Fig. 1.1). While their exact functions remain unresolved, the CARD may allow the c-IAPs to interact with additional CARD-containing proteins and appears to regulate their E3 ubiquitin ligase activity (Lopez et al., 2011), and the UBA domain has been shown to bind ubiquitin and has recently been found to facilitate recruitment of other factors involved in the ubiquitination process (Budhidarmo and Day, 2014).

Elevated expression of XIAP and c-IAP1 has been described in multiple cancer types, which has led to the targeting of the IAPs as a potential anti-cancer therapy. Additionally, high expression of certain IAPs has been correlated with poor survival rates in several cancers, including XIAP expression in colorectal cancer and diffuse large B cell lymphoma (Xiang et al., 2009; Hussain et al., 2010), c-IAP2 in pancreatic cancer and

colorectal cancer (Esposito et al., 2007; Krajewska et al., 2005), and c-IAP1 in cervical squamous cell carcinoma (Imoto et al., 2002). However, the precise role of the IAPs in the progression of cancer is still unclear and remains a focus of ongoing research.

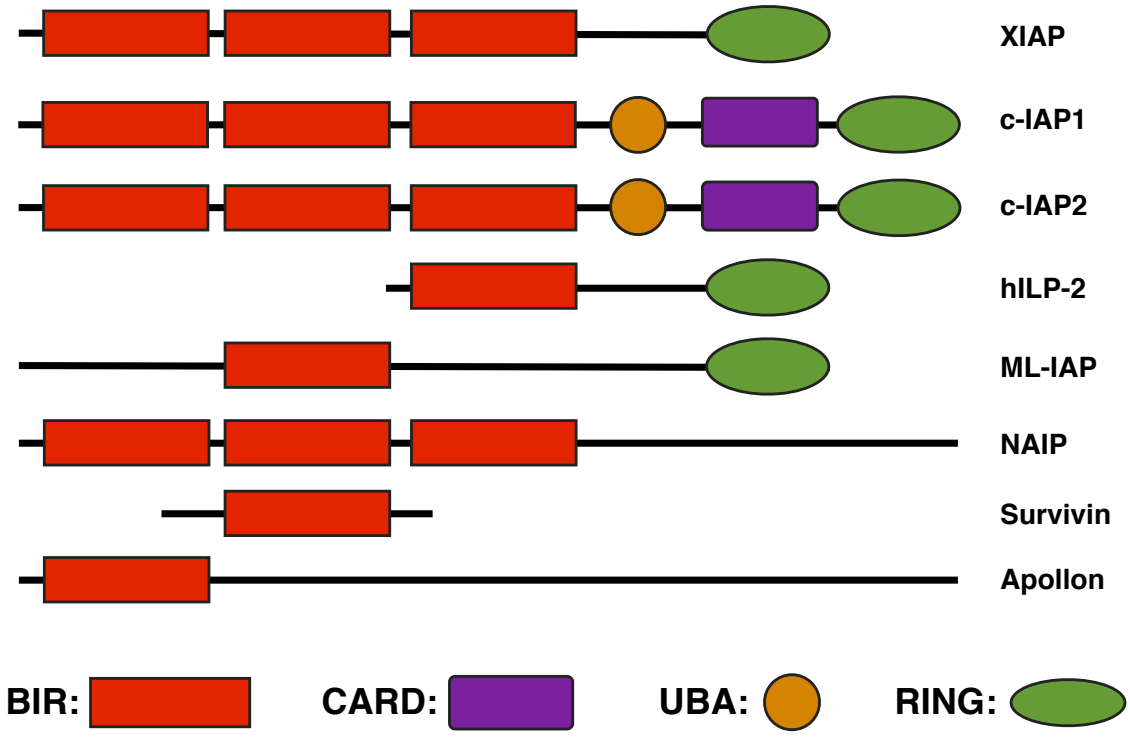


**Figure 1.1 Schematic depiction of the inhibitor of apoptosis (IAP) protein family.** BIR, baculovirus IAP repeat; CARD, caspase-associated recruitment domain; UBA, ubiquitin-associated domain; RING, really interesting new gene.

## IAPs and Regulation of Cell Death

The IAPs were originally described as regulators of cell death (Liu et al., 2000), a process that is vital in the development and maintenance of the immune system and its response to antigenic challenge. Furthermore, the ability to avoid cell death is a major characteristic of cancer (Hanahan and Weinberg, 2000). While many forms of cell death have been described (Ashkenazi and Salvesen, 2014; Danial and Korsmeyer, 2004), the

best-characterized type of cell death is apoptosis. Apoptosis, sometimes referred to as programmed cell death, is a highly regulated process that progresses through a series of clearly defined cellular events, including caspase activation, cell membrane blebbing, and fragmentation of chromosomal DNA, and can be initiated by a multitude of cues (Ashkenazi and Salvesen, 2014). Importantly, apoptosis, unlike other forms of cell death, is generally non-inflammatory, and this is because the process of apoptosis fragments the cell in a way in which the resulting apoptotic bodies are easily and quickly removed by phagocytic cells prior to the release of cellular components that can elicit an inflammatory response through a process called efferocytosis (Korns et al., 2011).

Apoptosis is important in the regulation of cancer progression and in normal development, including development and maintenance of the immune system, as well as during the immune response. For example, apoptosis is vital for the proper development of the T and B cell repertoire (Cohen and Duke, 1992), and cells that undergo nonproductive gene rearrangement and fail to produce a functional T cell receptor or B cell receptor are eliminated by apoptosis. Similarly, autoreactive cells are deleted by apoptosis during development and maturation (Marrack and Kappler, 2004). During the immune response, cytotoxic T cells and NK cells induce apoptosis in infected cells (Shresta et al., 1998), and following the clearance of pathogen, the expanded effector cell population is contracted through the induction of apoptosis (Cohen and Duke, 1992).

There are two major signaling cascades leading to apoptosis, the intrinsic and extrinsic death pathways, which are classified based on their distinct sources of lethal stimuli. The intrinsic cell death pathway is initiated by intracellular insults such as starvation, ultraviolet irradiation, chemotherapeutic drugs, and lack of necessary growth

factors (Tait and Green, 2010). Central to the intrinsic death pathway is the control of the structural integrity of the mitochondria. Major regulators of this event are the members of the B cell lymphoma-2 (Bcl-2) protein family, which can be categorized into two major groups: factors that promote apoptosis and those that inhibit apoptosis (Youle and Strasser, 2008). Following an apoptotic signal, the anti-apoptotic proteins Bcl-2 and Bcl-x long (Bcl-$x_L$) are sequestered by pro-apoptotic members of the Bcl-2 family, which allows the pro-apoptotic Bcl-2 proteins Bcl-2-antagonist/killer-1 (Bak) and Bcl-2-associated X protein (Bax) to oligomerize and insert themselves into the outer membrane of the mitochondria, forming a pore-like structure (Fig. 1.2) (Tait and Green, 2010). Once formed, these structures allow for the rapid release of components held in the inter-membrane space of the mitochondria, such as second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO), through an event known as mitochondrial outer membrane permeabilization, or MOMP (Tait and Green, 2010). Cytochrome c, a component of the electron transport chain, is also released from the mitochondria during this process (Ow et al., 2008), and once in the cytoplasm, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9 to form a structure called the apoptosome (Fig. 1.2). This complex activates caspase-9, which proceeds to cleave and activate the downstream effector caspases and subsequently results in cell death (Tait and Green, 2010).

The extrinsic pathway, in contrast, is death receptor-mediated, wherein a ligand binds a death receptor, such as Fas, on the surface of the cell. Binding of the trimeric ligand induces the trimerization of the receptor, which subsequently recruits the Fas-associated via death domain (FADD) protein. FADD, through its death domain (DD),

binds to DD within the cytoplasmic tail of the receptor (Park et al., 2007). Additionally, FADD contains a death effector domain (DED), which recruits pro-caspase-8 to the forming receptor signaling complex (Park et al., 2007). This FADD:caspase complex is oftentimes referred to as the death inducing signaling complex (DISC). In certain cells, termed Type I cells, the active caspases in the DISC proceed to directly cleave and activate downstream caspases, including caspase-3 and caspase-7, which then execute the cellular changes associated with apoptosis (Fig. 1.2) (Scaffidi et al., 1998). More commonly, however, cells require additional pro-apoptotic signals from the mitochondria. The extrinsic death pathway in these cells, called Type II cells, therefore overlaps significantly with signaling machinery of the intrinsic cell death pathway (Scaffidi et al., 1998). In these cells, formation of the DISC results in the caspase-8-mediated cleavage of a protein called Bcl-2 homology 3-interacting domain death agonist (Bid) to generate truncated Bid (tBid), which proceeds to initiate Bax/Bak oligomerization and results in MOMP, apoptosome formation, effector caspase activation, and eventually cell death (Fig. 1.2) (Wei et al., 2000; Eskes et al., 2000).

As described above, the IAPs were originally thought to regulate apoptosis through the inhibition of caspases. XIAP is the archetypical IAP protein that directly binds and inhibits caspases, specifically caspase-3, caspase-7, and caspase-9, preventing their downstream effector functions (Deveraux et al., 1998; Deveraux et al., 1997). This binding occurs through the BIR domains of XIAP and has been shown to inhibit cell death (Salvesen and Duckett, 2002; Duckett et al., 1998). Due to this described role, members of the IAP protein family have been generally considered direct inhibitors of cell death. However, the caspase inhibition exhibited by XIAP is suppressed following

the initiation of the mitochondria-dependent apoptotic pathways. When MOMP occurs, cytochrome c release is accompanied by the release of other mitochondrial proteins into the cytoplasm, including Smac and the serine protease Omi/HtrA2 (Tait and Green, 2010). These proteins possess IAP binding motifs (IBMs) and bind to XIAP in a manner that liberates the caspases, allowing them to be activated (Fig. 1.2). Furthermore, the IAP binding proteins can induce the ubiquitination and subsequent proteasome-dependent degradation of the bound IAPs (Csomos et al., 2009).

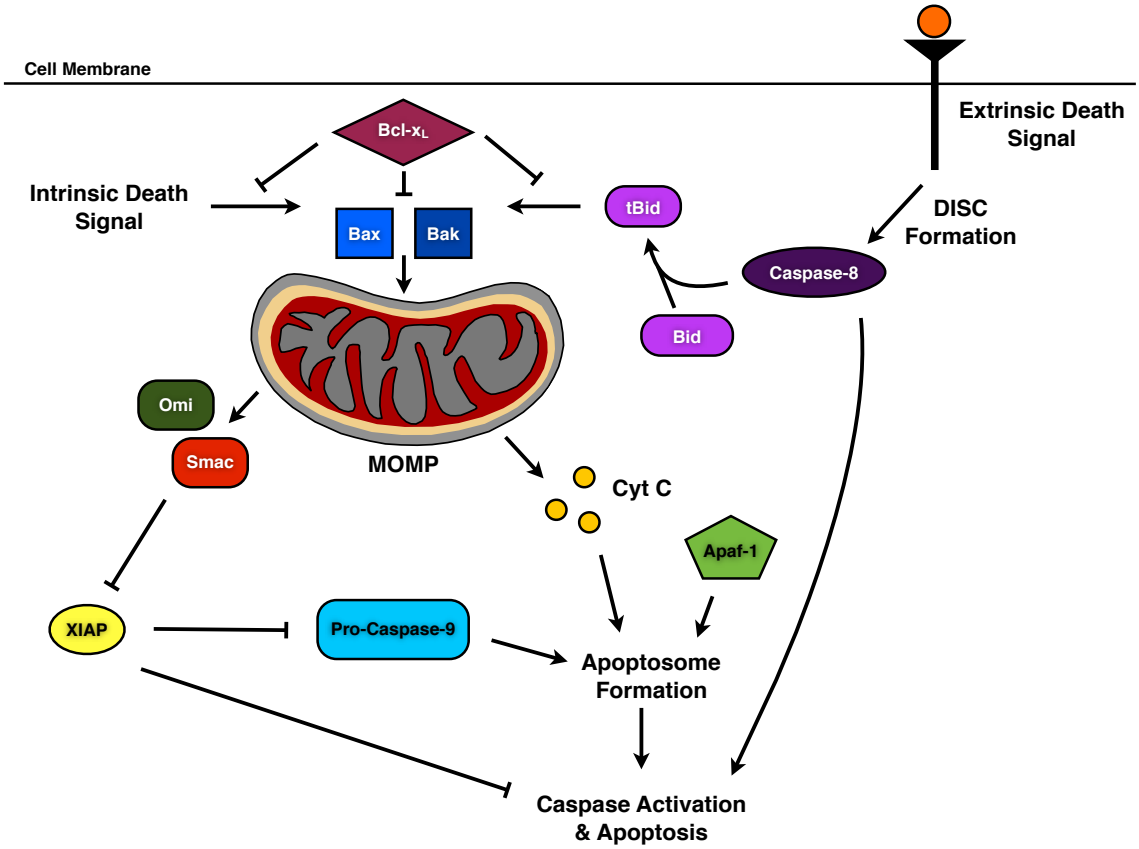


**Figure 1.2 The intrinsic and extrinsic apoptotic pathways.** The intrinsic cell death pathway is initiated by an intracellular death signal. This signal results in the oligomerization and translocation of Bax and Bak into the outer membrane of the mitochondria. This triggers mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c and IAP binding proteins. Cytochrome c forms the apoptosome with Apaf-1 and pro-caspase-9, which results in cell death. The IAP binding proteins, such as Smac, bind to XIAP and antagonize the caspase-binding function of XIAP. The extrinsic cell death pathway is receptor-mediated and results in the formation of the death inducing signaling complex (DISC). In certain cells, the DISC can directly activate downstream caspases, leading to cell death. However, in most cells, the DISC, through its caspase-8 component, cleaves Bid to form tBid, leading to Bax/Bak oligomerization, MOMP, apoptosome formation, and subsequent cell death.

It has been demonstrated that Smac is capable of binding c-IAP1/2, inducing their autoubiquitination and degradation (Samuel et al., 2006; Yang and Du, 2004; Du et al., 2000; Wu et al., 2000; Csomos et al., 2009). However, unlike XIAP, the c-IAPs do not

possess the ability to inhibit the apoptotic functions of caspases, despite their ability to bind caspases (Eckelman and Salvesen, 2006). This suggested that the c-IAPs might regulate cell death through other mechanisms. Recent work has implicated the c-IAPs in regulating other cell death-activating platforms. If the c-IAPs are removed by chemical, physiological, or genetic means, stimulation of tumor necrosis factor-receptor 1 (TNF-R1) forms a complex containing FADD, active caspase-8, and receptor-interacting serine/threonine protein kinase 1 (RIP1) to induce apoptosis (Silke and Brink, 2010). Additionally, the c-IAPs have been associated in a recently described form of cell death, designated necroptosis, which exhibits hallmarks of both apoptosis and the inflammation-inducing necrosis and occurs following the chemical degradation of the c-IAPs in conjunction with the inhibition of caspases (Feoktistova et al., 2011; Tenev et al., 2011). The death-activating platform involved in this pathway is called the ripoptosome and is comprised of RIP1, FADD, inactive caspase-8, and RIP3, a related protein to RIP1 (Feoktistova et al., 2011; Tenev et al., 2011). The formation of the ripoptosome is purportedly independent of receptor activation and is thought to occur in specific cases following genotoxic stress, certain inflammatory stimuli, and c-IAP degradation (Darding and Meier, 2012; Feoktistova et al., 2011; Tenev et al., 2011).

As the body of research grows, it has become more apparent that the IAPs play important functions in a host of cellular processes beyond their initially described function of caspase and cell death inhibition, implying that caspase-binding may, perhaps, only represent a minor facet of the IAPs. As such, the IAPs have come to be recognized as important regulators of intracellular signaling cascades, specifically the activation of nuclear factor-κB (NF-κB). The c-IAPs, as well as XIAP, have been

implicated in multiple pathways of NF-κB activation (Gyrd-Hansen and Meier, 2010), a family of transcription factors that play vital roles in a variety of signaling relevant to immunology and cancer as will be described in more detail below. Therefore, NF-κB can be, at times, considered a counterbalance to the death promoting signals within a cell. Even though they are poor inhibitors of caspase activity, the c-IAPs may contribute to the determination of cell fate through their regulation of NF-κB.

## IAPs as Regulators of Cell Signaling

The functional scope of the IAPs has expanded beyond caspase-binding and cell death inhibition. IAPs have been implicated in regulating intracellular copper levels (Burstein et al., 2004; Mufti et al., 2006; Brady et al., 2010), cell cycle regulation (Samuel et al., 2005; Cartier et al., 2011), and cell migration (Oberoi et al., 2012; Kenneth and Duckett, 2012), and have also been shown to be important signal transduction factors in a variety of receptor-mediated pathways, many of which activate NF-κB (Silke and Brink, 2010).

As discussed above, several members of the IAP family, including the c-IAPs and XIAP, possess RING domains, which have been shown to be vital in many of the signaling cascades in which the IAPs participate. In these signaling pathways, the IAPs, through their RING domains, function as E3 ubiquitin ligases, which are the final component in the ubiquitination enzymatic cascade. As a whole, the ubiquitination process involves ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes (Komander and Rape, 2012). The E1 enzyme is responsible for activating ubiquitin for the entire pool of downstream ubiquitin pathways (Heride et al.,

2014; Pickart, 2001), while the E2 enzymes are able to bind with the ubiquitination target protein and the E3 ligase (Deshaies and Joazeiro, 2009). The process culminates with the E3 ligase transferring the ubiquitin molecule from the E2 to the target substrate, resulting in a covalently linked ubiquitinated substrate (Deshaies and Joazeiro, 2009). Through this process and in addition to monoubiquitination, polyubiquitin chains can be constructed, marking target proteins for a variety of subsequent functional outcomes. There are at least eight distinct polyubiquitin chains that can be formed within a cell, and these chains are defined by their linkages (Heride et al., 2014). Two well-studied polyubiquitin chains are the K63-linked chain and the K48-linked chain, defined by their linkages at specific lysines within ubiquitin (Heride et al., 2014), and the IAPs have been shown to mediate both K48 and K63 ubiquitination (Yang et al., 2000; Huang et al., 2000; Li et al., 2002; Varfolomeev et al., 2008; Bertrand et al., 2008). The K48 ubiquitin chain is generally considered to be a degradation signal that targets the marked protein for proteasome-mediated destruction. In contrast, K63 polyubiquitination does not mediate proteasomal degradation, but rather acts as a scaffold for recruitment of additional factors to the signaling complex (Walczak, 2011). The c-IAPs are E3 ubiquitin ligases for multiple components in the NF-κB pathways, including RIP1 and NF-κB-inducing kinase (NIK), and the ability to mediate these forms of ubiquitination is integral to the functions of the IAPs in regulating the activation of NF-κB.

NF-κB is a family of transcription factors that plays important functions in many cellular responses. The family is comprised of five members: p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), and following activation, these factors dimerize to form the active transcription factor (Hayden and Ghosh, 2008). There are at least ten

distinct dimers, which each have different transcriptional properties (Smale, 2012). Each NF-κB subunit possesses a Rel homology domain (RHD) that allows for the binding to κB elements within DNA, and p65, RelB, and c-Rel also have transcriptional activation domains (TADs), which are associated with active transcription (Vallabhapurapu and Karin, 2009). In contrast, p50 and p52 do not possess a TAD, and homodimers of these subunits appear to regulate transcription through different mechanisms (Vallabhapurapu and Karin, 2009). Prior to activation, NF-κB dimers are sequestered in the cytoplasm of the cell, rendered inactive through the binding of IκB proteins. These proteins mask the nuclear localization sequence in NF-κB and prevent DNA binding through their ankyrin repeats (Sun, 2012). While the IκB protein family consists of multiple members, the most well characterized member is IκBα. Additionally, the precursor proteins of p50 and p52, p105 and p100, respectively, also possess ankyrin repeats within their C-terminal region and play similar functions to the other IκB proteins (Perkins, 2007). These precursor proteins are processed, liberating p50 and p52 to dimerize and modulate transcription. Similarly, IκBα is ubiquitinated and subsequently degraded by the proteasome following inititiation of the signaling cascade, allowing the NF-κB dimer to translocate into the nucleus.

The NF-κB pathways are activated by a multitude of stimuli and play important roles in cancer and the innate immune response. For example, the pattern recognition receptors, such as nucleotide-binding oligomerization domain-like receptors (NLRs), Toll-like receptors (TLRs), and retinoic acid-inducible gene 1-like receptors (RLRs), activate NF-κB following recognition of a pathogen (Oeckinghaus et al., 2011; Vallabhapurapu and Karin, 2009). Signaling involving the pro-inflammatory cytokine IL-

1β is also dependent on NF-κB, and, once active, induces NF-κB to further propagate an inflammatory response (Barker et al., 2011). Additionally, carcinogens and other tumor promoters can activate NF-κB, promoting inflammation and contributing to tumorigenesis (Aggarwall, 2004; Hanahan and Weinberg, 2011). Furthermore, NF-κB has been demonstrated to have important roles in the adaptive arm of immunity, including T and B cell development and their responses to immunological challenges. The B cell receptor and the T cell receptor activate NF-κB following encounter with antigen, and mice lacking NF-κB subunits display impaired B and T cell responses (Beinke and Ley, 2004). Additionally, in NF-κB deficient mice, the development of lymphoid organs is severely impaired. For example, mice lacking *Nfkb2* or *Relb* exhibit significant disruption of the architecture of the spleen and abnormal lymph node organization (Weih et al., 1995; Weih and Caamano, 2003), and ablation of *Nfkb2* or *Relb* also results in an increased incidence of T cell-mediated inflammatory disease (Weih et al., 1995; Zhu et al., 2006). In *Nfkb1*$^{-/-}$ mice, B cells fail to induce a proliferative response when exposed to lipopolysaccharide (LPS) and soluble CD40L (Snapper et al., 1996), and *Nfkb1* is also needed for T cell proliferation following antigen stimulation (Zheng et al., 2001), and differentiation into effector subsets (Beinke and Ley, 2004). Additionally, it is now believed that NF-κB is involved in thymic selection and development of T cells, though its exact role is still unclear (Gerondakis et al., 2014).

There are two major pathways of NF-κB signaling, the canonical pathway that involves the p65:p50 dimer and the non-canonical pathway that employs the RelB:p52 dimer (Hayden and Ghosh, 2008). Canonical NF-κB can be activated by a wide variety of stimuli, some of which involve the IAPs, and the resulting signaling cascades converge

on the IκB kinase (IKK) complex (Perkins, 2007). This complex has three components: IKKα (IKK1), IKKβ (IKK2), and NF-κB essential modulator (NEMO, also known as IKKγ). IKKα and IKKβ are the kinase subunits of the complex, while NEMO is a regulatory factor (Scheidereit, 2006). Once activated, the IKK complex phosphorylates IκBα, triggering its subsequent ubiquitination and proteasome-mediated degradation. As described above, this allows the canonical NF-κB dimer to translocate to the nucleus and initiate transcription. Non-canonical NF-κB, which is activated by a more limited set of stimuli, relies on a protein called NF-κB-inducing kinase (NIK). Prior to stimulation, NIK is constitutively degraded by a c-IAP-containing complex (Vallabhapurapu et al., 2008). Following activation, this complex is inhibited, resulting in an increase in NIK protein levels, which then leads to the activation of IKKα. IKKα phosphorylates the NF-κB precursor protein p100 at Ser-866 and Ser-870 (Sun, 2012), resulting in its proteasome-dependent cleavage and generation of the active p52 subunit. The RelB:p52 dimers can then translocate into the nucleus to modulate gene expression. While the IAPs have been shown to participate in a variety of NF-κB activation pathways, the c-IAPs have been implicated in the regulation of both canonical and non-canonical NF-κB following stimulation of members of the TNF receptor superfamily, playing both activating and inhibitory roles (Silke and Brink, 2010).

## The c-IAPs and the TNF Receptor Superfamily

The TNF receptor superfamily is comprised of a multitude of receptors that play important roles in the immune response (Silke and Brink, 2010), and these receptors can be broadly characterized into two major subsets based on their structural domains.

Receptors such as Fas and TNF-R1, which can be widely expressed, possess death domains within their cytoplasmic tails (Wertz and Dixit, 2010). These domains allow the receptor to recruit other DD-containing factors, building a receptor complex that can induce a variety of signaling. These receptors have important roles in inflammation, hematopoiesis, and the immune response (Walczak, 2011). In contrast, there are TNF receptor superfamily members that do not possess death domains and instead recruit signaling cofactors via TRAF-binding domains. These domains allow for the recruitment of members of the TNF receptor-associated factor (TRAF) family (Silke and Brink, 2010), and while TRAFs are implicated in signaling from both groups of receptors, direct TRAF binding to the receptor results in distinct consequences (Hacker et al., 2011). Additionally, the expression of these receptors is more limited, and many of these receptors are predominantly found on hematopoietic cells (Croft et al., 2012), and as such, many of these receptors have been shown to play important roles in immunity. Previous work has demonstrated that the survival of mature B cells is dependent on the B cell activating factor receptor (BAFF-R) (Claudio et al., 2002), and CD40 (TNFRSF5) has also been shown to have a critical role in the B cell-mediated immune response (Quezada et al., 2004; Elgueta et al., 2009; Coope et al., 2002). Likewise, T cells are also reliant on members of the TRAF-binding TNF receptor superfamily, which can be found on naïve, effector, and memory T cells (Croft, 2003). For example, CD27 (TNFRSF7) can be found on naïve, effector, and memory T cells and is believed to be involved in promoting the initial expansion of the naïve T cell population, potentially by regulating the cell cycle (Agematsu et al., 1994; Hendriks et al., 2000; Croft, 2003). OX40 (TNFRSF4) is expressed on effector T cells, and its absence results in increased cell

death several days after T cell activation, suggesting that OX40 provides signals that promote the survival of effector T cells during the immune response (Rogers et al., 2001; Croft, 2003). CD30 (TNFRSF8), which will be discussed in more detail below, is also found on effector T cells and may provide additional co-stimulatory signals during the T cell response (Croft, 2003).

CD30, a member of the direct TRAF-binding subset of the TNFR superfamily, can be expressed on a subset of activated T cells (Del Prete et al., 1995; Bowen et al., 1996; Nakamura et al., 1997; Croft, 2003), and while the exact physiological role of the receptor is still unclear, the mechanism of CD30 signaling is more clearly understood. CD30 has been shown to activate both canonical and non-canonical NF-κB (Wright et al., 2007; Csomos et al., 2009), as well as MAPK pathways (Younes and Aggarwall, 2003). Furthermore, prolonged stimulation of CD30 has been shown to induce cell cycle arrest (Wright et al., 2007; Buchan and Al-Shamkhani, 2012), and CD30 activation has been linked to both inducing apoptosis and promoting proliferation and survival (Croft, 2003; Wright et al., 2007). The cytoplasmic tail of CD30 contains two distinct TRAF binding domains, with residues 561-573 binding TRAFs 1, 2, 3, and 5, while residues 578-586 specifically bind TRAFs 1 and 2 (Gedrich et al., 1996; Aizawa et al., 1997; Duckett et al., 1997; Duckett and Thompson, 1997; Buchan and Al-Shamkhani, 2012). As discussed in more detail below, the TRAF binding domains are vital for the c-IAP-dependent regulation of NF-κB activation. However, CD30 has been shown to activate NF-κB in the absence of its TRAF binding domains, indicating that upstream regions of the cytoplasmic tail of CD30 may also contribute to signaling (Duckett et al., 1997; Buchan and Al-Shamkhani, 2012). While the exact physiological role of CD30 remains unclear, it

has been realized that CD30 is highly expressed on certain types of cancers, including anaplastic large cell lymphoma (ALCL), Hodgkin's lymphoma, multiple myeloma, and certain T cell leukemias (Stein et al., 1985; Chiarle et al., 1999; Younes and Aggarwall, 2003). Due to its association with certain cancers, recent work has used CD30 as a means to target antibody-drug conjugates to malignant cells (Younes et al., 2010; Foyil and Bartlett, 2011). However, the full scope of the consequences of CD30-mediated NF-κB activation and its relationship to disease progression remains to be investigated.

The c-IAPs are important factors in signaling from both categories of the TNF receptor superfamily, playing distinct roles for each subset of the superfamily. In the context of NF-κB activation by TNF-R1, a DD-containing receptor, the c-IAPs actively participate in the recruitment of proteins for signal transduction. Upon ligand binding to TNF-R1, the adaptor molecule TNF-R1-associated death domain protein (TRADD) is recruited to the DD of TNF-R1 (Hsu et al., 1995). TRADD binding, in turn, recruits additional proteins, including TRAF2, c-IAP1/2, and RIP1 to form the initial receptor signaling complex (Fig 1.3) (Hsu et al., 1996a; Hsu et al., 1996b; Shu et al., 1996; Micheau and Tschopp, 2003; Vince et al., 2009). The c-IAPs polyubiquitinate RIP1 in a K63-dependent manner, forming a scaffold for the recruitment of additional factors, including transforming growth factor-β activating kinase 1 (TAK1), TAK1 binding protein 2 (TAB2), and TAB3, which preferentially binds to K63-linked ubiquitin chains (Fig 1.3) (Wang et al., 2001; Varfolomeev et al., 2008; Bertrand et al., 2008; Walczak, 2011). Additionally, the c-IAPs become polyubiquitinated in a K63-dependent manner through autoubiquitination, and these ubiquitin chains are thought to recruit the tripartite linear ubiquitin chain assembly complex (LUBAC), comprised of Sharpin, HOIL-1, and

HOIP (Haas et al., 2009; Gerlach et al., 2011; Walczak, 2011). LUBAC modifies the NEMO subunit of the IKK complex with linear ubiquitin chains, which is believed to impact its function (Gerlach et al., 2011). IKKβ is phosphorylated by the TAK:TAB complex, activating the IKK complex and resulting in the subsequent downstream activation of NF-κB (Fig 1.3) (Yang et al., 2001; Takaesu et al., 2003).

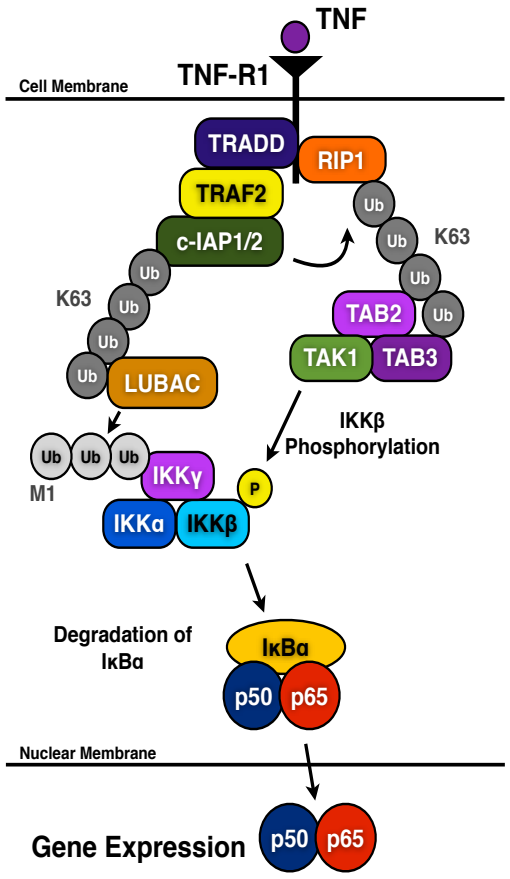


**Figure 1.3 The role of c-IAP1/2 in TNF-R1-mediated NF-κB activation.** Upon ligand binding to TNR-R1, death domain-containing factors, such as RIP1 and TRADD are recruited to the cytoplasmic tail of the receptor. This, in turn, recruits additional factors, including TRAF2 and the c-IAPs. The c-IAPs undergo autoubiquitination and ubiquitinate RIP1. The ubiquitin chains act as scaffolds to recruit LUBAC and the TAB:TAK complex. The IKK complex is modified with a linear ubiquitin chain by LUBAC and is activated by phosphorylation by the TAB:TAK complex. The IKK complex phosphorylates IκBα, resulting in its subsequent proteasome-dependent degradation and the translocation of the canonical NF-κB dimer into the nucleus.

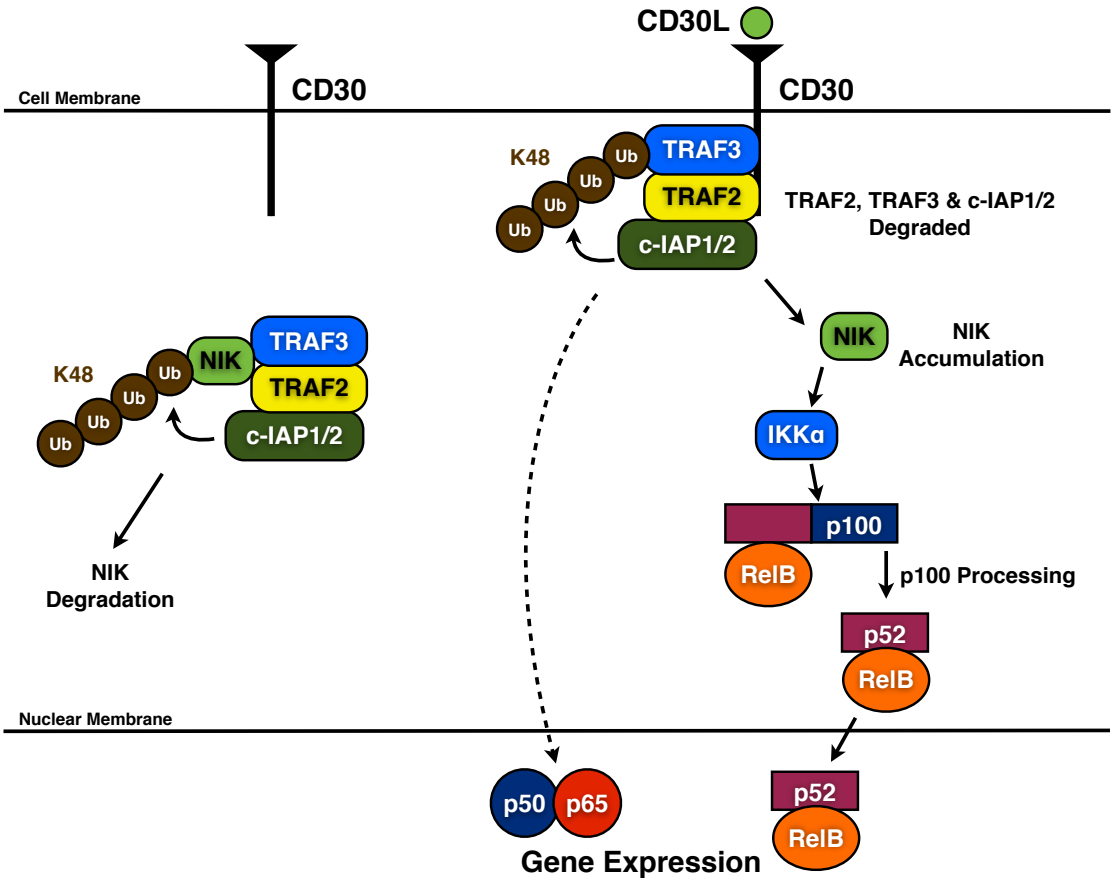


**Figure 1.4 The role of c-IAP1/2 in CD30-mediated NF-κB activation.** Prior to stimulation, the c-IAPs form a complex with TRAF2 and TRAF3 and ubiquitinate NIK in a K48-dependent manner, resulting in the constitutive degradation of NIK. Following receptor activation, the TRAF:c-IAP complex is recruited to the cytoplasmic tail of CD30, where the c-IAPs ubiquitinate TRAF3, inducing its degradation. TRAF2 and c-IAP1/2 are also degraded, allowing for the accumulation of NIK. NIK activates IKKα, which phosphorylates p100. Subsequently, p100 is cleaved, allowing the active non-canonical NF-κB dimer to translocate into the nucleus. Receptor-mediated canonical NF-κB activation also occurs, albeit through a poorly defined mechanism.

While the E3 ubiquitin ligase activity of the c-IAPs is involved in the propagation of the TNF-R1 signaling cascade, the c-IAPs also play an inhibitory role in regulating non-canonical NF-κB in the context of CD30-mediated signaling. Prior to receptor activation, the c-IAPs form a complex with TRAF2 and TRAF3. This complex binds

NIK, which is constitutively expressed (Zarnegar et al., 2008; Vallabhapurapu and Karin, 2009). Both TRAF2 and TRAF3 have been shown to directly bind NIK (Malinin et al., 1997; Liao et al., 2004), and the c-IAPs modify NIK with K48-linked ubiquitin chains, resulting in its proteasome-dependent degradation (Varfolomeev et al., 2007; Zarnegar et al., 2008; Vallabhapurapu et al., 2008; Hacker et al., 2011), thereby repressing non-canonical NF-κB activation (Fig. 1.4). Following receptor activation, the TRAF:c-IAP complex is recruited to the cytoplasmic tail of CD30 via its TRAF-binding domains. After this recruitment, the c-IAPs modify TRAF3 with K48-linked ubiquitin chains, leading to the degradation of TRAF3 (Fig. 1.4) (Duckett and Thompson, 1997; Zarnegar et al., 2008; Vallabhapurapu et al., 2008). TRAF2 and c-IAP1/2 are then subsequently degraded following translocation to a detergent insoluble fraction (Duckett and Thompson, 1997; Wright et al., 2007; Csomos et al., 2009; Silke and Brink, 2010). The absence of the c-IAP:TRAF complex allows NIK levels to accumulate, resulting in the activation of non-canonical NF-κB (Fig. 1.4). While the role of c-IAP1/2 in other aspects of CD30 signaling remains undefined, CD30-mediated degradation of the c-IAPs is an important regulatory event for the activation of non-canonical NF-κB.

## Synthetic IAP Antagonism

Physiological degradation of c-IAP1/2 is dependent on the activation of a limited set of receptors, as discussed above. However, over the last decade, a class of small molecule compounds has been developed that possess the ability to induce the degradation of the IAPs. These compounds are based on the IAP binding motif (IBM) of Smac (Schimmer et al., 2004; Li et al., 2004; Sun et al., 2004), and the IBM of Smac is a

four residue sequence of Alanine-Valine-Proline-Isoleucine (AVPI) that allows the protein to interact with the BIR domains of the IAPs (Liu et al., 2000; Wu et al., 2000). Since the caspase:IAP interaction occurs via the BIR domains, binding of Smac interrupts this interaction and liberates the caspases for downstream activity. Furthermore, this binding of Smac to an IAP can induce the autoubiquitination and degradation of the IAP (Csomos et al., 2009). Likewise, the Smac mimetics (SMs), which are also known as IAP antagonists, are able to bind the IAPs and induce their degradation (Varfolomeev et al., 2007; Vince et al., 2007; Darding et al., 2011). Due to the connection between the IAPs and cell death, SMs have been actively studied as potential therapeutics against cancer.

The ability to evade cell death is a characteristic of cancer, and therefore, methods to induce cell death in malignant cells have been an active field of study in cancer research (Hanahan and Weinberg, 2000). As discussed above, the precise role of the IAPs in the progression of cancer is still being studied. However, multiple cancers are associated with higher expression of the IAPs, which also correlates with poorer survival rates (Xiang et al., 2009; Hussain et al., 2010; Esposito et al., 2007; Krajewska et al., 2005; Imoto et al., 2002). For these reasons, the IAPs have been considered prime targets for therapeutic strategies.

There are currently two major categories of SMs: monovalent compounds, which mimic a single AVPI motif, and bivalent compounds that mimic two AVPI motifs. Both classes of SMs have been shown to induce cell death in certain cancers, though bivalent compounds are often significantly more potent (Varfolomeev et al., 2007; Vince et al., 2007; Petersen et al., 2007; Lu et al., 2008). This is thought to be due to their increased ability to dimerize the IAPs, inducing their degradation (Varfolomeev et al., 2007; Vince

et al., 2007; Lu et al., 2008). Alternatively, the increased potency of bivalent SMs may be due to their ability to interact with both BIR2 and BIR3 of XIAP, resulting in more efficient caspase activation (Gao et al., 2007; Varfolomeev et al., 2009). There are currently a variety of SMs, both monovalent and bivalent, in preclinical and clinical development (Fulda and Vucic, 2012).

While the ability of SMs to liberate caspases from XIAP may contribute to the observed induction of cell death, this is now believed to represent only one aspect of their mechanism. SMs have been shown to preferentially target and degrade the c-IAPs over XIAP (Varfolomeev et al., 2007; Lu et al., 2008), and while this does not preclude the inhibition of XIAP, it suggested a more prominent role for the c-IAPs in regulating SM-induced cell death. It is currently thought that SM-induced killing is dependent on TNF (Varfolomeev et al., 2007; Vince et al., 2007). More specifically, following SM treatment, the c-IAPs are degraded, and TNF is produced in an autocrine or paracrine manner, activating TNF-R1. However, in the absence of c-IAP1/2, the ubiquitin scaffolds that help form the receptor signaling complex cannot be properly formed, leading RIP1 to then associate with TRADD, FADD, and caspase-8 to form a death inducing signaling complex, resulting in caspase activation and cell death (Fig. 1.5) (Varfolomeev et al., 2008). Currently, cells can be divided into two major categories: those that are killed by SM treatment alone, and cells that are not killed by SM treatment alone but are sensitized to exogenous TNF (Petersen et al., 2007; Tenev et al., 2011). Cells in the former category are able to produce autocrine TNF through a NF-κB-dependent manner (Petersen et al., 2007), while cells in the latter category, conversely, do not produce TNF following SM treatment, and therefore require an additional source of TNF (Vince et al., 2007). More

recently, another mechanism of cell death has been proposed which involves the formation of the ripoptosome following SM treatment (Fig. 1.5). The mechanism of the complex has been described above, but importantly, ripoptosome-mediated cell death is independent of TNF and the mitochondrial death pathways (Tenev et al., 2011; Feoktistova et al., 2011), indicating that SM-induced death may not always be reliant on TNF.

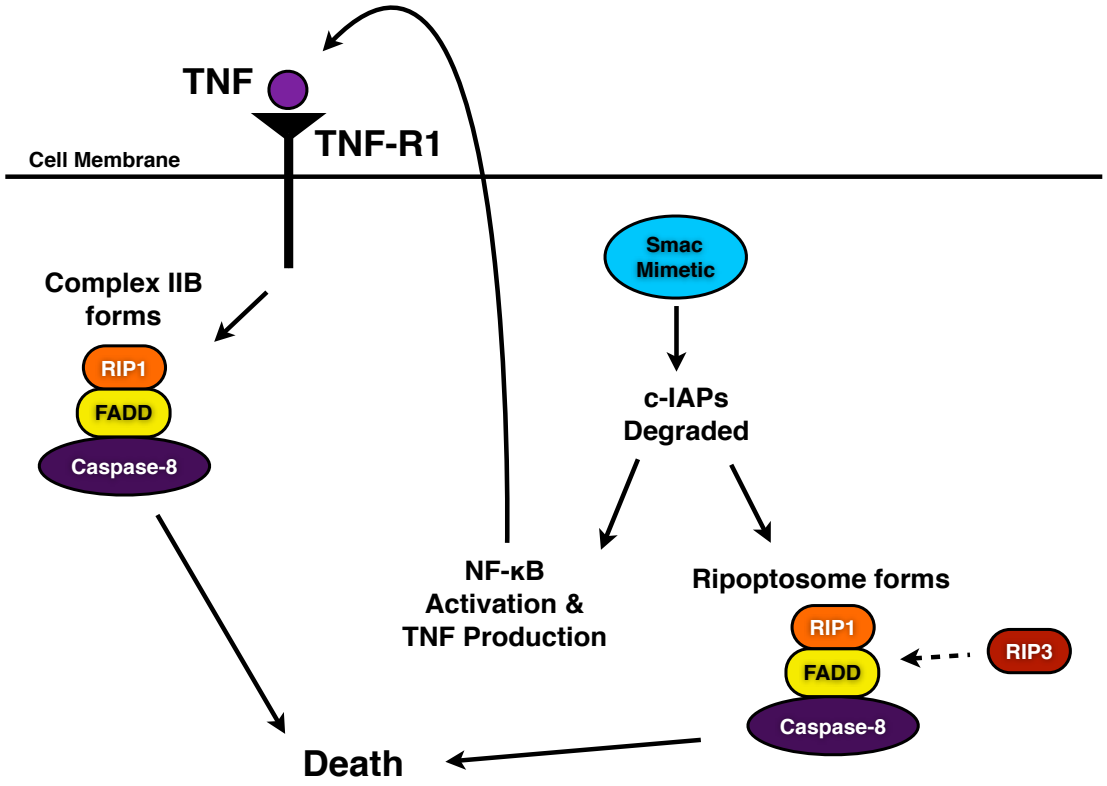


**Figure 1.5 Current model of Smac mimetic-induced cell death.** Smac mimetic treatment results in the degradation of c-IAP1/2. In certain cells, this leads to the NF-κB-dependent production of autocrine TNF. In the absence of c-IAP1/2, normal TNF-R1 signaling is impaired, resulting in the formation of a death inducing signaling complex called Complex IIB. This complex then activates downstream caspases and cell death. More recently, another mechanism of SM-induced cell death has been described. Degradation of c-IAP1/2 results in the receptor-independent formation of a complex called the Ripoptosome. This complex can induce apoptosis or necroptosis, depending on the status of caspase-8 activation and the presence of RIP3.

While the targeting of the IAPs may be a promising therapeutic strategy for certain cancers, in other malignancies, the absence of properly functioning IAPs appears to contribute to the disease. Deletions of c-IAP1 and c-IAP2 were found in multiple myeloma and resulted in constitutive activation of non-canonical NF-κB, potentially contributing to disease progression (Keats et al., 2007; Annunziata et al., 2007). Additionally, a t(11;18)(q21;q21) chromosomal translocation frequently occurs in mucosa-associated lymphoid tissue (MALT) lymphoma, resulting in a fused protein of the BIR domains of c-IAP2 and the paracaspase function of MALT translocation protein 1 (MALT1), leading to constitutive activation of both canonical and non-canonical NF-κB (Dierlamm et al., 1999; Rosebeck et al., 2011; Rosebeck et al., 2014). These data suggest that the IAPs have multifaceted, and perhaps contradictory, functions in cancer, and therefore, targeting the IAPs may be a successful strategy in certain cases, but may exacerbate the disease in other situations. SM-induced degradation of c-IAP1/2 would allow NIK to accumulate, leading to the constitutive activation of non-canonical NF-κB and potentially contributing to disease progression and initiation of cytokine release syndrome, which has been observed in early clinical trials with SMs (Infante et al., 2014). Therefore, the consequences of IAP antagonism warrant additional study.

CD30 activation and SM treatment represent physiological and synthetic means, respectively, to induce the degradation of c-IAP1/2 and therefore represent useful tools for investigating the consequences of c-IAP1/2 activity. While both stimuli result in c-IAP degradation, it is not clear if the downstream effects of this event are similar. Likewise, it is known that both stimuli activate NF-κB, but the full extent of how the

cellular context impacts the activity of c-IAP1/2 and its regulation of intracellular signaling is not fully understood.

## Dissertation Objectives

The goal of this dissertation was to further explore the role of ubiquitin in signaling and specifically investigate the multifaceted roles and outcomes of c-IAP1/2 activity in key intracellular signaling pathways. Their role in regulating NF-κB was initially examined by comparing the effects of physiological and synthetic IAP antagonism by receptor activation and Smac mimetic treatment, respectively. The NF-κB signaling pathways regulated by c-IAP1/2 were then further investigated, characterizing the role of these pathways in different cellular contexts. This dissertation has been organized into the following specific aims:

**Specific Aim 1 (Chapter II):** Compare physiological and synthetic IAP antagonism and investigate and compare the downstream consequences following each stimulus.

**Specific Aim 2 (Chapter III):** Investigate the cell type-specificity of the downstream effects of IAP antagonism, asking how the cellular context contributes to the efficacy of SM function.

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# CHAPTER II

## Effects of Physiological and Synthetic IAP Antagonism on c-IAP-dependent Signaling

### Summary

The ubiquitin ligases c-IAP1/2 play central roles in signal transduction mediated by numerous receptors that participate in inflammatory and immune responses. Their degradation is concomitant with the activation of certain pathways and is physiologically induced by activation of receptors. A number of synthetic compounds have been developed that also target the c-IAPs and induce their degradation. However, the extent of a synthetic IAP antagonist's ability to mirror the transcriptional program by a physiological signal remains unclear. A systems approach was used to compare the transcriptional programs triggered by activation of CD30, a receptor previously shown to degrade the c-IAPs, to SM-164, a synthetic IAP antagonist that specifically triggers c-IAP degradation. Employing a technique that allows for the specific analysis of newly transcribed RNA, comparative transcriptome profiles for CD30 activation and SM-164 treatment were generated, revealing novel functions of IAP antagonists and consequences of c-IAP1/2 degradation that included a role for c-IAP1/2 in the regulation of the ribosome and protein synthesis. These findings expand our knowledge of the roles of c-IAP1/2 in signaling and provide insight into the mechanism of synthetic IAP antagonists, furthering our understanding of their therapeutic potential.

## Introduction

As discussed in Chapter I, the IAP proteins are central mediators of a divergent group of cellular signaling pathways, largely involved in immune and inflammatory responses (Salvesen and Duckett, 2002). Two of these proteins, c-IAP1/2, have been implicated as key regulators of NF-κB, a protein family comprised of five different members that dimerize to form active transcription factors (Hayden and Ghosh, 2008; Smale, 2012). The best-characterized NF-κB dimers are p65:p50 and RelB:p52, which are often referred to as canonical and non-canonical NF-κB, respectively (Vallabhapurapu and Karin, 2009).

The canonical and non-canonical NF-κB pathways are activated by members of the tumor necrosis factor (TNF) receptor superfamily and play important roles in the immune response, inflammation, and cancer (DiDonato et al., 2012; Perkins, 2012). While c-IAP1/2 have a demonstrated role in the activation of canonical NF-κB following TNF treatment, their degradation is required to activate the non-canonical NF-κB pathway, a signaling cascade that physiologically occurs following activation of a limited subset of the TNF receptor superfamily that exhibit direct binding to TRAF adaptor proteins, including CD30, CD40, and TNF-R2 (Hacker et al., 2011; Wright et al., 2007; Wright and Duckett, 2009). Prior to stimulation, c-IAP1/2 form a complex with TRAF2 and TRAF3, and this complex is thought to bind and degrade the constitutively expressed NIK (Vallabhapurapu et al., 2008). Following ligand binding, the receptor recruits the TRAF:c-IAP1/2 complex through the direct binding of the TRAFs. This interaction subsequently triggers the degradation of c-IAP1/2 and results in the gradual accumulation of NIK. This, in turn, begins a signaling cascade that leads to the phosphorylation and

processing of the NF-κB precursor p100 to the active NF-κB subunit p52. The p52 moiety can dimerize with RelB to form the non-canonical NF-κB transcription factor that subsequently regulates an incompletely defined list of genes involved in multiple cellular processes, including the immune response (Smale, 2012; Vallabhapurapu et al., 2008; Vallabhapurapu and Karin, 2009; Csomos et al., 2009).

As described in Chapter I, the degradation of c-IAP1/2 can be recreated experimentally using a class of synthetic, small molecule compounds known as Smac mimetics (SMs) or IAP antagonists (Varfolomeev et al., 2007; Vince et al., 2007; Lu et al., 2008). These compounds are structurally based on the IAP binding motif (IBM) of a physiological binding partner of the IAPs known as second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low isoelectric point (Smac/DIABLO). These compounds bind to c-IAP1/2 and trigger their autoubiquitination and subsequent degradation, resulting in the processing of p100 to p52 and the activation of non-canonical NF-κB (Csomos et al., 2009; Varfolomeev et al., 2007; Vince et al., 2007; Lu et al., 2008; Darding et al., 2011). Additionally, SM treatment has been shown to activate canonical NF-κB under some circumstances, though the exact mechanism remains undefined (Varfolomeev et al., 2007; Vince et al., 2007). While SMs have been shown to replicate aspects of receptor activation, the extent of the functional overlap between the two classes of stimuli remains unclear.

In this chapter, a systems approach was used to extensively compare the downstream effects of physiological and synthetic IAP antagonism. This was done in a cellular system in which both receptor activation and SM treatment lead to the degradation of c-IAP1/2 and result in canonical and non-canonical NF-κB activation.

While stimulation of the receptor also induced additional signaling pathways, such as JNK and ERK, SM treatment did not, demonstrating that these pathways were independent of c-IAP1/2. To characterize the transcriptional consequences of IAP antagonism-induced NF-κB activation, Bru-seq, a recently developed technique that specifically analyzes newly transcribed RNA, was used to identify gene expression profiles following each stimulus. While overlapping, the resulting transcriptome profiles revealed differences between the two modes of IAP antagonism, suggesting that consequences of c-IAP degradatiom may be context-dependent. Furthermore, analysis of the transcriptome data revealed novel functions of c-IAP1/2 degradation and sequelae of SM treatment. One novel consequence of c-IAP1/2 degradation was the decreased expression of genes related to the ribosome and translation. In support of this finding, biological assays found that SM treatment resulted in decreased protein synthesis. These findings identify novel functions for the c-IAPs, and provide insight into the mechanism of SMs and their therapeutic potential.

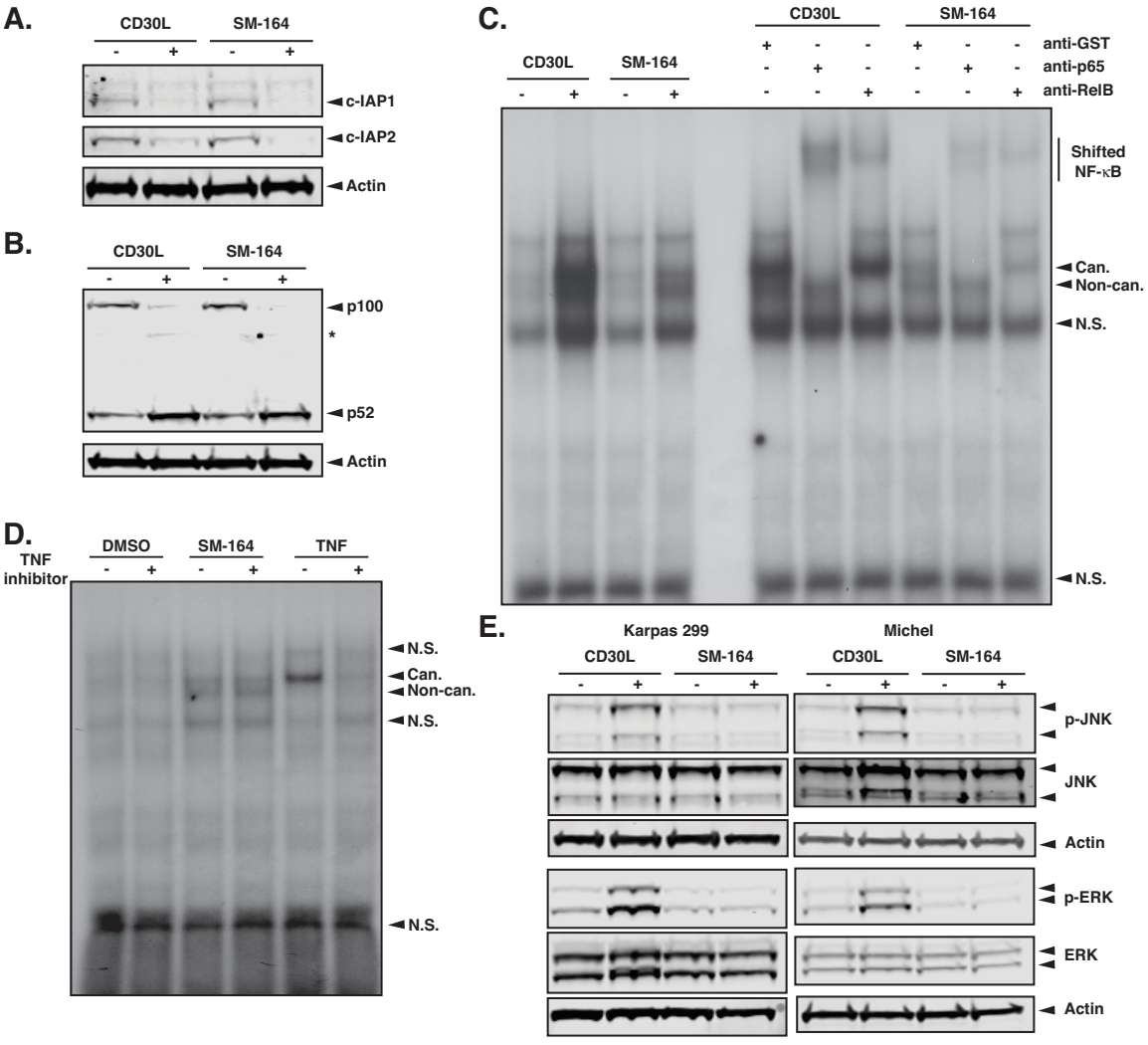
## Results

### Smac mimetic treatment models CD30-mediated c-IAP1/2 degradation and NF-κB activation

To evaluate the consequences of c-IAP degradation, the effects of a Smac mimetic were compared to those of a cell surface receptor previously shown to induce the degradation of the c-IAPs upon activation. As discussed in Chapter I, CD30 is a member of the TNF receptor superfamily that binds and degrades c-IAP1/2 as part of a larger complex. CD30 is highly expressed on the surface of certain lymphoma and leukemia cells, including anaplastic large cell lymphoma (ALCL) and Hodgkin's lymphoma cells

(Chiarle et al., 1999; Mir et al., 2000). In healthy individuals, CD30 expression is restricted to a small subset of activated T and B cells, and while its physiological role remains poorly defined, the ability of the receptor to degrade c-IAP1/2 following activation has been previously documented (Csomos et al., 2009). Using a cell-based system for CD30 stimulation (Wright et al., 2007) and the ALCL cell lines Karpas 299 and Michel (Tian et al., 1995), the consequences of Smac mimetic (SM) treatment and CD30 activation were compared. Both stimuli resulted in the degradation of c-IAP1 and c-IAP2 (Fig. 2.1A, 2.2A) and induced the processing of p100 to p52, a widely used marker of non-canonical NF-κB activation (Fig. 2.1B, 2.2A). Both CD30 stimulation and SM treatment resulted in nuclear translocation of DNA-binding canonical and non-canonical NF-κB, as detected by electrophoretic mobility shift assay (EMSA) and confirmed by supershift analysis (Fig. 2.1C), indicating that SM treatment models CD30-mediated NF-κB activation. While SM treatment has previously been shown to induce autocrine TNF production (Varfolomeev et al., 2007; Vince et al., 2007), pretreatment with the TNF inhibitor Enbrel did not prevent the NF-κB activation (Fig. 2.1D), indicating that SM-induced NF-κB was independent of TNF. In addition to NF-κB activation, members of the TNFR superfamily can trigger other signaling cascades, including the JNK and ERK pathways (Silke, 2011). To test if SM treatment and CD30 stimulation activated these pathways, Karpas 299 and Michel cells were briefly treated with SM or stimulated with CD30L, and phosphorylation of JNK and ERK was assessed. While receptor stimulation activated these pathways, SM treatment did not (Fig. 2.1E). Additionally, inhibition of JNK and ERK did not affect NF-κB activation, indicating that this occurred independently of CD30-mediated JNK and ERK signaling (Fig. 2.1F).

Collectively, these results demonstrate that the ability of SM treatment to model receptor signaling is limited to activation of NF-κB.



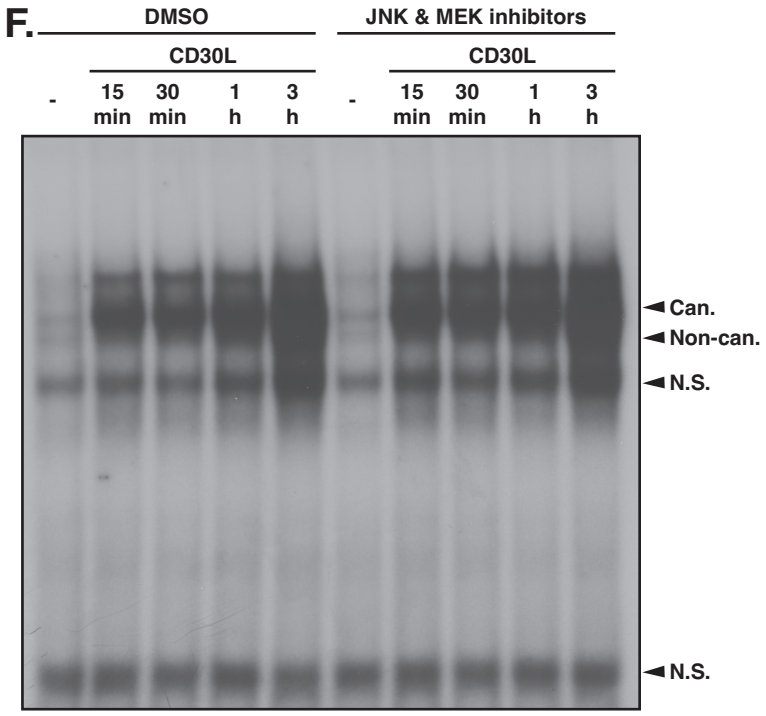
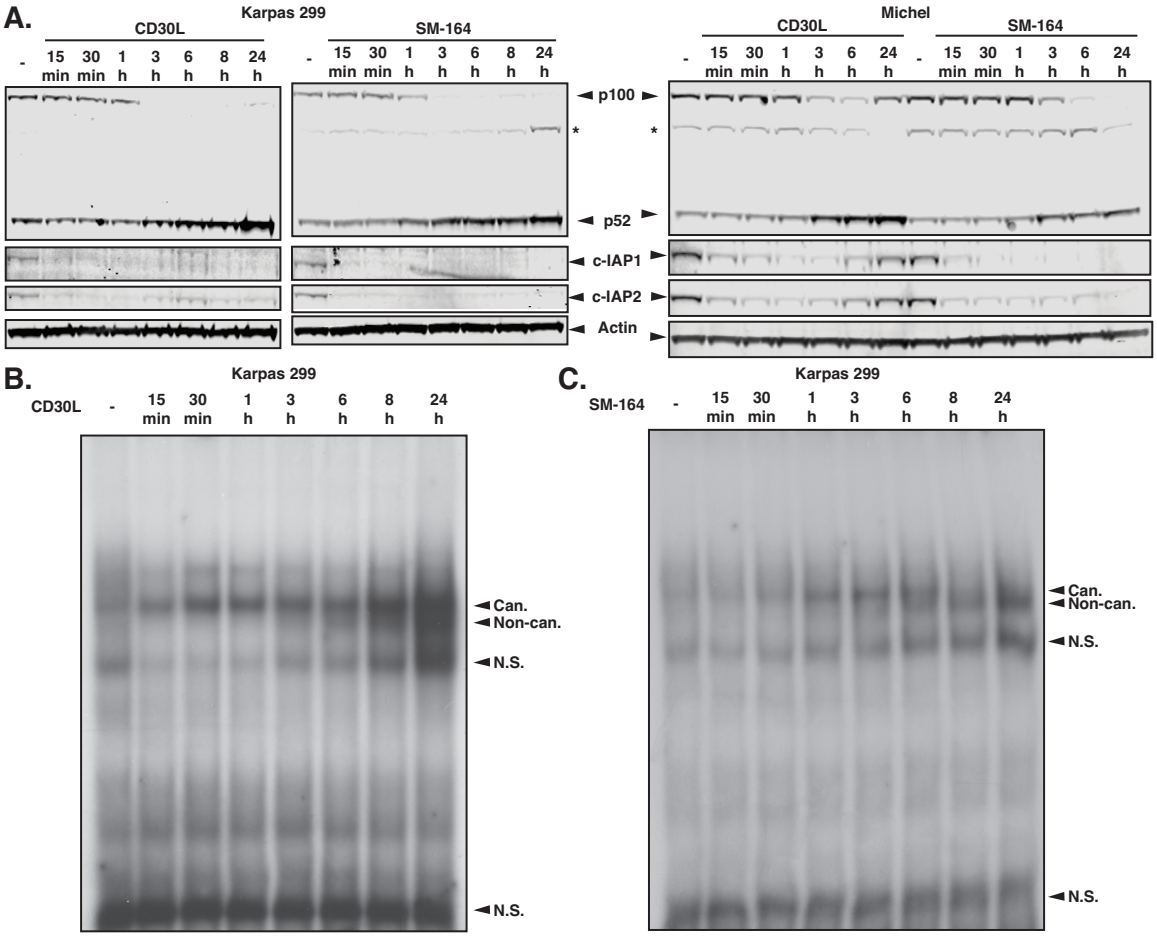



**Figure 2.1 Smac mimetic treatment models CD30-mediated NF-κB activation**
**A.** Karpas 299 cells were treated with 100 nM SM-164 or exposed to CHO cells expressing CD30L for 3 h. Control samples were treated with DMSO or exposed to control CHO cells. Whole cell lysates were prepared, and c-IAP1/2 degradation was assessed by Western blot. **B.** Karpas 299 cells were stimulated as in A. Whole cell lysates were prepared, and p100 processing was analyzed by Western blot. Bands marked with an asterisk (*) are non-specific. **C.** Karpas 299 cells were treated as in A, nuclear extracts were collected, and NF-κB activation was analyzed by EMSA. To identify the NF-κB bands, the CD30L and SM-164 treated samples were incubated with antibodies against the indicated NF-κB subunits or GST as a control and used in a supershift assay. **D.** Karpas 299 cells were pretreated with the TNF inhibitor Enbrel at 10 μg/mL for 1 h. The cells were then treated with 100 nM SM-164 for 3 h or 500 U/mL TNF for 30 min. Nuclear extracts were isolated, and NF-κB activation was analyzed by EMSA. **E.** Karpas 299 and Michel cells were incubated with CHO cells expressing CD30L or treated with 100 nM SM-164 for 15 min. Whole cell lysates were collected and the activation status of the indicated MAP kinase pathways was determined by Western blot. **F.** Karpas 299 cells were pretreated with 25 μM of the JNK inhibitor SP600125 and 50 nM of the MEK inhibitor Trametinib for 1h. The cells were then exposed to CD30L for the indicated times. Following treatment, nuclear extracts and whole cell lysates were collected from the samples. The nuclear extracts were used to assess NF-κB activation by EMSA. Can. NF-κB, canonical NF-κB; Non-can. NF-κB, non-canonical NF-κB; N.S., non-specific.

**Kinetics of NF-κB induction by receptor activation and SM treatment**

As described above, both CD30 activation and SM treatment were found to trigger the degradation of c-IAP1/2 and to activate canonical and non-canonical NF-κB. However, it was unclear if these processes occurred with similar kinetics. Time course experiments were performed with each stimulus to examine this question. Degradation of c-IAP1/2 occurred rapidly following CD30 stimulation and SM treatment, and substantial loss of protein was observed within 15 min of treatment in both ALCL cell lines (Fig. 2.2A). Additionally, both stimuli resulted in p100 processing to the active non-canonical NF-κB subunit p52 at 3 h (Fig. 2.2A), consistent with previous reports describing the delayed kinetics of non-canonical NF-κB activation (Vallabhapurapu et al., 2008). Notably, CD30 consistently appeared to be a stronger inducer of NF-κB than SM treatment (Fig. 2.2), and this observation may be due to the presence of less nuclear NF-κB or weaker DNA-binding activity of NF-κB following SM treatment. A non-canonical NF-κB nuclear protein complex was detected at 3 h by EMSA (Fig. 2.2B-D), indicating that CD30 stimulation and SM treatment activate non-canonical NF-κB with similar kinetics. Activation of canonical NF-κB, however, differed between the two stimuli. CD30-mediated canonical NF-κB occurred rapidly, being observed 15-30 min following receptor activation, and remained observable throughout the entire time course (Fig. 2.2B, D). Conversely, SM-induced canonical NF-κB was delayed and initially observed at 1 h (Fig. 2.2C, D), and the level of active canonical NF-κB triggered by SM peaked around 6 h before decreasing throughout the remaining time points. This decrease in canonical NF-κB coincided with activation of non-canonical NF-κB (Fig. 2.2C, D). Taken together, these data indicated that CD30 activation and SM treatment induced c-

IAP1/2 degradation and activated the same NF-κB pathways, albeit with differing rates, suggesting potential mechanistic and functional differences.



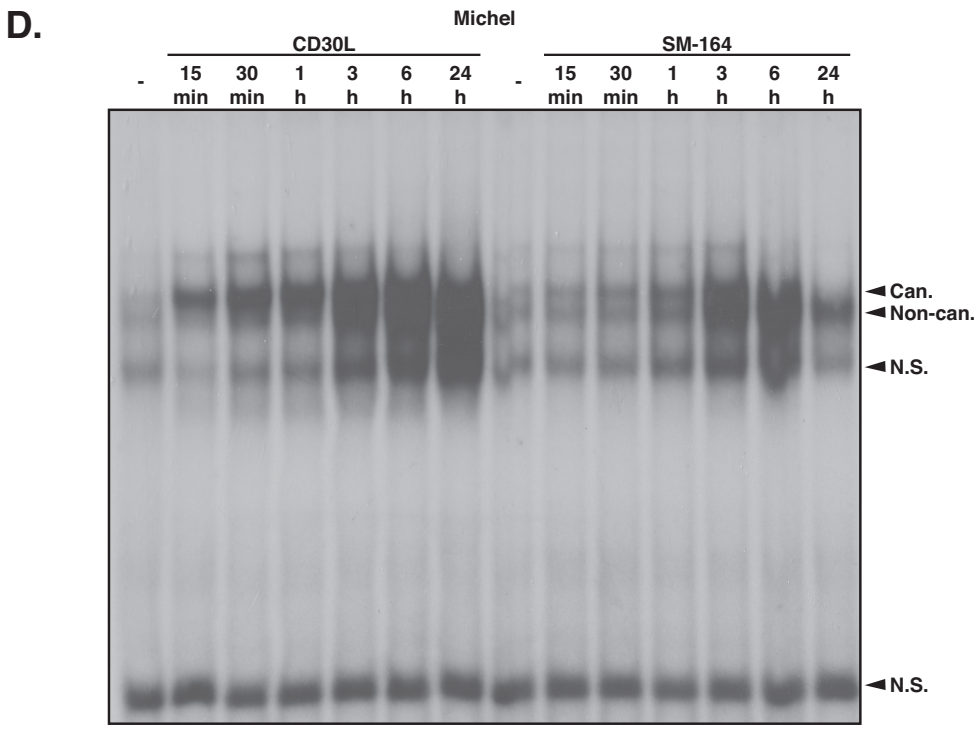


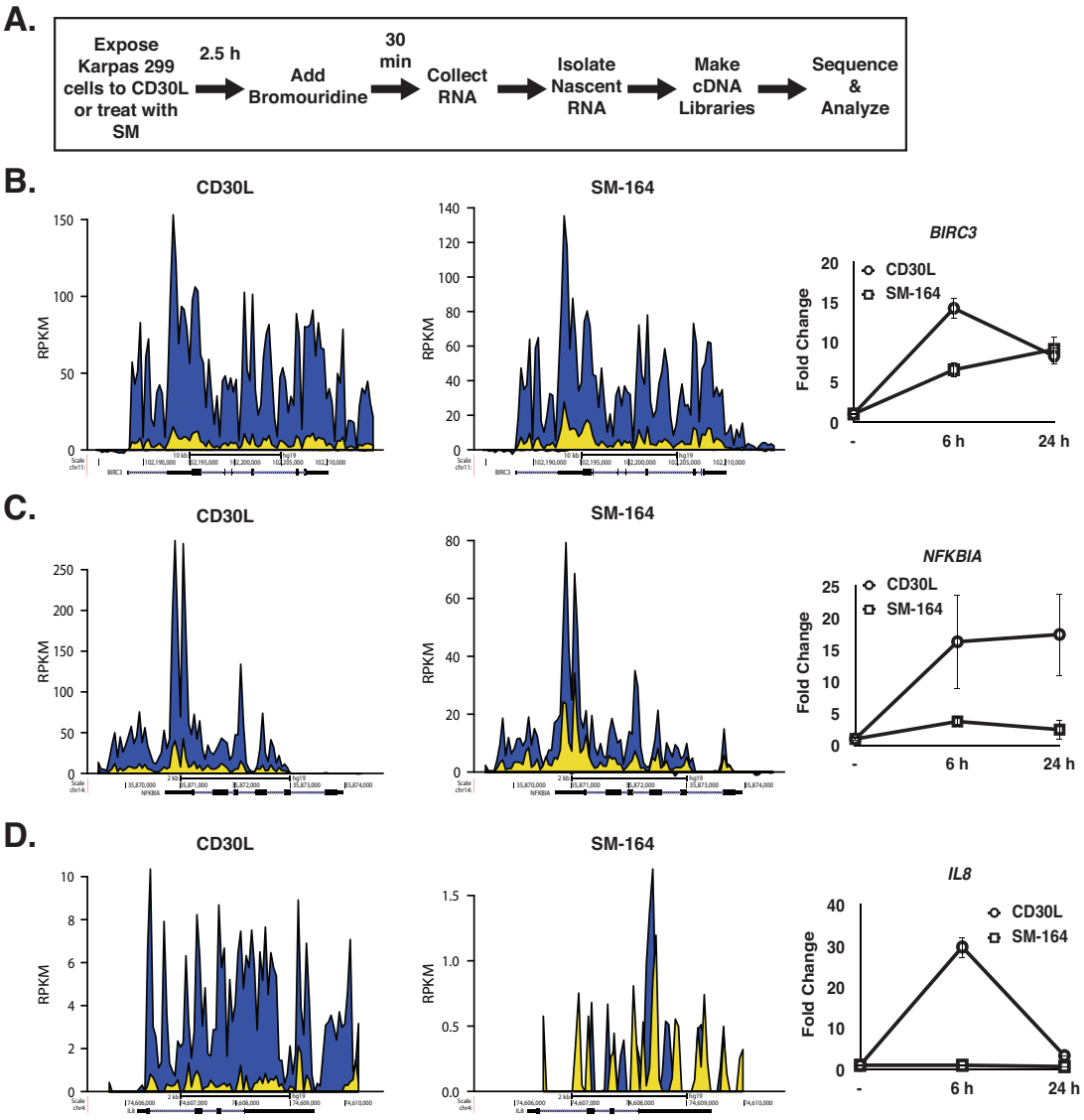

**Figure 2.2 Kinetics of NF-κB induction by receptor activation and SM treatment.** **A.** Karpas 299 and Michel cells were exposed to CD30L-expressing CHO cells or control CHO cells (A) or treated with 100 nM SM-164 or DMSO (B) for the indicated times. Whole cell lysates were collected and used to assess p100 processing and c-IAP1/2 degradation by Western blot. Bands marked with an asterisk (*) are non-specific. **B-D**. Karpas 299 (B, C) or Michel (D) cells were exposed to CD30L or incubated with 100 nM SM-164 for the indicated times. Nuclear extracts were prepared and NF-κB activation was measured by EMSA.

## Comparison of gene regulation induced by physiological and synthetic IAP antagonism

While the ability of SMs to activate NF-κB has been previously reported (Varfolomeev et al., 2007; Vince et al., 2007), it is not known if this resulted in the regulation of a similar gene expression profile as receptor signaling. To address this question, a recently developed technique, Bru-seq, that allows for transcriptome analysis of newly transcribed RNA was employed (Paulsen et al., 2013; Paulsen et al., 2014). As depicted schematically in Figure 2.3A, Karpas 299 cells were treated with SM or exposed to CD30L, incubated with bromouridine, and the bromouridine-labeled RNA was isolated

and converted into cDNA libraries for deep sequencing. In the initial analysis of the Bru-seq data, we compared the expression of two well-characterized NF-κB gene targets: *BIRC3*, which encodes the c-IAP2 protein, and *NFKBIA*, the gene encoding IκBα (Csomos et al., 2009; Paulsen et al., 2013; Kenneth et al., 2014). The profiles of the mapped sequencing reads were similar for both stimuli (Fig. 2.3B, C), indicating that the stimuli produced similarly processed transcripts. Transcription of both genes was induced upon each treatment, with *BIRC3* being the most highly transcribed gene in both cases. Compared to an unstimulated sample, transcription of *BIRC3* was induced 12-fold following CD30 activation, while SM treatment resulted in a 7-fold increase in *BIRC3* transcription (Fig. 2.3B). The Bru-seq results were mirrored by qRT-PCR experiments that also illustrated a more robust expression of genes following CD30 stimulation. Similar to *BIRC3*, expression of *NFKBIA* was also markedly higher following CD30L than SM (12-fold increase and 7-fold increase, respectively) (Fig. 2.3C). Since both stimuli degraded c-IAP1/2 to the same degree (Fig. 2.1A) and with similar rates (Fig. 2.2A, B), these results indicate that the receptor may provide additional signals that strengthen the magnitude of NF-κB activation. Notably, *IL8*, the gene encoding the interleukin-8 cytokine and a canonical NF-κB gene target (Paulsen et al., 2013), exhibited the second highest expression following CD30 stimulation (12-fold increase), but was not significantly affected by SM treatment (Fig. 2.3D), indicating different functional consequences of NF-κB activation by the two stimuli. Karpas 299 cells exposed to the control CHO cells exhibited a somewhat different pattern of basal gene expression compared to the cells treated with DMSO. However, this technical aspect did not appear to significantly affect the observed stimulus-induced gene expression profiles.

To better visualize and compare the stimuli-induced transcriptomes, the change in transcription of genes following SM treatment was plotted against the change in transcription of genes following CD30 activation (Fig. 2.3E). While certain genes were affected by both stimuli, such as *BIRC3* and *NFKBIA*, the transcription of other genes, like *IL8*, were modified by only one of the stimuli. Collectively, these data demonstrate that the transcriptional consequences of IAP antagonism by SM reflect aspects of receptor-induced signaling, while also providing evidence of functional differences between the two forms of IAP antagonism.



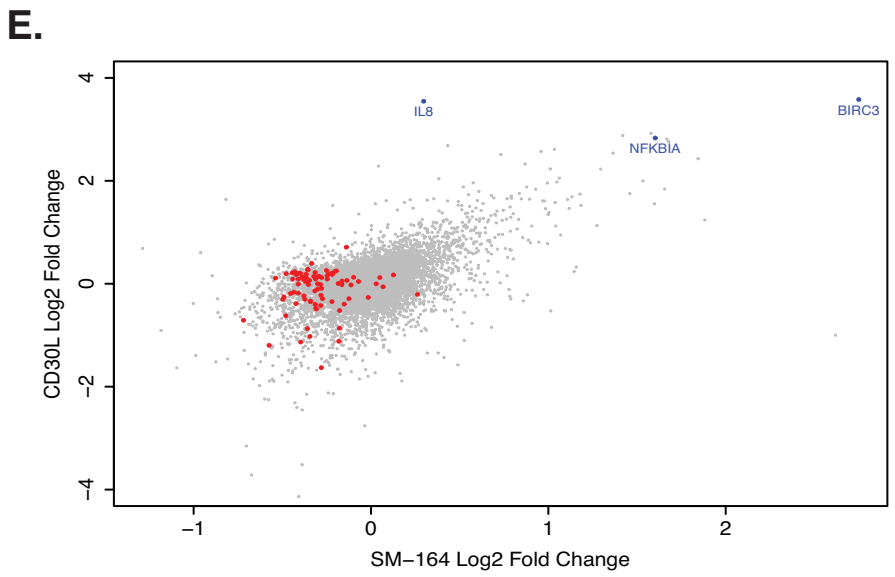
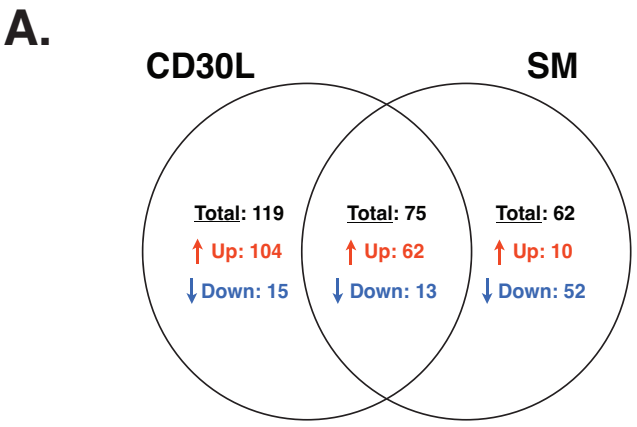



**Figure 2.3 Comparison of gene regulation induced by physiological and synthetic IAP antagonism.**
**A.** Diagram of the Bru-seq procedure. Karpas 299 cells were exposed to CD30L or treated with 100 nM SM-164 for the indicated times. **B-D.** Sequencing reads from nascent RNA expressed as reads per thousand base pairs per 1 million reads (RPKM) and mapped to the *BIRC3* gene (B), *NFKBIA* gene (C), and *IL8* gene (D) with reference sequence annotation below. The exons and UTRs are denoted as black lines. The CD30L and SM-164 treated samples are shown in blue, and the unstimulated control samples are shown in yellow. For the qRT-PCR, Karpas 299 cells were exposed to CD30L or treated with 100 nM SM-164 for the indicated times. RNA was isolated and converted to cDNA, and the expression of the indicated genes was measured. **E.** The log2 fold change of genes from the SM-164 treated Bru-seq sample were plotted against the log2 fold change of genes from the CD30L Bru-seq sample. The location of *BIRC3*, *NFKBIA*, and *IL8* are in blue, and the red dots represent the genes in the KEGG_RIBOSOME gene set. These experiments were performed with the help of Dr. Mats Ljungman and his lab.

### Gene set analysis reveals novel roles for c-IAP1/2 and IAP antagonists

The initial analysis of the transcriptome profiles generated by each stimulus highlighted the variety of genes regulated by c-IAP1/2 degradation. Due to the initial complexity of classifying these genes with their wide functional diversity, gene set enrichment analysis (GSEA) was performed, allowing for the identification of groups of genes that exhibit similar changes in expression using gene sets that have been

categorized based on shared, biologically relevant characteristics, such as belonging to a common enzymatic pathway or presence in the same cellular compartment (Subramanian et al., 2005). GSEA has advantages over traditional strategies of gene expression analysis, including the ability to detect biologically significant processes involving groups of genes that show only modest changes in expression (Subramanian et al., 2005). Analysis of the GSEA data indicated that CD30 activation and SM treatment collectively modulated 256 gene sets (Fig. 2.4A). There were 119 CD30-specific gene sets identified (Fig. 2.4A), and it was expected that CD30-specific gene sets would be identified since the receptor activated multiple pathways that were not activated by SM (Fig. 2.1). Examples of CD30-specific gene sets are shown in Figure 2.4B. Additionally, 62 SM-specific gene sets were identified (Fig. 2.4A) even though SM treatment was thought to mimic receptor signaling by degrading c-IAP1/2, suggesting that the SM may have additional, uncharacterized effects. These effects may be regulated by additional IAPs, such as X-linked inhibitor of apoptosis (XIAP), which can be antagonized by SMs (Varfolomeev et al., 2007; Vince et al., 2007; Lu et al., 2008). Notably, the majority of the SM-specific gene sets were down-regulated by the compound and several gene sets were functionally related to metabolism and protein synthesis (Fig. 2.4C).



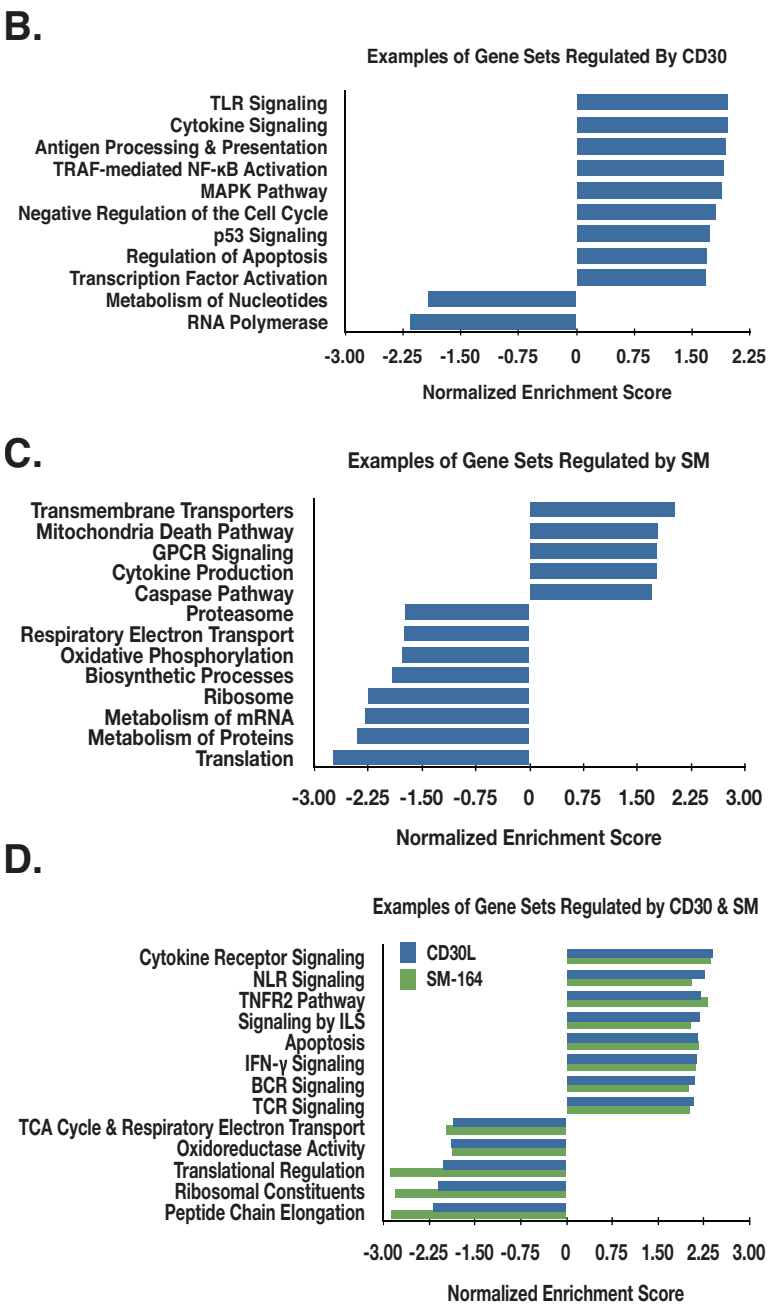



**Figure 2.4 Gene set analysis reveals novel roles for c-IAP1/2 and IAP antagonists.** **A.** A summary of the results from gene set enrichment analysis (GSEA) performed with the Bru-seq data. The numbers are gene sets modulated by the designated stimulus. The number of gene sets up and down-regulated are noted. **B-D.** Examples of gene sets with false discovery rates (FDR) < 0.05 that are regulated by CD30 alone (B), SM treatment alone (C), or regulated by both stimuli (D). The bars represent normalized enrichment score for the gene set. These experiments were performed with the help of Dr. Mats Ljungman and his lab.

The GSEA data identified 75 gene sets shared between CD30 activation and SM treatment (Fig. 2.4A). Many of these gene sets were expected, as they were related to functions shared between the two stimuli, such as regulation of NF-κB or involvement in TNF-R2 and cell death signaling cascades (Fig. 2.4D). Conversely, it was unexpected that there would be gene sets significantly down-regulated by both stimuli. Functionally, many of these gene sets were involved the regulation of the ribosome and translation, with SM treatment resulting in a more substantial down-regulation of the transcription of these genes and gene sets (Fig. 2.3E, 2.4D). Experiments with a different Smac mimetic, Birinapant, did not affect the expression of ribosomal genes to the extent of SM-164 (Fig. 2.5A). However, Birinapant was a weaker inducer of c-IAP2 degradation and NF-κB activation (Fig. 2.5B), suggesting that the effect on ribosomal gene expression is dependent on efficient degradation of both c-IAP1 and c-IAP2, as well as the subsequent activation of NF-κB. Additionally, treatment with the caspase inhibitor z-VAD-fmk abrogated the SM-induced down-regulation of ribosomal genes (Fig. 2.6), indicating a potential regulatory role for caspases in this process. While further work is needed to elucidate the exact mechanism, these analyses indicate that IAP antagonism by receptor activation or treatment with a synthetic compound has diverse functional consequences, including newly identified potential roles in the regulation of ribosome biogenesis and protein synthesis.

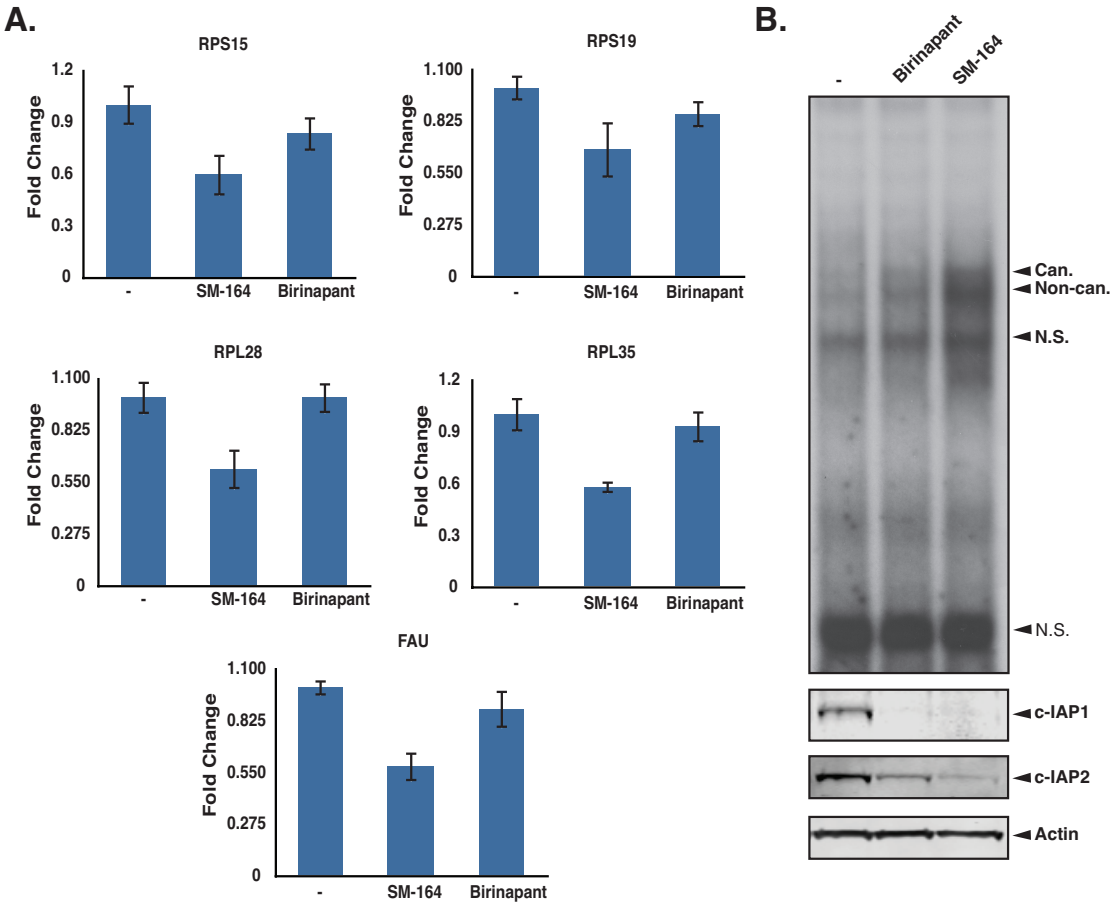



**Figure 2.5 Birinapant is a weak inducer of c-IAP2 degradation, NF-κB activation, and down-regulation of ribosomal gene expression.**
**A.** Karpas 299 cells were treated with 100 nM Birinapant or 100 nM SM-164 for 6 h. RNA was then isolated and converted to cDNA. The expression of the indicated ribosomal genes was measured by qRT-PCR. **B.** Karpas 299 cells were treated with 100 nM SM-164 or 100 nM Birinapant for 3 h. Nuclear extracts and whole cell lysates were then prepared from the samples. The nuclear extracts were used to assess NF-κB activation by EMSA, and the whole cell lysates were used to assess c-IAP degradation. Can. NF-κB, canonical NF-κB; Non-can. NF-κB, non-canonical NF-κB; N.S., non-specific.

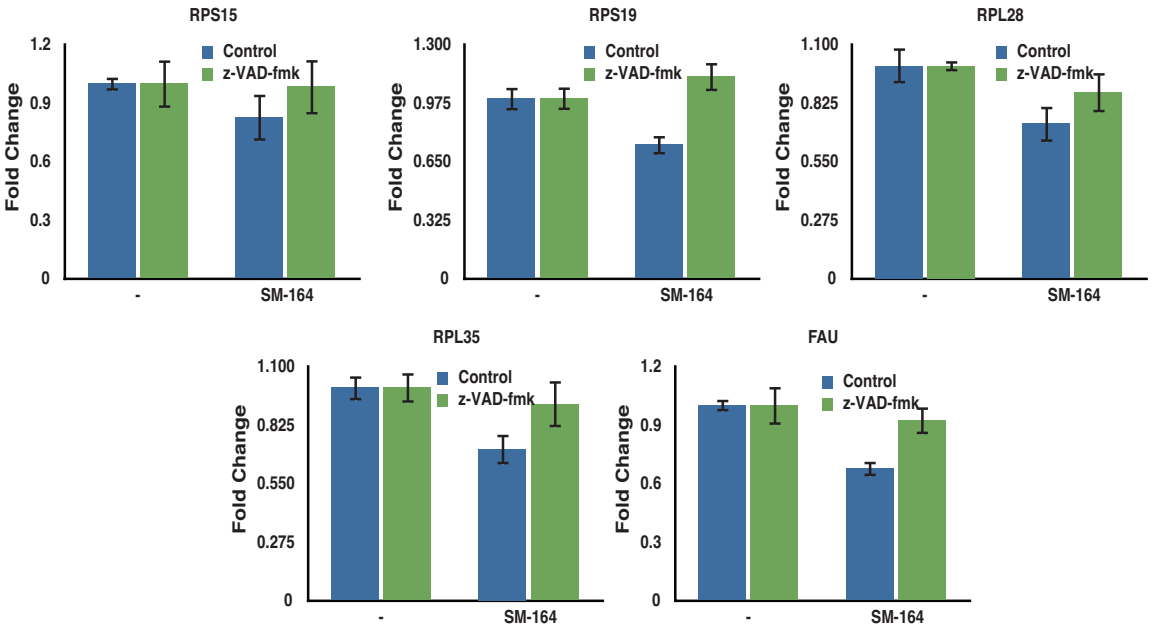



**Figure 2.6. Caspase inhibition prevents down-regulation of ribosomal gene expression.**
Karpas 299 cells were pretreated with 10 μM z-VAD-fmk for 1 h. The cells were then treated with 100 nM SM-164 for 6 h. RNA was then harvested and converted to cDNA. The expression of the indicated ribosomal genes was measured by qRT-PCR.

### IAP antagonism results in decreased protein synthesis

As described above, the GSEA results indicated novel roles for c-IAP1/2 in the regulation of genes related to the ribosome and translation. To test if the observed transcriptome results had a cellular consequence, protein synthesis was measured following CD30 activation and SM treatment using an assay based on incorporation of a methionine analogue (Signer et al., 2014; Zhang et al., 2014). Karpas 299 cells were treated with the designated stimulus and then incubated in L-methionine-free medium that had been supplemented with L-methionine or the methionine analogue L-homopropargylglycine (HPG). The incorporation of HPG was then assessed by flow cytometry. SM treatment resulted in a measurable decrease in protein synthesis by 3 h, and this decrease continued with longer treatment times (Fig. 2.7A-C). Importantly,

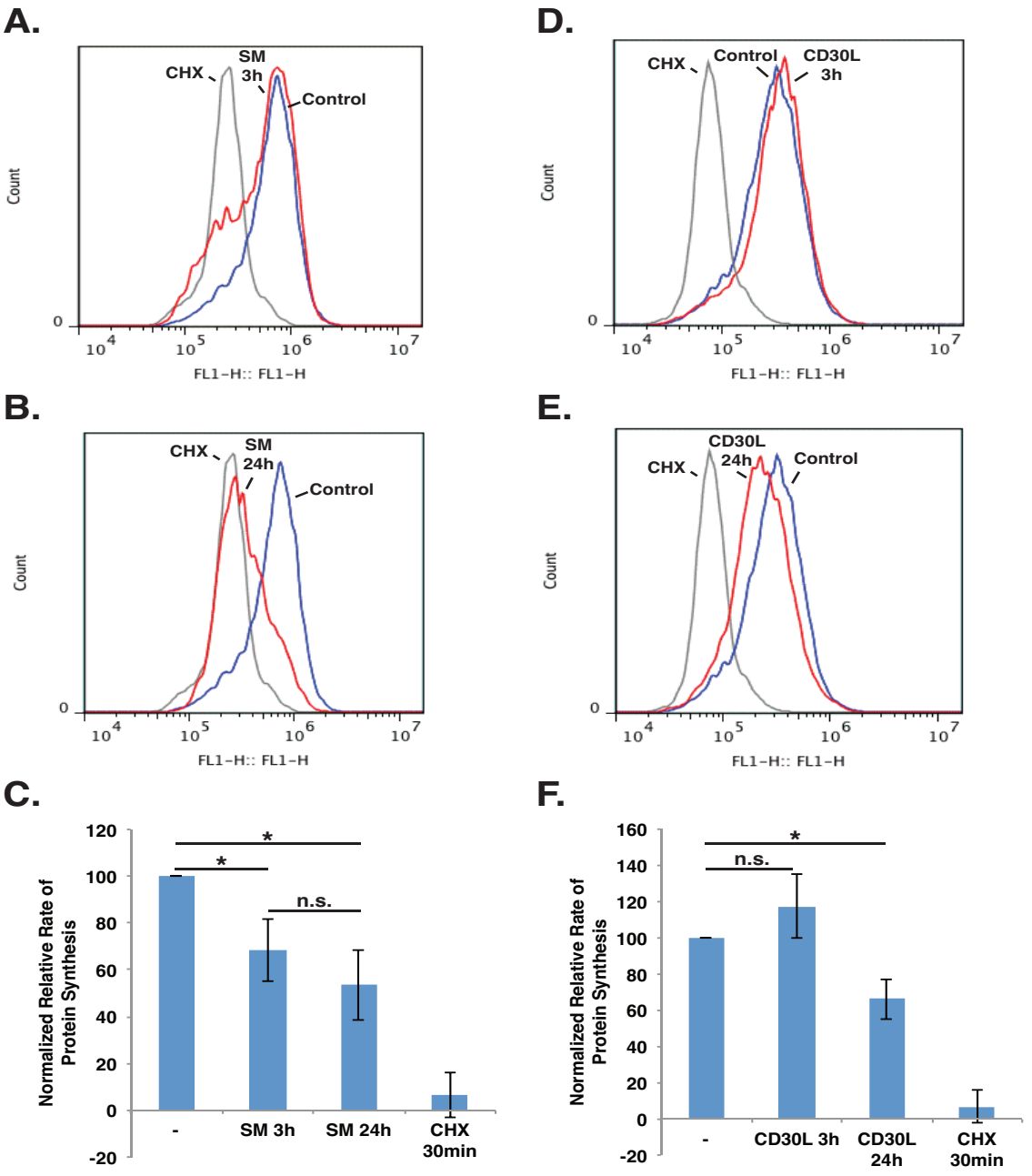



**Figure 2.7 IAP antagonism results in decreased protein synthesis.**
**A-C.** Karpas 299 cells were incubated with 100 nM SM-164 or 100 µg/mL cycloheximide (CHX) for the indicated times. The medium was then replaced with methionine-free medium supplemented with methionine or the methionine analogue HPG, and the cells were incubated for 1 h. HPG incorporation was measured by flow cytometry. **D-F.** Karpas 299 cells were exposed to CD30L or 100 µg/mL CHX for the indicated times. The medium was then replaced with methionine-free medium supplemented with methionine or HPG, and the cells were incubated for 1 h. HPG incorporation was measured by flow cytometry. Data represent the mean ± standard deviation of at least three independent experiments (*$P < 0.01$).

the decrease in protein synthesis was initially observed prior to any substantial SM-induced cell death (Fig. 2.8). In contrast, CD30 activation had a minor effect on protein synthesis at early time points and only had a substantial effect by 24 h (Fig. 2.7D-F), and similar results were also observed in Michel cells (Kocab et al., 2015). These findings indicate that IAP antagonism, in the system tested, has an inhibitory impact on global protein synthesis, revealing a novel regulatory consequence for c-IAP1/2 degradation and SM treatment.

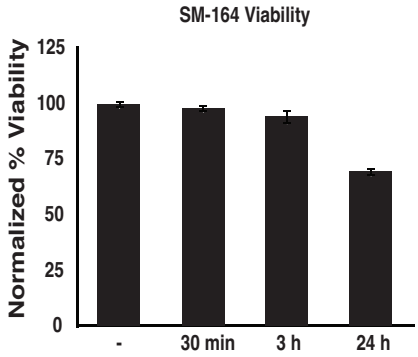



**Figure 2.8 Viability following Smac mimetic treatment.**
Karpas 299 cells were treated with 100 nM SM-164 for the indicated times, and cell viability was measured by propidium iodide exclusion and flow cytometry.

## Discussion

In this chapter, the consequences of IAP antagonism on gene regulation by physiological and synthetic stimuli were examined, and genes and pathways unique to each stimulus were identified, as were novel processes affected by both inducers of c-IAP1/2 degradation. One finding of particular interest was the down-regulation of genes related to the ribosome and translation. This observation was identified following SM treatment and CD30 activation, and resulted in decreased protein synthesis following both stimuli, though treatment with the Smac mimetic had an earlier observable effect on

protein synthesis than CD30 stimulation, which only became apparent at 24 h (Fig. 2.7). It has been previously established that SMs induce death in certain cells, and that this killing is dependent on TNF (Varfolomeev et al., 2007; Vince et al., 2007). However, this study provides evidence suggesting an additional mechanism of SM killing. In this model, SM triggers the degradation of c-IAP1/2 and results in the shutdown of protein synthesis, partially mimicking the effects of cycloheximide. The lethality of TNF would then be due, at least in part, to the inability to synthesize pro-survival proteins, similar to established mechanisms of TNF killing (Vandenabeele et al., 2010; Darding and Meier, 2012; Oeckinghaus et al., 2011).

The consequences of CD30-mediated degradation of c-IAP1/2 differed from the IAP antagonism induced by SM treatment, as CD30 activation did not exert as rapid of an effect on protein synthesis as compared to SM treatment. Furthermore, the decrease in protein synthesis was observed at 24 h, which coincided with previous reports of CD30-mediated cell cycle arrest at that time point (Wright et al., 2007; Buchan and Al-Shamkhani, 2012a). Since there is an established connection between cell cycle arrest and decreased protein synthesis (Ruggero and Pandolfi, 2003), this observation raises the intriguing possibility that c-IAP1/2 may regulate cell cycle arrest through their control of protein synthesis.

As noted above, CD30 and SM affected protein synthesis at different rates, and this may be due to several reasons, such as variable expression of CD30 on the cells, additional pathways activated by receptor stimulation such as MAPK pathways (Fig. 2.1), unique genes regulated by the receptor (Fig. 2.3, 2.4), or a combination thereof. Similarly, the unique consequences of SM treatment may contribute to the observed

differences. One potentially important difference may be the mechanism of canonical NF-κB activation by CD30, which appears to differ from SM-induced canonical NF-κB. CD30, like its close TNFR superfamily relatives, is thought to activate NF-κB through its TRAF binding domains (Buchan and Al-Shamkhani, 2012a; Matsuzawa et al., 2008; Duckett et al., 1997). However, it has also been shown that CD30 can activate NF-κB in the absence of its TRAF binding domains (Buchan and Al-Shamkhani, 2012b; Duckett et al., 1997), suggesting that CD30 may activate multiple NF-κB pathways with undefined functions. Less is known, however, about SM-induced canonical NF-κB. Since SM-induced canonical NF-κB appears to be due to c-IAP1/2 degradation, it may be reliant on the accumulation of NIK, a protein normally associated with non-canonical NF-κB activation but that also has a reported ability to activate canonical NF-κB (Malinin et al., 1997; Zarnegar et al., 2008). The potential mechanistic difference in canonical NF-κB activation is supported by the delayed kinetics of NF-κB activation after SM treatment (Fig. 2.2), possibly suggesting a reliance on the accumulation of NIK. Furthermore, SM treatment resulted in a weaker NF-κB signal (Fig. 2.2), potentially indicating additional mechanistic divergence from receptor signaling. Moreover, the canonical NF-κB activated by CD30 may be functionally distinct from the NF-κB activated by SM, potentially explaining the unique gene regulation by each stimulus.

In addition to differences in downstream gene targets, the mechanisms of receptor-induced and pharmacological IAP antagonism are intrinsically different. Receptor-mediated c-IAP1/2 degradation occurs in parallel with TRAF2 degradation (Csomos et al., 2009; Duckett and Thompson, 1997) and is dependent on translocation of the c-IAP:TRAF2 complex to an insoluble cellular fraction (Wright et al., 2007). In

contrast, SMs directly bind to c-IAP1/2 and selectively trigger their autoubiquitination and subsequent degradation without degrading TRAF2 (Darding et al., 2011; Sun et al., 2007; Csomos et al., 2009). This highlights another facet of SMs not shared with receptor signaling that may have downstream consequences, as it has been reported that TRAF2 overexpression can trigger p100 processing (Csomos et al., 2009). Furthermore, SMs have been shown to inhibit XIAP, and while SM-induced degradation of XIAP is not always observed (Lu et al., 2008), the SM may still bind and inhibit XIAP, a component in multiple signaling pathways (Galban and Duckett, 2010; Kenneth and Duckett, 2012), potentially resulting in cellular consequences distinct from CD30 activation, which is not thought to affect XIAP. Notably, caspase inhibition appeared to negate SM-induced ribosomal gene regulation (Fig. 2.6), potentially supporting a role for XIAP and its caspase-binding activity. In addition to providing insight into the functions of c-IAP1/2 in signaling, these findings highlight unique aspects of Smac mimetics that should be useful for defining their therapeutic value, including their regulation of protein synthesis.

## Materials and Methods

**Cell lines and culture conditions.** Karpas 299 and Michel cells were grown in RPMI 1640 (Mediatech, Herndon, VA, USA) medium supplemented with 10% FBS and 2 mM L-glutamine. The generation of the $CD30L^+$ Chinese hamster ovary (CHO) cells has been previously described (Wright et al., 2007). CHO cells and $CD30L^+$ CHO cells were cultured in F-12 nutrient medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS and 2 mL L-glutamine. All cells were maintained at 37°C in an atmosphere of 5% $CO_2$.

**Materials.** The following primary antibodies were used in this study: anti-p100/p52 (Millipore); anti-c-IAP1 (Enzo Life Sciences, Farmingdale, NY, USA); anti-c-IAP2 (Cell Signaling, Danvers, MA, USA); anti-GST, anti-p65, and anti-RelB (Santa Cruz Biotechnology, San Diego, CA, USA); anti-phospho-JNK, anti-JNK, anti-phospho-ERK, and anti-ERK (Cell Signaling); and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). The Smac mimetic SM-164 (Sun et al., 2007) was a kind gift from Dr. Shaomeng Wang (University of Michigan). Materials used in this study were: Birinapant (ChemieTek, Indianapolis, IN, USA), Enbrel (University of Michigan Hospital pharmacy), z-vad-fmk (Cayman Chemical, Ann Arbor, MI, USA), Trametinib (LC Laboratories, Woburn, MA, USA) and SP600125 (SelleckChem, Houston, TX, USA).

**CD30 stimulation.** As described previously (Wright et al., 2007), Karpas 299 cells were exposed for the times indicated to either CHO cells (negative control) or $CD30L^+$ CHO cells which had been seeded in 10 cm plates. Following the CD30 stimulation, the Karpas 299 cells were collected from the CHO cells with gentle pipetting.

**Cell lysate and nuclear extract preparation.** Cells were treated as described in the figure legends. Following treatment, the cells were collected and centrifuged at 100 × g for 5 min. Medium was then aspirated, and cells were resuspended in PBS. The cell suspension was then divided into tubes, and whole cell lysates and nuclear extracts were subsequently prepared. Whole cell lysates were prepared using RIPA lysis buffer in a process that has been described previously (Kenneth et al., 2014). For the nuclear extract

preparation, cells were washed once with buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, and 0.5 mM DTT) and pelleted by centrifugation at 1500 × g for 1 min. The supernatant was aspirated, and the cell pellet was resuspended in 15 µL of cold buffer A supplemented with 0.1% NP-40. The samples were incubated on ice for 5 min before being centrifuged at 16000 × g in a microfuge at 4°C for 15 min. The supernatant was removed, and the pellet was thoroughly resuspended in 10 µL of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM PMSF, 0.6 mM DTT, and 25% glycerol) and incubated at 4°C for 20 min with occasional mixing. The samples were then centrifuged at 16000 × g at 4°C for 15 min. The supernatant was transferred to a fresh tube containing 60 µL of modified buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 20% glycerol). The nuclear extracts were stored at -80°C.

**Immunoblot analysis.** Protein concentrations of whole cell lysates were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Lysates of equal protein concentrations were prepared in LDS sample buffer (Invitrogen, Carlsbad, CA, USA), separated on denaturing NuPAGE 4-12% polyacrylamide gradient gels (Invitrogen), and transferred to nitrocellulose membranes (GE Healthcare, Amersham, UK). Membranes were blocked in a 1:1 mixture of Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) and Tris-buffered saline (TBS). Membranes were then incubated with primary antibodies in a 1:1 mixture of Odyssey blocking buffer and TBS containing 0.1% Tween 20 (Fisher BioReagents, Waltham, MA, USA) overnight at 4°C. Following washing with TBS with 0.1% Tween 20, membranes were incubated with

IRDye secondary antibodies (Li-Cor) for 1 h at room temperature. Membranes were then washed with TBS and analyzed using the Odyssey CLx infrared imaging system (Li-Cor) according to the manufacturer's instructions.

**Electrophoretic mobility shift assays.** Two complimentary oligonucleotides containing NF-κB consensus binding sites (5'-GATCCAGGGACTTTCCGCTGGGGACTTTCCA-3' and 5'-GATCTGGAAAGTCCCCAGCGGAAAGTCCCTG-3') were annealed and radiolabeled using T4 polynucleotide kinase (New England BioLabs, Ipswich, MA, USA) in the presence of [$\gamma$-$^{32}$P] ATP. The radiolabeled probe was then purified using illustra Microspin G-25 Columns (GE Healthcare) according to the manufacturer's instructions. The presence of NF-κB in the nuclear extracts was assessed by incubating 2 μL of the nuclear extract in modified buffer D without glycerol along with 1 μg of poly(dI-dC)•poly(dI-dC), 1 μL of Bluejuice loading dye (Invitrogen), and 0.1 μL of the radiolabeled probe for a total volume of 20 μL. The prepared samples were then separated on a non-denaturing 4% polyacrylamide gel. Supershift assays were performed by adding 2 μL of antibodies against GST, p65, or RelB (Santa Cruz Biotechnologies) to the reaction mixture. Samples were incubated for 20 min at room temperature prior to running on the gel. For all assays, free probe was run off the gel to obtain maximum resolution. Autoradiography was conducted overnight at -20°C.

**Quantitative real-time PCR.** Cells were treated as indicated in the figure legends. Following treatment, the cells were washed with PBS, and total RNA was isolated using the RNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacture's

instructions. 1 μg of total RNA was converted to cDNA using a reverse transcription reaction with random hexamer primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). 1 μL of the resulting cDNA was analyzed for the indicated target genes using the ViiA 7 Real-Time PCR System (Applied Biosystems). Each target assay was normalized to β-actin or 18*S* levels.

**Transcriptome analysis by Bru-seq.** Karpas 299 cells were incubated with the treatments indicated in the figure legends. To label nascent RNA, 2 mM bromouridine (Bru) was added to the media for the final 30 min of treatment time. The Bru-seq procedure has been previously described in detail (Paulsen et al., 2013; Paulsen et al., 2014). Briefly, total RNA was collected from the treated cells using TRIzol reagent (Invitrogen), and the Bru-labeled, nascent RNA was isolated using anti-BrdU antibodies (BD Biosciences, San Jose, CA, USA) conjugated to magnetic beads (Invitrogen). The isolated RNA was converted into cDNA libraries, which were sequenced at the University of Michigan Sequencing Core using an Illumina HiSeq 2000 sequencer. The sequencing and read mapping were performed as previously described (Paulsen et al., 2013; Paulsen et al., 2014). GSEA was used to identify up-regulated and down-regulated gene sets by determining which associated genes were significantly enriched in each gene set (Subramanian et al., 2005). The log fold change in expression of genes greater than 1kb and expressed above 0.5 RPKM was used as the ranking metric by GSEA. The gene sets were obtained from version 4.0 of the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The gene sets used were canonical pathways (KEGG, Reactome, and BioCarta) and gene ontologies (biological

processes, molecular functions, and cellular compartment). Gene sets with FDR corrected P-values lower than 0.01 were considered to be significantly enriched and were used in the analysis. The primary sequencing data files from this study have been deposited in the NCBI Gene Expression Omnibus (GEO accession number: GSE64927).

**Measurement of protein synthesis.** Cells were stimulated as indicated in the figure legends. Following the designated times, the cells were collected, and centrifuged at 100 × g for 5 min. The supernatant was removed, and the cells were resuspended in L-methionine free RPMI (Invitrogen) supplemented with 1 mM L-methionine or HPG (50 μM final concentration), and were incubated at 37°C and 5% $CO_2$ for 1 h. The cells were then harvested, washed with PBS, and fixed in 50% ice cold ethanol overnight at -20°C. The samples were then processed using the Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and protein synthesis was measured by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences). Relative rates of protein synthesis were calculated by normalizing the treated HPG sample to the control HPG sample after subtracting the auto-fluorescence background.

**Viability assays.** Following the indicated treatments, cells were harvested, washed with PBS and subsequently resuspended in PBS with 2 μg/mL propidium iodide (PI). The cell viability of the PI-stained cells was assessed by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

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# CHAPTER III

## Orchestration and Consequences of Canonical and Non-canonical NF-κB Activation by Synthetic IAP Antagonism

### Summary

Smac mimetics (SMs) have been shown to degrade the c-IAPs and activate NF-κB, but are known to exhibit varying effects in different cellular contexts. To better understand the consequences of SM-induced signaling in different contexts, this signaling node was further investigated, finding that SM-induced activation of NF-κB was cell type-specific, and activation of canonical NF-κB corresponded to SM-induced cell death. Notably, p100 processing occurred in all tested cells, but nuclear, DNA-binding non-canonical NF-κB was cell type-specific, suggesting that p100 processing is a poor surrogate marker for non-canonical NF-κB activation. Additionally, suppression of the non-canonical NF-κB subunit RelB resulted in a prolonged canonical NF-κB activation following extended treatment with SM, and this was associated with increased cell death and suppressed gene expression, collectively illustrating regulatory crosstalk between the two NF-κB signaling pathways. In contrast, prolonged SM treatment in cells possessing RelB resulted in gene expression and cell viability, suggesting that RelB-dependent gene expression was necessary to abrogate the DNA binding of canonical NF-κB. These results identify key consequences of both NF-κB signaling pathways, and further our understanding of the therapeutic potential of Smac mimetics.

## Introduction

As described in Chapter I, the c-IAPs are highly expressed in a variety of cancers, and amplification of the IAPs has been associated with poor survival outcomes in cancer, including colorectal, pancreatic, and cervical squamous cell carcinoma (Esposito et al., 2007; Krajewska et al., 2005; Imoto et al., 2002). For these reasons, the c-IAPs are actively being investigated as potential therapeutic targets. A class of small molecule compounds based on the IAP binding protein, Smac, has been developed to target the IAPs. These Smac mimetics bind and induce the autoubiquitination and subsequent degradation of the c-IAPs, and this synthetic IAP antagonism has been demonstrated to result in cell death in a subset of cells (Vince et al., 2007; Varfolomeev et al., 2007; Lu et al., 2008). As discussed in Chapter I, these compounds have also been shown to activate NF-κB, a family of transcription factors that regulates genes involved in a variety of processes, including inflammation, cancer, and immune responses (Vince et al., 2007; Varfolomeev et al., 2007; DiDonato et al., 2012; Perkins, 2012). In mammalian cells, NF-κB is comprised of five members, p65/RelA, RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), that dimerize to form the active DNA-binding transcription factors (Ghosh and Hayden, 2012; Smale, 2012).

The canonical NF-κB heterodimer p50:p65 has been extensively investigated (Vallabhapurapu and Karin, 2009), and activation of canonical NF-κB is triggered by a diverse set of stimuli, including DNA damage and stimulation of innate and adaptive immune receptors. As illustrated in more detail in Chapter I, prior to activation, the NF-κB subunits are sequestered in the cytoplasm by the inhibitor of κB (IκB) proteins (Hayden and Ghosh, 2008), but upon stimulation, the IκB proteins are phosphorylated by

the IκB kinase (IKK) complex and targeted for ubiquitination, resulting in their proteasome-mediated degradation and the subsequent translocation of the NF-κB dimers into the nucleus to effect gene expression (Ghosh and Hayden, 2012). The c-IAPs mediate the activation of canonical NF-κB following stimulation of certain members of the tumor necrosis factor receptor (TNF) receptor superfamily, such as TNF-R1, and the absence of the c-IAPs induced by RNA interference or SM treatment abrogates signaling from these receptors, demonstrating a dependency on active c-IAP participation in this cascade (Varfolomeev et al., 2008). In contrast to TNF-R1-mediated signaling, SM-induced NF-κB activation occurs in a receptor-independent manner through c-IAP degradation and modulation of NF-κB inducing kinase (NIK) protein levels, which has been shown to activate both canonical and non-canonical NF-κB (Malinin et al., 1997; Zarnegar et al., 2008).

In contrast to our knowledge of canonical NF-κB activation, our understanding of the non-canonical NF-κB dimer p52:RelB is much less extensive. While canonical NF-κB is activated by many stimuli, there are a more limited number of activators of non-canonical NF-κB, and the best-characterized activators of this pathway are a subgroup of receptors within the TNF receptor superfamily that include CD30, CD40, and TNF-R2 (Hacker et al., 2011; Wright et al., 2007; Wright and Duckett, 2009). As discussed in Chapter I, ligand binding causes these receptors to recruit and induce the degradation of TNF receptor-associated factor 2 (TRAF2), TRAF3, and c-IAP1/2 (Vallabhapurapu and Karin, 2009; Csomos et al., 2009; Vallabhapurapu et al., 2008). Prior to stimulation, c-IAP1/2 inhibit non-canonical NF-κB activation by constitutively ubiquitinating and degrading NIK, but after their receptor-mediated degradation, NIK accumulates and

triggers phosphorylation of IKKα, which in turn phosphorylates p100, leading to the proteasome-dependent processing of p100 to the p52 form. The p52 subunit can then dimerize with RelB to form the active transcription factor (Vallabhapurapu and Karin, 2009; Sun, 2012).

In Chapter II, the consequences of two forms of IAP antagonism, SM treatment and CD30 activation, were compared, leading to the identification of novel functions for the c-IAPs, including regulation of protein synthesis (Kocab et al., 2015). While the studies in Chapter II compared CD30 and SM-induced NF-κB activation, previous work has connected NF-κB activation to SM-mediated cell death, illustrating that cells were either sensitive or resistant to SM-induced cell death and that this cell death was linked to NF-κB activation (Vince et al., 2007; Varfolomeev et al., 2007; Tenev et al., 2011; Petersen et al., 2007). Therefore, it was unclear if the findings in Chapter II would be universally observed in other cell lines. To address this, multiple cell lines were treated with the bivalent Smac mimetic, SM-164. The data presented in this chapter demonstrated that SM-induced c-IAP degradation results in cell specific consequences, and that SM-induced activation of canonical and non-canonical NF-κB was limited to a subset of cell lines. Furthermore, transcriptome analysis of SM-treated cells revealed major differences between the cell lines, potentially identifying novel consequences of SM treatment. Additionally, while not all cell lines activated non-canonical NF-κB following SM treatment, an effect that may be due to the absence of receptors or mutation of proteins in the signaling cascade, suppression of RelB in cells competent to activate non-canonical NF-κB resulted in a heightened sensitivity to SM-induced death, indicating that non-canonical NF-κB provided a protective advantage following SM

treatment. The protective effect conferred by non-canonical NF-κB stemmed from a modulation of the DNA binding of canonical NF-κB, and subsequent transcriptome analysis demonstrated that RelB was necessary to reverse the effects of SM-induced canonical NF-κB through the RelB-dependent expression of regulators of canonical NF-κB. Collectively, the findings presented in this chapter identify key characteristics of non-canonical NF-κB, and further our understanding of the mechanism of SMs, as well as their therapeutic potential.

## Results

### Smac mimetic-induced NF-κB activation is cell type-specific and corresponds with cell death

Smac mimetic treatment has been previously shown to induce NF-κB activation, which has been linked to SM-induced cell death (Varfolomeev et al., 2007; Vince et al., 2007; Lu et al., 2008), and this earlier work with SMs collectively demonstrated that SM treatment either triggers cell death alone in some cell types, or sensitizes cells to TNF-mediated cell death (Varfolomeev et al., 2007; Vince et al., 2007; Tenev et al., 2011; Petersen et al., 2007). In Chapter II, the outcomes of SM treatment were investigated in the anaplastic large cell lymphoma (ALCL) cell line Karpas 299, characterizing canonical and non-canonical NF-κB activation and identifying new functional aspects of c-IAP1/2 (Kocab et al., 2015). To further investigate SM-induced NF-κB activation and its consequences, activation of NF-κB after SM treatment was assessed in multiple cell lines with varying sensitivity to SM-induced cell death (Tenev et al., 2011). The presence of nuclear, DNA-binding NF-κB following SM treatment was determined by electrophoretic

mobility shift assay (EMSA), and the results indicated that activation of NF-κB varied among the cell lines tested (Fig. 3.1A). Canonical NF-κB was robustly activated by SM treatment in the breast cancer cell line MDA-MB-231 and in Karpas 299 cells, but was not induced in HEK293 or in the colon cancer cell line HCT116. Additionally and in agreement with previously published reports, the treated cell lines exhibited differing degrees of sensitivity to SM-induced cell death (Fig. 3.1B) with cell death being observed in Karpas 299 cells and MDA-MB-231 cells, and resistance to SM-induced death being exhibited by HEK293 and HCT116 cells, indicating the requirement for additional stimuli to induce cell death in these cells. Taken together, these data support the proposed connection between canonical NF-κB activation and SM-induced cell death through the canonical NF-κB-dependent production of autocrine TNF (Vince et al., 2007; Varfolomeev et al., 2007).

To further test the role of canonical NF-κB in promoting SM-induced cell death, MDA-MB-231 cells, which were sensitive to SM-induced death (Fig. 3.1B) and activated canonical NF-κB following SM treatment but not non-canonical NF-κB (Fig. 3.1A) were examined. SM treatment of MDA-MB-231 cells with suppressed expression of p65, a subunit of the canonical NF-κB dimer, resulted in higher viability as compared to the control cells, suggesting that activation of canonical NF-κB contributed to SM-induced cell death (Fig. 3.1C).

While differences in activation of canonical NF-κB supported the previous reports describing that SM-induced cell death is dependent on canonical NF-κB (Vince et al., 2007; Varfolomeev et al., 2008), differences in non-canonical NF-κB activation were also observed. While SM treatment has been previously shown to activate non-canonical

NF-κB as indicated by p100 processing (Varfolomeev et al., 2007; Vince et al., 2007), activation of nuclear, DNA-binding non-canonical NF-κB following SM treatment was found to be cell type-specific, as the SM-induced nuclear, DNA-binding non-canonical NF-κB RelB:p52 heterodimer was only observed in the Karpas 299 cells (Fig. 3.1A). To further investigate this, processing of p100 to p52, a major event following c-IAP1/2 degradation that is widely used as a surrogate marker for non-canonical NF-κB activation (Vallabhapurapu et al., 2008; Csomos et al., 2009; Vince et al., 2007; Varfolomeev et al., 2007), was assessed in order to determine if this event occurred globally or was also cell type-specific. In contrast to the EMSA data that illustrated the cell type-specific activation of non-canonical NF-κB, p100 processing was universally observed in the multiple cell types (Fig. 3.1D), suggesting that p100 processing was a poor surrogate assay for non-canonical NF-κB activation. Additionally, as expected, SM treatment triggered the degradation of c-IAP1 and c-IAP2, but, notably, did not degrade XIAP under the conditions used here (Fig. 3.1D), suggesting that the observed effects were due to the degradation of the c-IAPs and not due to XIAP degradation. Collectively, these data demonstrate that SM treatment results in nuclear, DNA-binding canonical and non-canonical NF-κB in a cell type-specific manner.

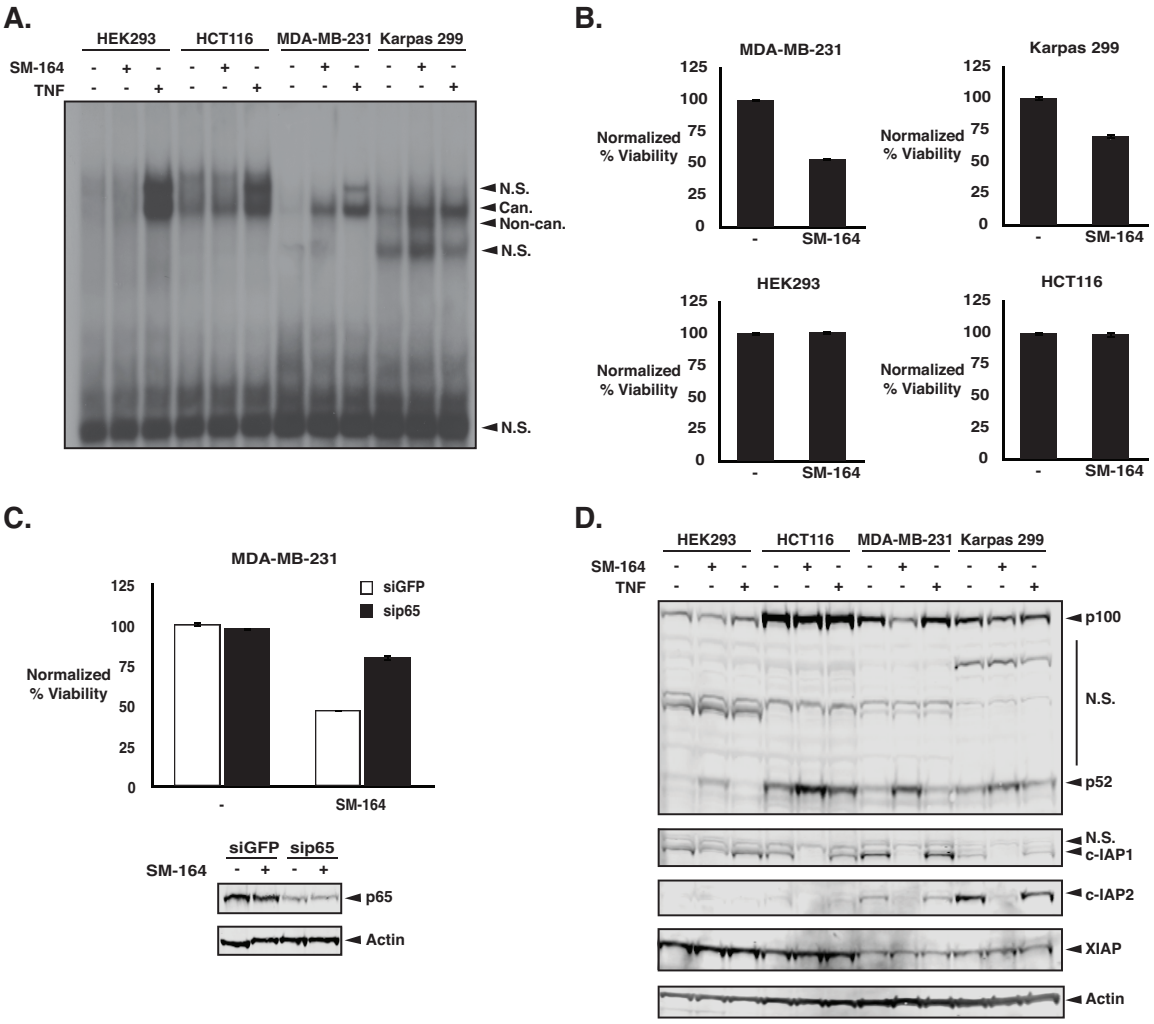


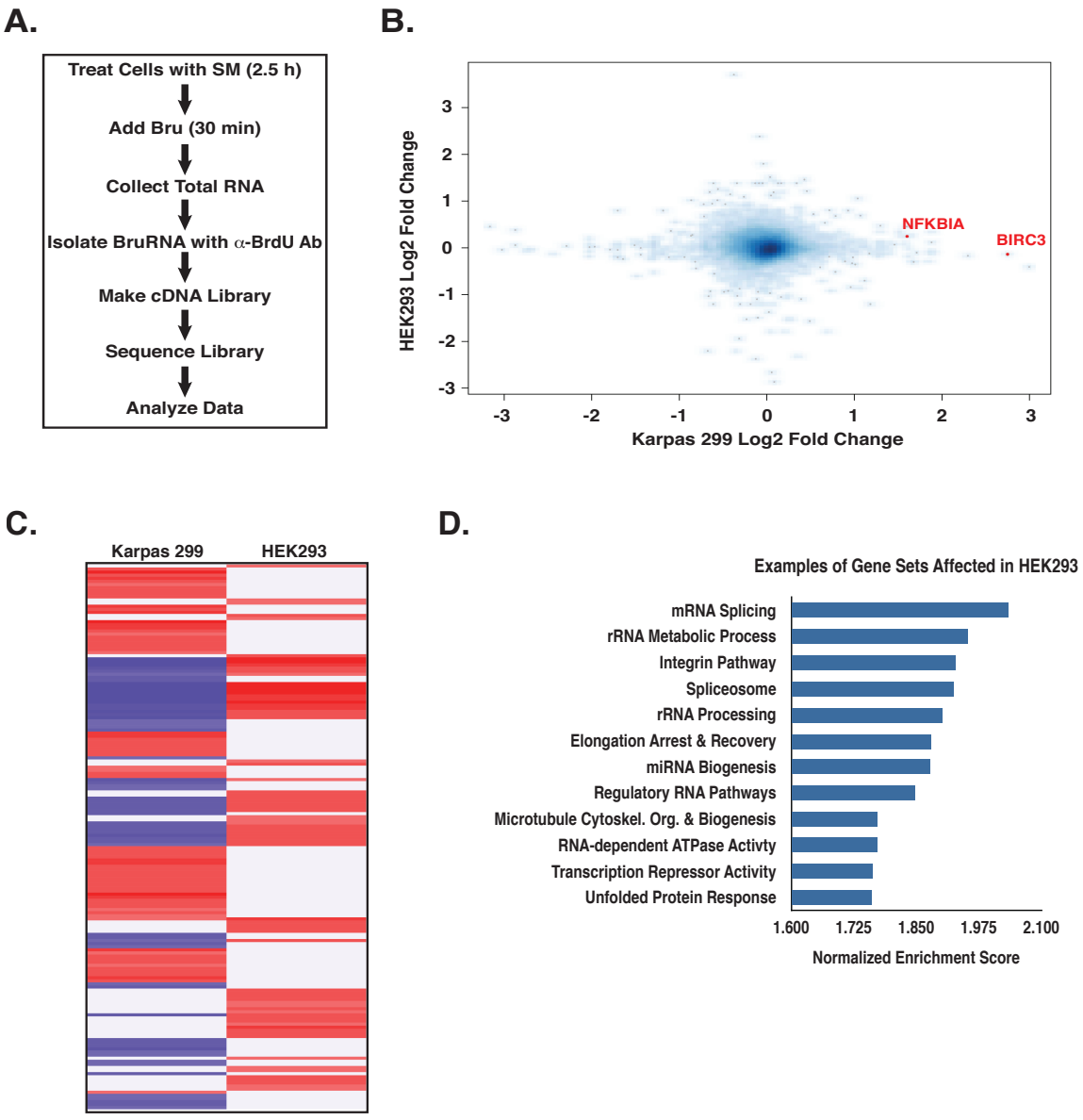

**Figure 3.1 SM-induced NF-κB activation is cell type-specific and corresponds with cell death**

**A.** HEK293, HCT116, MDA-MB-231, and Karpas 299 cells were treated with 100 nM SM-164 for 3 h or 500 U/mL TNF for 30 min. Nuclear extracts were prepared, and NF-κB was analyzed by EMSA. **B.** HEK293, HCT116, MDA-MB-231, and Karpas 299 cells were treated with 100 nM SM-164 for the indicated time. Following incubation, cell viability was measured by propidium iodide (PI) exclusion and flow cytometry. **C.** MDA-MB-231 cells were transfected with siRNA targeting p65 or GFP (control). Cells were treated with 100 nM SM-164 for 24 h. Following treatment, whole cell lysates were prepared and analyzed by Western blot, and cell viability was measured using PI exclusion and quantitated by flow cytometry. **D.** HEK293, HCT116, MDA-MB-231, and Karpas 299 cells were treated with 100 nM SM-164 for 3 h or 500 U/mL TNF for 30 min. Whole cell lysates were prepared and analyzed by Western blot using the specified antibodies. Can. NF-κB, canonical NF-κB; Non-can. NF-κB, non-canonical NF-κB; N.S., non-specific.

**Gene expression profiles differ between cell lines following Smac mimetic treatment**

While activation of NF-κB was found to be cell-type specific, it was unclear if this specificity was also applicable to the downstream consequences of SM treatment. To investigate the functional consequences of SM-induced NF-κB, a recently developed technique, Bru-seq, was employed to characterize the transcriptome of SM-treated cells through the analysis of newly transcribed RNA (Paulsen et al., 2013; Paulsen et al., 2014). As described in Chapter II, Bru-seq was used to compare the outcomes receptor-mediated and SM-induced c-IAP antagonism in Karpas 299 cells (Kocab et al., 2015), identifying novel consequences of IAP antagonism. To test if the effects observed following SM treatment were universal, HEK293 cells, which did not activate NF-κB following SM treatment (Fig. 3.1A), were treated with SM-164, and transcriptome profiles were generated by Bru-seq and compared to the Karpas 299 data. HEK293 cells were treated with SM in the same experimental conditions as for the Karpas 299 cells, incubated with bromouridine, and the resulting bromouridine-labeled RNA was isolated and converted into cDNA libraries, which were subsequently sequenced (Fig. 3.2A). The resulting data were compared to the previously published Karpas 299 data (Kocab et al., 2015), and in the initial analysis, the change in gene expression in SM-treated HEK293 was plotted against the change in gene expression in SM-treated Karpas 299 cells (Fig. 3.2B). As expected, expression of established NF-κB target genes, such as *NFKBIA*, which encodes for IκBα, and *BIRC3*, the gene for c-IAP2, was barely changed in the SM-treated HEK293 cells, but was highly increased in the Karpas 299 cells, which also exhibited activated NF-κB, indicating a role for NF-κB following SM treatment. As observed previously, SM treatment resulted in small changes in expression in a variety of

genes in Karpas 299 and, perhaps more notably, in HEK293 cells (Kocab et al., 2015). To classify these genes and better understand the biological implications of their regulation, gene set enrichment analysis (GSEA) was performed, allowing for the characterization of groups of genes with modest expression changes using gene sets that have been established based on shared, biologically relevant characteristics (Subramanian et al., 2005). Using this analysis allows for the identification of biologically significant processes involving genes with small changes in expression (Subramanian et al., 2005), and while the resulting GSEA data from SM-treated Karpas 299 cells showed a significant number of down-regulated gene sets (Kocab et al., 2015), SM-treatment resulted in the enrichment of numerous gene sets in HEK293 cells (Fig. 3.2C). Of the gene sets modified by SM treatment, the HEK293-specific sets involved RNA processing, suggesting that the c-IAPs may have a NF-κB-independent role in regulating RNA metabolism (Fig. 3.2D). Additionally, SM treatment did not affect gene sets related to NF-κB signaling and apoptosis regulation in HEK293 cells, as these were restricted to Karpas 299 cells (Fig. 3.2E), suggesting the necessity of NF-κB activation for regulation of these gene sets. There were, however, gene sets shared between the cell lines that were modified by SM treatment, but the enrichment of these gene sets was universally divergent (Fig. 3.2F). For example, Karpas 299 cells exhibited down-regulation of gene sets related to the ribosome and protein translation, as well as gene sets involved in metabolism. In contrast, these gene sets were all up-regulated in HEK293 cells following SM treatment, further emphasizing the cell type-specific effects of SM treatment and collectively illustrating key differences between cells that activate NF-κB and those that do not following SM treatment.


A.
Treat Cells with SM (2.5 h)
Add Bru (30 min)
Collect Total RNA
Isolate BruRNA with α-BrdU Ab
Make cDNA Library
Sequence Library
Analyze Data
B.
HEK293 Log2 Fold Change
Karpas 299 Log2 Fold Change
NFKBIA
BIRC3
C.
Karpas 299
HEK293
D.
Examples of Gene Sets Affected in HEK293
mRNA Splicing
rRNA Metabolic Process
Integrin Pathway
Spliceosome
rRNA Processing
Elongation Arrest & Recovery
miRNA Biogenesis
Regulatory RNA Pathways
Microtubule Cytoskel. Org. & Biogenesis
RNA-dependent ATPase Activty
Transcription Repressor Activity
Unfolded Protein Response
Normalized Enrichment Score

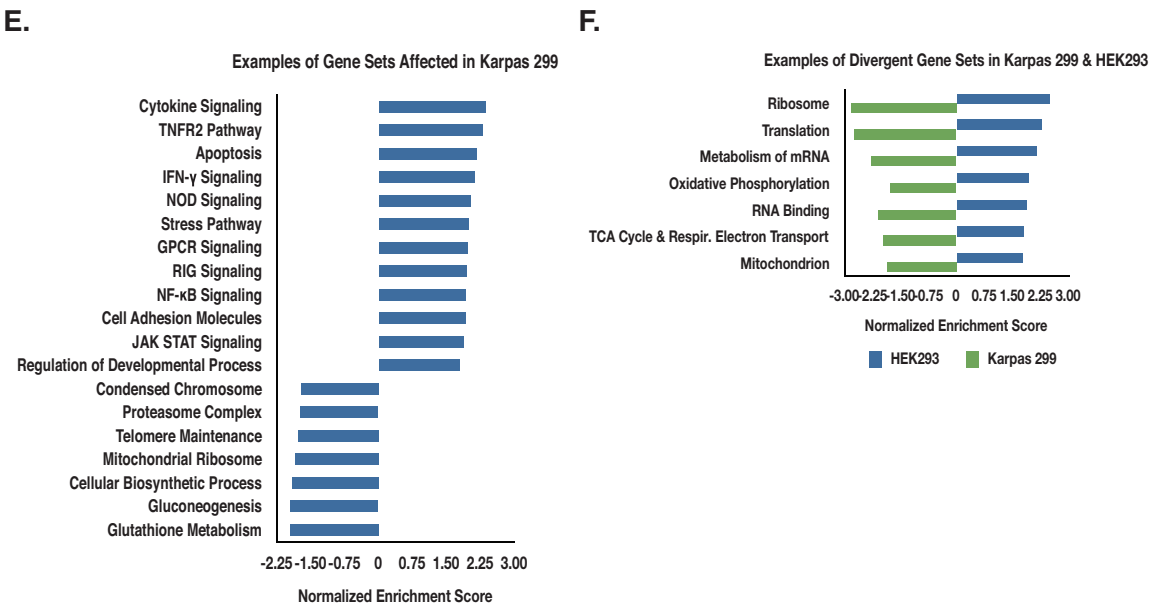



**Figure 3.2 Gene expression profiles differ between cell lines following Smac mimetic treatment**

**A.** Schematic of the Bru-seq experimental process. Karpas 299 and HEK293 cells were treated with 100 nM SM-164 for the indicated times. **B.** Plot of the Bru-seq data plotting the log2 fold change in gene expression from the SM-treated Karpas 299 against the log2 fold change in gene expression of the SM-treated HEK293 cells. The location of the canonical NF-κB gene targets *NFKBIA* and *BIRC3* are marked by red dots. **C.** Global depiction of the gene set enrichment analysis (GSEA) that reveals up- and down-regulated gene sets in the SM-treated cell lines. **D-F.** Examples of gene sets with false discovery rates (FDR) <0.05 that are regulated following SM treatment in HEK293 cells alone (D), in Karpas 299 cells alone (E), or regulated in both cell lines (F). The bars represent the normalized enrichment score for the indicated gene set. These experiments were performed with the help of Dr. Mats Ljungman and his lab.

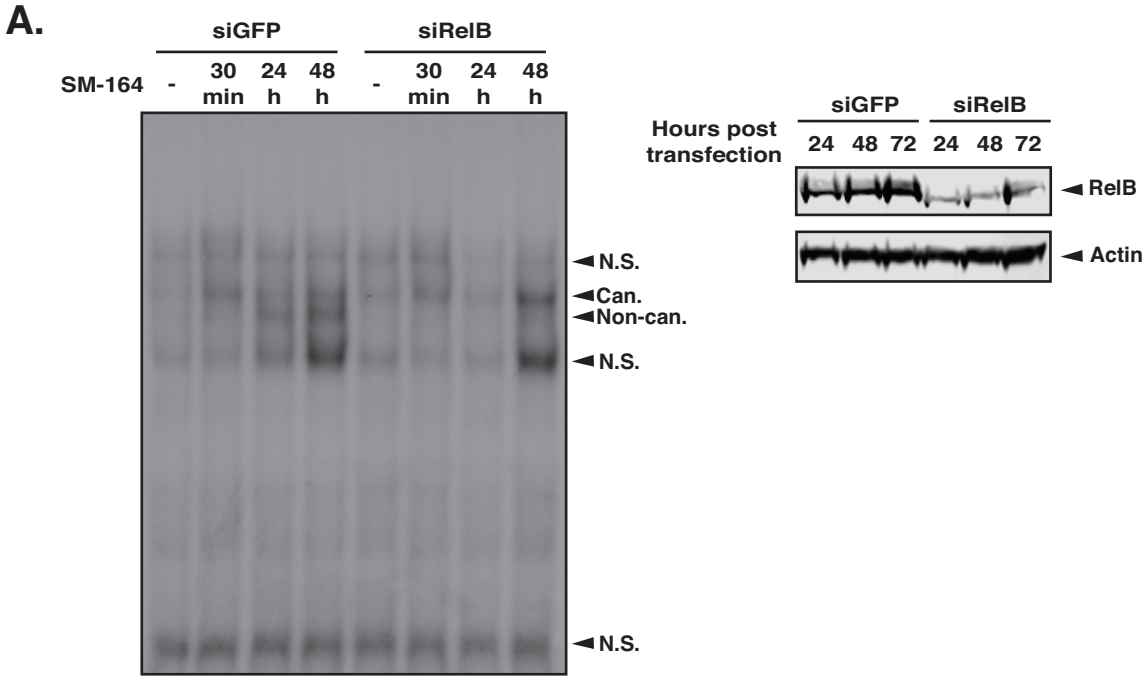
## Suppression of RelB results in prolonged nuclear presence of SM-induced canonical NF-κB

As described above, SM treatment exhibited cell type-specific effects, including NF-κB activation. However, the biological significance of non-canonical NF-κB activation remained unclear. To examine the role of this signaling pathway, expression of the non-canonical NF-κB subunit RelB was suppressed by siRNA in Karpas 299 cells, the only cell line tested that exhibited SM-induced non-canonical NF-κB activation. Non-

canonical NF-κB was not observed in Karpas 299 cells with reduced RelB expression, but was strongly detected in the control cells following SM treatment (Fig. 3.3A). Notably, a significant level of nuclear, DNA-binding canonical NF-κB was identified in RelB-suppressed cells, suggesting that activation of non-canonical NF-κB modulates canonical NF-κB activity (Fig. 3.3A). A previously observed transition from canonical NF-κB to non-canonical NF-κB following SM treatment was described in Chapter II, indicating a potential regulatory role for non-canonical NF-κB (Kocab et al., 2015), and in support of this hypothesis, the data presented in chapter suggest that RelB is necessary for this transition.

Since non-canonical NF-κB has been previously reported to impart a pro-survival phenotype (Gardam et al., 2011), the effect of reduced RelB expression on SM-induced cell death was consequently explored. SM treatment resulted in limited cell death in cells transfected with control siRNA, and the viability of these cells stabilized by 48 h of treatment. A similar initial decrease in cell viability was observed following SM treatment of cells with suppressed RelB expression, but decreased viability was observed at 48 h in these cells, thereby indicating that RelB promoted cell viability following SM treatment (Fig. 3.3B). The notion that non-canonical NF-κB provides a protective advantage was supported by a comparison of Karpas 299 cells to MDA-MB-231 cells, which do not activate non-canonical NF-κB and only activate canonical NF-κB following SM treatment (Fig. 3.1A). The Karpas 299 cells exhibited limited cell death at 24 h, but viability was unchanged at 48 h (Fig. 3.3C). In contrast, prolonged treatment with SM resulted in continued cell death in the MDA-MB-231 cells (Fig. 3.3C), and this pattern of cell death strongly resembled the death observed in the RelB-suppressed Karpas 299 cells

(Fig. 3.3B), suggesting a role for non-canonical NF-κB in promoting cell survival. Collectively, these data demonstrate that the absence of non-canonical NF-κB activation in cells sensitive to SM-killing results in a persistent DNA binding of nuclear, death-promoting canonical NF-κB and continued susceptibility to SM-induced cell death.



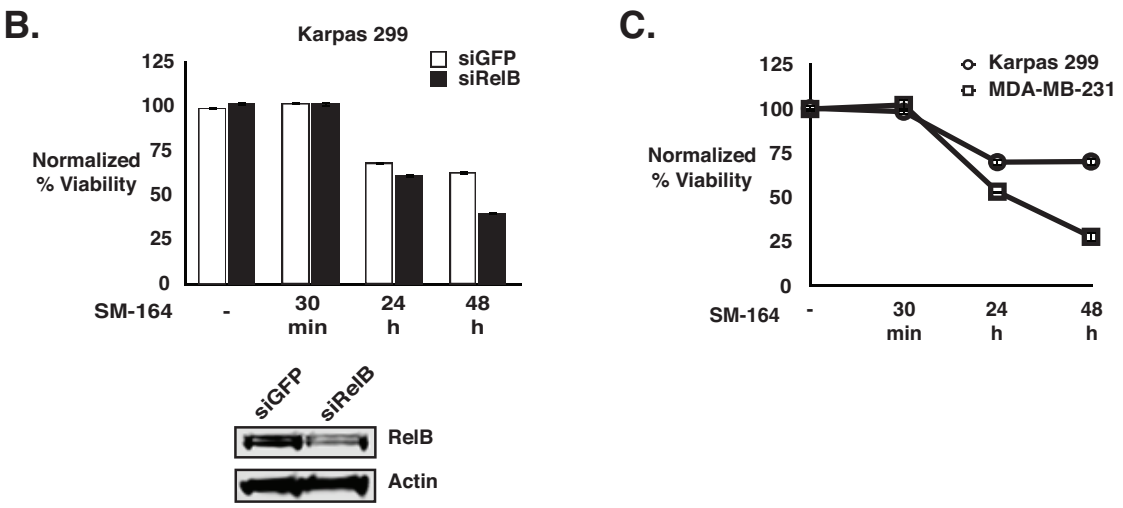



**Figure 3.3 Suppression of RelB results in prolonged nuclear presence of SM-induced canonical NF-κB**

**A.** Karpas 299 cells were transfected with siRNA targeting RelB or GFP (control). Cells were then treated with 100 nM SM-164 for the indicated times. Nuclear extracts were prepared, and NF-κB was analyzed by EMSA. Over the course of the experiment, whole cell lysates were collected and were used to analyze RelB expression. **B.** Karpas 299 cells were transfect with siRNA targeting RelB or GFP (control). Cells were then treated with 100 nM SM-164 for the indicated times. Cell viability was measured by propidium iodide exlusion and flow cytometry. Whole cell lystates collected following treatment were analyzed for RelB expression levels. **C.** MDA-MB-231 and Karpas 299 cells were treated with 100 nM SM-164 for the indicated times. Following incubation, cell viability was measured by propidium iodide exclusion and flow cytometry. Can., canonical; Non-can., non-canonical; N.S., non-specific.

### RelB reverses the effects of SM-induced canonical NF-κB

To better understand the relationship between canonical and non-canonical NF-κB following SM treatment, transcriptome analysis following prolonged treatment with SM was performed. RelB-suppressed Karpas 299 cells were treated with SM for 24 h in parallel with control cells. As expected, suppression of RelB prevented SM-induced non-canonical NF-κB and resulted in a strong canonical NF-κB band observable by EMSA (Fig. 3.4A). Additionally, canonical NF-κB corresponded to down-regulation of gene sets as determined by GSEA in these samples (Fig. 3.4B), and many of these gene sets

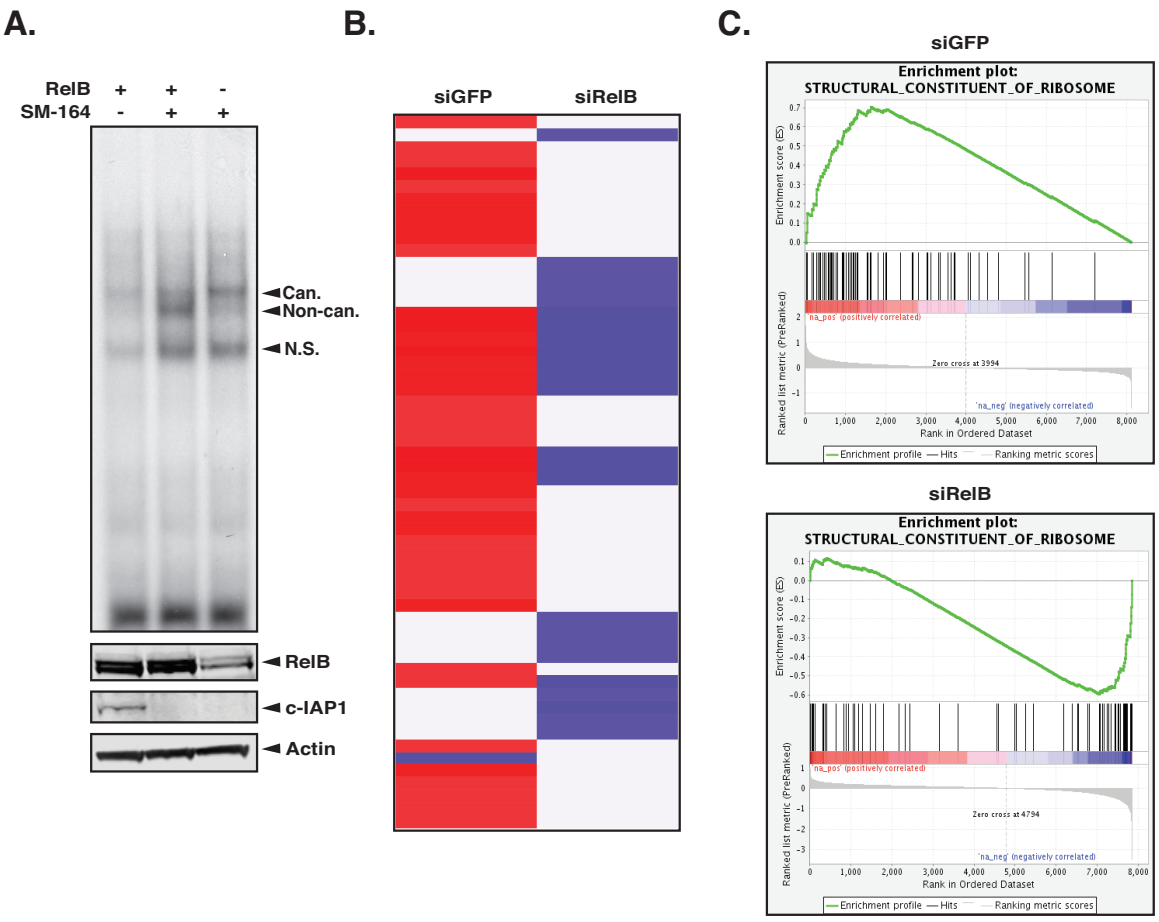



**Figure 3.4 RelB reverses the effects of SM-induced canonical NF-κB**
Karpas 299 cells were transfected with siRNA against RelB or GFP (control). The cells were treated with 100 nM SM-164 for 24 h. For the final 30 min of treatment, the cells were incubated with bromouridine in preparation for Bru-seq. **A.** Nuclear extracts were prepared from treated cells, and NF-κB activation was assessed by EMSA. **B.** Depiction of the GSEA from the Bru-seq results reveals the gene set regulation by RelB in the SM-treated cells. **C.** Example enrichment plots from the treated samples. The distribution of genes is listed according to rank position. FDR < 0.05. These experiments were performed with the help of Dr. Mats Ljungman and his lab.

had also been down-regulated after 3 h of SM treatment (Fig. 3.2C, F), suggesting a potential inhibitory role for canonical NF-κB. In contrast to the cells with suppressed RelB expression, the GSEA results for the cells transfected with control siRNA were markedly different. These cells, which possessed RelB, had a multitude of up-regulated gene sets, many of which had been down-regulated in the RelB-suppressed cells (Fig.

3.4B). One example of this observation was the gene set for the structural constituents of the ribosome, which had a normalized enrichment score of 2.32 in the control cells, but in the RelB-suppressed cells, the normalized enrichment score was -1.89 (Fig. 3.4D), illustrating the divergent gene set regulation in the absence of RelB. Furthermore, the data presented in Chapter II showed that SM treatment can down regulate gene expression of ribosomal proteins and inhibit protein synthesis, postulating that this could contribute to SM-induced cell death (Kocab et al., 2015). The current data support the hypothesis that down-regulation of ribosomal gene sets resulted in sustained SM-killing, as the RelB-suppressed cells exhibited down-regulated ribosomal gene sets and increased sensitivity to SM-induced death. Overall, these data illustrate a role for non-canonical NF-κB in reversing the effects of SM-induced canonical NF-κB, thereby contributing to a pro-survival phenotype.

### RelB-dependent expression of regulatory factors abrogates nuclear, death-promoting canonical NF-κB

As described above, the presence of RelB abrogated the DNA binding of SM-induced canonical NF-κB and its effects on gene regulation. In addition to this functional reversion of gene set regulation, cells with decreased RelB expression also exhibited long-term nuclear presence of the SM-induced canonical NF-κB dimer, but this did not result in significant gene expression (Fig. 3.5A). Conversely, gene expression was observed in the control cells that exhibited primarily non-canonical NF-κB following 24 h of SM treatment (Fig. 3.5A), suggesting that RelB contributed to increased gene expression. Analysis of mRNA levels (Fig. 3.5B) and protein expression (Fig. 3.5C) showed decreased expression of the canonical NF-κB regulators A20 and IκBα in RelB-

suppressed cells, demonstrating that these proteins were poorly expressed following suppression of RelB levels. These results suggest that RelB was responsible for gene expression that included the transcription of NF-κB regulatory factors, which are needed to inhibit SM-induced canonical NF-κB in the nucleus and thereby prevent increased cell death. Furthermore, these results highlight the crosstalk between the NF-κB pathways and illustrate a canonical NF-κB regulatory function for non-canonical NF-κB.

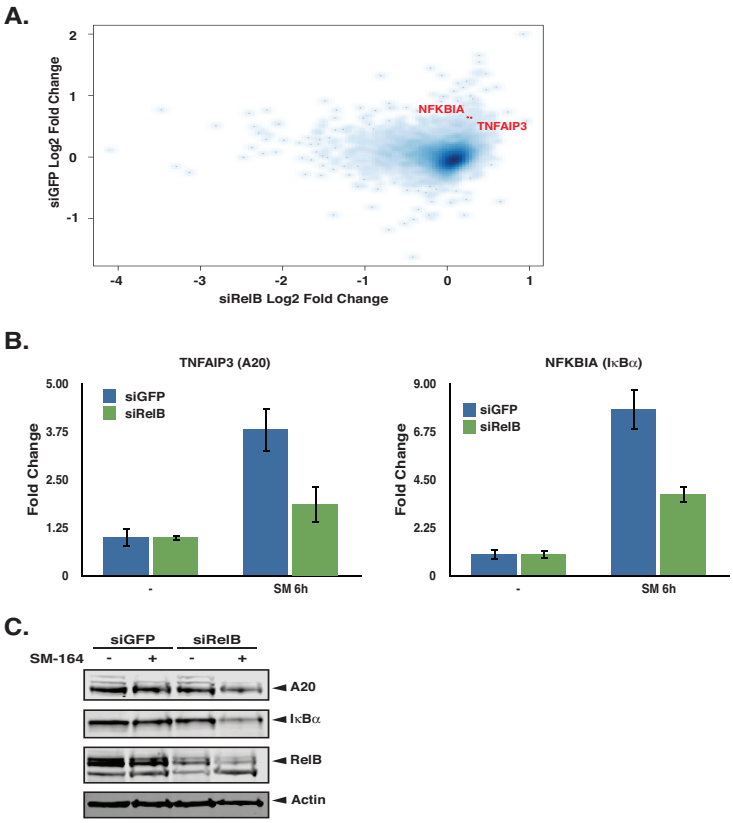



**Figure 3.5 RelB-dependent expression of NF-κB regulatory factors abrogates nuclear, death-promoting canonical NF-κB**
**A.** Plot of the log2 fold change in gene expression of the siGFP control Karpas 299 cells against the log2 fold change in gene expression of the siRelB cells following 24 h SM treatment. The location of *TNFAIP3* and *NFKBIA* are marked by red dots. **B.** Karpas 299 cells were transfected with siRNA against RelB or GFP (control), and were subsequently treated with 100 nM SM-164 for 6 h. Following treatment, RNA was isolated and converted to cDNA, and the expression of the indicated genes was measured. **C.** Whole cell lystates from cells prepared as in B were collected and the expression of the indicated proteins was assessed by Western blot.

## Discussion

In this chapter, the consequences of SM-induced NF-κB activation were investigated, and the results indicated that SM treatment resulted in different outcomes in a cell type-specific manner. More specifically, NF-κB activation was not universally observed, as canonical NF-κB activation was restricted to a subset of cell types, and the observation of non-canonical NF-κB activation was even more limited. These differences in NF-κB activation may be attributed to differential expression of other components in the signaling cascade. A scenario could be envisioned wherein cells that do not rely on physiological degradation of the c-IAPs for signaling would lack aspects of the downstream signaling cascade, potentially explaining why non-canonical NF-κB was only observed in Karpas 299 cells, a cell line that possesses a receptor that mediates physiological IAP antagonism. Notably, however, p100 processing to the active non-canonical NF-κB subunit p52 was observed in all SM-treated cells. While this indicates that assaying p100 processing to p52 is not an optimal surrogate marker for non-canonical NF-κB activation since it does not detect the presence of nuclear, DNA-binding NF-κB, it also elicits a provocative question regarding the function of p52: if SM-induced p100 processing is conserved across all cells, but activation of non-canonical NF-κB is cell type-specific, why is p100 processing to p52 universally observed? The pool of p52 may be functionally inert in cells that lack the capacity to activate non-canonical NF-κB, but the possibility of p52 having a different role cannot be excluded. It has, for example, been reported that p52 homodimers can interact with p53 to repress or stimulate gene expression (Schumm et al., 2006). While the precise functions of p52 are still being defined, in the context of SM treatment and the absence of non-canonical NF-κB

activation, p52 may interact with other NF-κB subunits and proteins to modulate the expression of various genes and contribute to SM-mediated cell death, therefore representing intriguing avenues for future investigations.

While SM treatment did not result in the universal activation of NF-κB, it still affected gene expression, indicating a transcriptional consequence of SM treatment that is independent of NF-κB signaling. SM treatment did not activate NF-κB in HEK293 cells, but it still resulted in minor changes in gene expression and up-regulation of certain gene sets. While it is unclear if this observed up-regulation of gene sets has a functional effect on cellular processes like proliferation and survival, it may suggest that c-IAP1/2 degradation triggers additional signaling events that culminate in gene regulation. Alternatively, SM treatment may lead to consequences that are independent of its ability to induce the degradation of the c-IAPs and activate NF-κB. However, it remains to be seen if these effects are limited to HEK293 or whether they represent a more general mechanism of SMs. Notably, SM treatment resulted in divergent regulation of certain gene sets in HEK293 compared to Karpas 299 cells, and since Karpas 299 cells activated NF-κB following SM treatment and HEK293 did not, NF-κB may have an important role in regulating these genes.

In cells that were competent to activate NF-κB, canonical NF-κB activation promoted cell death. The observation of a death-promoting canonical NF-κB supports the previously proposed mechanism of SM-mediated cell death, which is based on the canonical NF-κB-dependent production of TNF (Varfolomeev et al., 2007; Vince et al., 2007; Petersen et al., 2007). In addition to its role in TNF production, canonical NF-κB may have additional, undiscovered functions, including the regulation of additional genes

that may contribute to cellular fate, an idea that is supported by previous data showing that canonical NF-κB regulated the expression of both pro-apoptotic and pro-survival genes in the context of the DNA damage response (Campbell et al., 2004; Wu and Miyamoto, 2008). Additionally, while previous evidence indicates that receptor-mediated canonical NF-κB is activated by a different mechanism that is not dependent of c-IAP degradation (Duckett et al., 1997; Hacker et al., 2011; Matsuzawa et al., 2008), there have been reports of a NIK-dependent activation of canonical NF-κB through an IKKα-mediated pathway (Zarnegar et al., 2008; A et al., 2000), suggesting a potential mechanism for SM-induced activation of canonical NF-κB signaling. It remains to be determined how these potential mechanistic differences in activation affect the gene targets and overall functionality of the resulting canonical NF-κB.

In this chapter, *bona fide* non-canonical NF-κB activation was only observed in Karpas 299 cells, even though p100 processing was observed in all tested cell lines. This suggests that Karpas 299 cells, which express a receptor that mediates c-IAP degradation, possess a functional signaling cascade that may also include additional, unspecified cofactors that are required for full activation of non-canonical NF-κB. Furthermore, while previous work demonstrated that mice lacking the non-canonical NF-κB subunits had severe developmental defects of the secondary lymphoid organs, (Weih and Caamano, 2003), the role of non-canonical NF-κB signaling remains poorly characterized. The data presented in this chapter demonstrated that the absence of non-canonical NF-κB prolonged the DNA binding activity of canonical NF-κB and coincided with continued SM-induced cell death, consequently indicating that non-canonical NF-κB exerts its protective effect, at least in part, by regulating the kinetics of canonical NF-κB through

the expression of canonical NF-κB regulators. This model could explain the observed pattern of SM-induced cell death in Karpas 299 cells as well as the kinetics of NF-κB activation described in Chapter II (Kocab et al., 2015): the initial cell death corresponded to early activation of canonical NF-κB, and by later time points, non-canonical NF-κB was fully activated and had replaced canonical NF-κB in the nucleus, inducing the expression of genes that inhibited the death signals and contributed to cell survival.

Taken together, the results presented in this chapter provide evidence that the consequences of SM treatment are cell type-specific and, therefore, may have important therapeutic implications for SMs as they advance through clinical trials. By identifying and characterizing these differences, we may better understand how a SM therapeutic regimen may affect a patient and whether it should be used in combination with other drugs. It has already been established that targeting the c-IAPs may not be the best strategy for all cancers, as constitutive NF-κB signaling may promote survival and/or lead to a cytokine storm and systemic inflammation (Annunziata et al., 2007; Rosebeck et al., 2011; Rosebeck et al., 2014; Infante et al., 2014). In the context of the work presented in this chapter, assaying NF-κB activation in cells may predict the efficacy of the drug. Cells that do not activate NF-κB would be resistant to the therapy, while SM treatment may be most efficacious in cells that activate only canonical NF-κB since SM-induced canonical NF-κB promotes cell death. In contrast, in cells that also activate non-canonical NF-κB, the therapy may only have a limited effect as prolonged exposure to the drug may result in a non-canonical NF-κB-dependent pro-survival phenotype, potentially exacerbating the severity of the disease. In summary, these results collectively provide

valuable insight into the functionality of SM treatment and contribute to our understanding of their therapeutic value.

## Materials and Methods

**Cell lines and culture conditions.** Human embryonic kidney (HEK) 293 cells were cultured in DMEM (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HCT116 cells were maintained in McCoy's 5A (Gibco, Carlsbad, CA, USA) medium supplemented with 10% FBS and 2 mM L-glutamine. MDA-MB-231 cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 2 mM nonessential amino acids solution (Gibco), and 1% penicillin-streptomycin (Gibco). Karpas 299 cells have been described previously (Mir et al., 2000) and were maintained in RPMI 1640 (Mediatech) medium supplemented with 10% FBS and 2 mM L-glutamine. All cells were cultured at 37°C in an atmosphere of 5% $CO_2$.

**Materials.** Human tumor necrosis factor (TNF) was purchased from Roche (Nutley, NJ, USA), and the siRNA transfection reagent INTERFERin was purchased from Polyplus Transfection (New York, NY, USA). The following primary antibodies were used in this study: anti-p100/p52 (Millipore, Billerica, MA, USA); anti-c-IAP1 and anti-XIAP (Enzo Life Sciences, Farmingdale, NY, USA); anti-c-IAP2 (Cell Signaling, Danvers, MA, USA); anti-RelB (Santa Cruz Biotechnology, San Diego, CA, USA); and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). A FlexiTube GeneSolution containing small interfering RNAs (siRNAs) against RelB was purchased from Qiagen (Valencia, CA, USA) along with a control siRNA against GFP. The siRNA used in this study targeting

p65 has been previously described (Wright et al., 2007). SM-164 was a kind gift from Dr. Shaomeng Wang (University of Michigan).

**Cell lysate and nuclear extract preparation.** Cells were treated as described in the figure legends. HEK293 cells were removed from the plate with gentle washing. Medium from HCT116 and MDA-MB-231 cells was collected, and the cells were washed with PBS and removed with trypsin, which was then inactivated using the collected medium. Karpas 299 cells were simply transferred to a conical tube. For all cell lines, cells were centrifuged at 100 × g for 5 min. Medium was then aspirated, and cells were resuspended in PBS. The cell suspension was then divided into tubes for different preparations so as to have corresponding whole cell lysates, nuclear extracts, and/or samples for viability assays. Whole cell lysates were prepared using RIPA lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors. Incubation for 30 min on ice ensured complete lysis. For the nuclear extract preparation, cells were washed once with Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, and 0.5 mM DTT) and pelleted by centrifugation at 1500 × g for 1 min. The supernatant was aspirated, and the cell pellet was resuspended in 15 μL of cold Buffer A supplemented with 0.1% NP-40. The samples were incubated on ice for 5 min before being centrifuged at 16000 × g in a microfuge at 4°C for 15 min. The supernatant was removed, and the pellet was thoroughly resuspended in 10 μL of Buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM PMSF, 0.6 mM DTT, and 25% glycerol) and incubated at 4°C for 20 min with occasional mixing. The samples were then centrifuged at 16000 × g at 4°C for 15 min. The

supernatant was transferred to a fresh tube containing 60 μL of modified Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 20% glycerol). The nuclear extracts were flash frozen and stored at -80°C.

**Immunoblot analysis.** Protein concentrations of whole cell lysates were determined by BCA. Lysates of equal protein concentrations were prepared in LDS sample buffer, separated on denaturing NuPAGE 4-12% polyacrylamide gradient gels (Invitrogen, Carlsbad, CA, USA), and transferred to nitrocellulose membranes (GE Healthcare, Amersham, UK). Membranes were blocked in a 1:1 mixture of Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) and Tris-buffered saline (TBS). Membranes were then incubated with primary antibodies in a 1:1 mixture of Odyssey blocking buffer and TBS containing 0.1% Tween 20 (Fisher BioReagents, Waltham, MA, USA) overnight at 4°C. Following washing with TBS with 0.1% Tween 20, membranes were incubated with IRDye secondary antibodies (Li-Cor) for 1 h at room temperature. Membranes were then washed with TBS and analyzed using the Odyssey CLx infrared imaging system (Li-Cor) according to the manufacturer's instructions.

**Electrophoretic mobility shift assays.** Two complimentary oligonucleotides containing NF-κB consensus binding sites (5'-GATCCAGGGACTTTCCGCTGGGGACTTTCCA-3' and 5'-GATCTGGAAAGTCCCCAGCGGAAAGTCCCTG-3') were annealed and radiolabeled using T4 polynucleotide kinase (New England BioLabs, Ipswich, MA, USA) in the presence of [$\gamma$-$^{32}$P] ATP. The radiolabeled probe was then purified using illustra Microspin G-25 Columns (GE Healthcare) according to the manufacturer's

instructions. The presence of NF-κB in the nuclear extracts was assessed by incubating 2 μL of the nuclear extract in modified buffer D without glycerol along with 1 μg of poly(dI-dC)•poly(dI-dC), 1 μL of Bluejuice loading dye (Invitrogen), and 0.1 μL of the radiolabeled probe for a total volume of 20 μL. The prepared samples were then separated on a non-denaturing 4% polyacrylamide gel. Supershift assays were performed by adding 2 μL of antibodies against GST, p65, or RelB (Santa Cruz) to the reaction mixture. Samples were incubated for 20 min at room temperature prior to running on the gel. Autoradiography was conducted overnight at -20°C.

**Viability assays.** Following the indicated treatments, cells were harvested, washed with PBS and subsequently resuspended in PBS with 2 μg/mL propidium iodide (PI). The cell viability of the PI-stained cells was assessed by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences).

**Transcriptome analysis by Bru-seq.** HEK293 or siRNA transfected Karpas 299 cells were incubated in the treatment conditions indicated in the figure legends. To label nascent RNA, 2 mM bromouridine (Bru) was added to the media for the final 30 min of treatment time. The Bru-seq procedure has been previously described in detail (Paulsen et al., 2013; Paulsen et al., 2014). Briefly, total RNA was collected from the treated cells using TRIzol reagent (Invitrogen), and the Bru-labeled, nascent RNA was isolated using anti-BrdU antibodies (BD Biosciences, San Jose, CA, USA) conjugated to magnetic beads (Invitrogen). The isolated RNA was converted into cDNA libraries, which were sequenced at the University of Michigan Sequencing Core using an Illumina HiSeq 2000

sequencer. The sequencing and read mapping were performed as previously described (Paulsen et al., 2013; Paulsen et al., 2014). GSEA was used to identify up-regulated and down-regulated gene sets by determining which associated genes were significantly enriched in each gene set (Subramanian et al., 2005). The log fold change in expression of genes greater than 1kb and expressed above 0.5 RPKM was used as the ranking metric by GSEA. The gene sets were obtained from version 4.0 of the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The gene sets used were canonical pathways (KEGG, Reactome, and BioCarta) and gene ontologies (biological processes, molecular functions, and cellular compartment). Gene sets with FDR corrected P-values lower than 0.01 were considered to be significantly enriched and were used in the analysis. The data generated from the HEK293 cells was compared to the published data with Karpas 299 cells and SM-164 (GEO accession number: GSE64927). The primary sequencing data files from this study will be submitted to the NCBI Gene Expression Omnibus upon acceptance of the manuscript.

**Quantitative real-time PCR.** Cells were treated as described in the figure legends. Following treatment, the cells were washed with PBS, and total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacture's instructions. 1 μg of total RNA was converted to cDNA using a reverse transcription reaction with random hexamer primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). 1 μL of the resulting cDNA was analyzed for the indicated target genes using the ViiA 7 Real-Time PCR System (Applied Biosystems). Each target assay was normalized to 18*S* levels.

**Transfections.** Karpas 299 cells were transfected with siRNA using a Bio-Rad Gen Pulser II electroporator (Hercules, CA, USA) at 300 V and 975 μF. MDA-MB-231 cells were transfected with siRNA using the INTERFERin siRNA transfection reagent according to the manufacturer's instructions.

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# CHAPTER IV

## Conclusions

The IAPs were originally thought to mainly function as regulators of apoptosis through their direct inhibition of caspases (Salvesen and Duckett, 2002; Deveraux et al., 1998; Liu et al., 2000), but over time, the IAPs have been implicated in numerous signaling pathways, indicating that they possess important functions unrelated to their originally described role in direct inhibition of apoptosis. The focus of this dissertation was on the regulation of signaling by the c-IAPs, ubiquitin ligases that have been previously shown to have contradictory roles in TNF receptor superfamily signaling (Silke and Brink, 2010). In the signaling cascades of certain receptors, such as TNF-R1, the c-IAPs have well-established roles in actively propagating the signal, leading to the activation of canonical NF-κB (Varfolomeev et al., 2008; Vince et al., 2009; Shu et al., 1996). In contrast, the c-IAPs constitutively inhibit the activation of non-canonical NF-κB through the degradation of NIK (Vallabhapurapu and Karin, 2009; Zarnegar et al., 2008b), but upon activation of certain receptors, such as CD30, the c-IAPs are degraded, allowing for the activation of non-canonical NF-κB (Silke and Brink, 2010; Wright et al., 2007; Csomos et al., 2009). Additionally, as discussed in Chapter I, degradation of the c-IAPs can be induced by synthetic means, including treatment with a Smac mimetic (Vince et al., 2007; Varfolomeev et al., 2007; Darding et al., 2011). However, the downstream consequences of c-IAP activity have not been well defined, and it is also

unclear if synthetic and physiological forms of IAP antagonism are truly comparable. The findings presented in this dissertation reveal that the biological properties of c-IAP1/2 are context-dependent. Specifically, while receptor-mediated and Smac mimetic-induced c-IAP degradation activated similar pathways and generated similar gene expression profiles, there were also substantial differences between the two stimuli, differences that will warrant future study. However, characterization of the similarities in the gene expression profiles following each stimulus revealed a novel function for the c-IAPs in the regulation of ribosomal gene expression and protein synthesis. Additionally, data presented in this dissertation demonstrated that SM-induced activation of NF-κB, an important outcome of c-IAP degradation, did not universally occur in all cell types, supporting the notion of context-dependent c-IAP1/2 activity. Due to the connection between NF-κB activation and SM-induced cell death, these findings also helped elucidate the mechanistic determinants of the efficacy of the drug, and may therefore have clinical impacts.

## Comparison of Physiological and Synthetic IAP Antagonism

As described in earlier chapters, the canonical and non-canonical NF-κB pathways are activated by members of the TNFR superfamily and play important roles in the immune response, inflammation, and cancer (Walczak, 2011; Silke and Brink, 2010). A major aspect of Chapter II was to study the function of the c-IAPs in this context, as they have been shown to play important roles in the activation of NF-κB following activation of members of the TNFR superfamily. The c-IAPs have a demonstrated role in the activation of canonical NF-κB following TNF treatment (Varfolomeev et al., 2008;

Vince et al., 2009; Shu et al., 1996), and this contrasts with the receptor-induced degradation of c-IAP1/2 that is a required event in the activation of the non-canonical NF-κB pathway, a signaling cascade that physiologically occurs following activation of a limited subgroup of the TNFR superfamily, including CD30, CD40, and TNFR2 (Vallabhapurapu and Karin, 2009; Zarnegar et al., 2008b). Additionally, Chapter II aimed to study c-IAP1/2 degradation by a class of synthetic, small molecule compounds called Smac mimetics (SMs), with the ultimate goal of comparing the physiological and synthetic forms of IAP antagonism in order to better understand the cellular roles of the c-IAPs. SMs, which are based on the IAP binding motif of Smac, bind to the c-IAPs and induce their autoubiquitination and subsequent degradation, resulting in NF-κB activation (Vince et al., 2007; Varfolomeev et al., 2007; Darding et al., 2011). While SM treatment has been shown to replicate aspects of receptor activation, it was unclear how extensively the consequences of SM treatment overlap with receptor-mediated degradation of the c-IAPs. Therefore, to investigate this question, the work presented in Chapter II explored the consequences of IAP antagonism following physiological and synthetic stimuli. Through these studies, genes and pathways unique to each stimulus were identified, as were novel processes affected following both forms of c-IAP degradation. One commonality between the two inducers of c-IAP degradation was the down-regulation of genes related to the ribosome and translation, and further analysis demonstrated that this effect on gene expression led to a decrease in protein synthesis following each stimulus as well. These results potentially suggest an additional mechanism of SM killing, wherein SM-induced c-IAP degradation triggers the shutdown of protein synthesis, mimicking the

effects of cycloheximide and rendering the cell more susceptible to TNF lethality (Fig. 4.1).

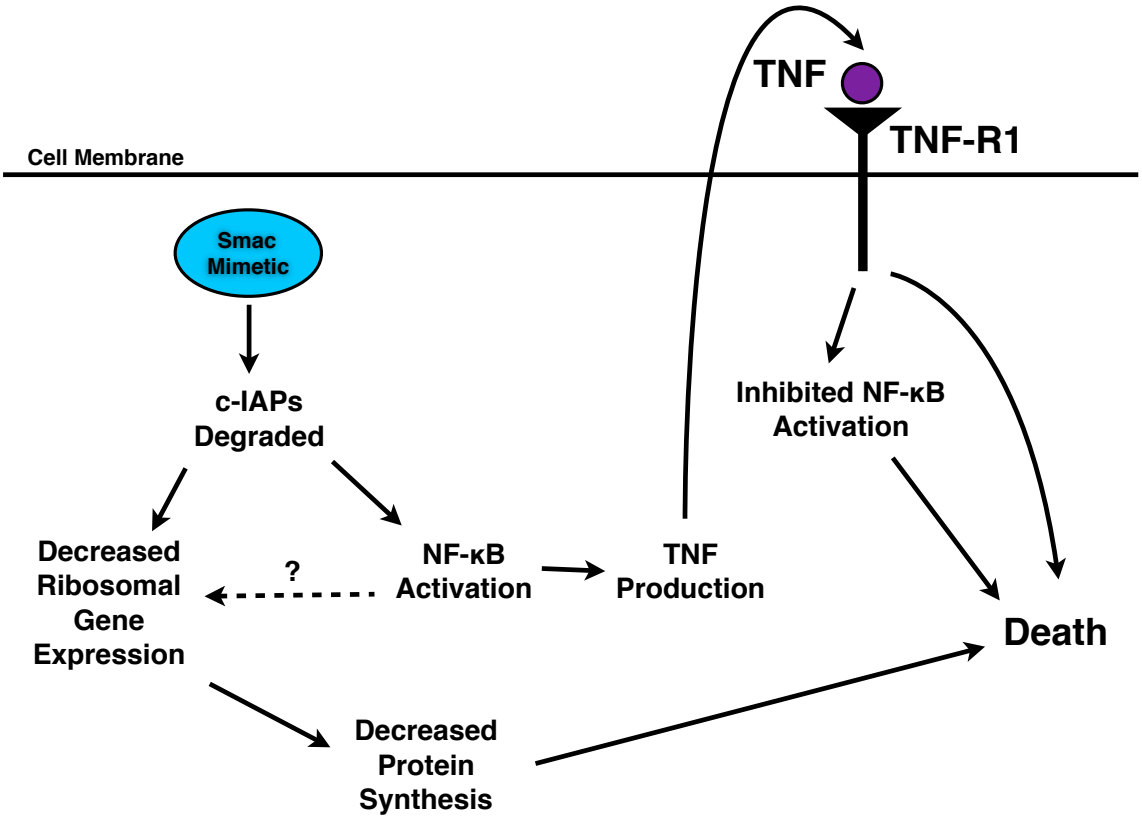


**Figure 4.1 Model of Smac mimetic-induced cell death.**
SM treatment results in the degradation of the c-IAPs, which activates NF-κB and leads to decreased gene expression of ribosomal proteins. Decreased gene expression of ribosomal proteins results in inhibited protein synthesis, mimicking the effect of cycloheximide and increasing the susceptibility to TNF-mediated cell death. While it is unclear if NF-κB has a role in regulating ribosomal gene expression, activation of NF-κB induces the production of autocrine TNF. Activation of TNF-R1 fails to activate NF-κB in the absence of the c-IAPs, preventing the expression of pro-survival factors and leading to TNF-R1-mediated cell death. Collectively, these events contribute to SM-induced cell death.

While both CD30 activation and SM treatment had an effect on protein synthesis, activation of the receptor did not affect protein synthesis as rapidly as SM treatment did. This may have been due to the ability of the receptor to activate additional signaling

pathways, including the ERK signaling cascade. ERK has been shown to be a regulator of protein synthesis through its regulation of mTORC1 (Mendoza et al., 2011), and its early activation following CD30 stimulation may have countered any initial down-regulation of ribosomal genes induced by c-IAP degradation. As ERK signaling was eventually terminated, the signal balance would have shifted, resulting in the down-regulation of the ribosomal genes and potentially explaining the delayed observation of decreased protein synthesis following CD30 activation.

The data presented in Chapter II also hinted at a potential connection between c-IAP degradation and cell cycle arrest, as the decrease in protein synthesis following CD30 activation was observed at 24 h, coinciding with previous reports of CD30-mediated cell cycle arrest occurring at the same time point (Buchan and Al-Shamkhani, 2012; Wright et al., 2007). Since there is an established connection between decreased protein synthesis and cell cycle arrest (Saracino et al., 2004; Verbin and Farber, 1967; Medrano and Pardee, 1980; Morinaga et al., 2008), the c-IAPs may regulate the cell cycle through their control of protein synthesis. In the context of CD30 signaling, c-IAP degradation-meditated cell cycle arrest may help control the expansion of activated T cells, preventing unchecked proliferation and preparing the cells for the contraction phase of the immune response by sensitizing the cells to death ligands. Additionally, if c-IAP degradation does regulate cell cycle, then future studies could focus on the effect of SMs on cell cycle, as it may represent another aspect of their mechanism. In the context of previous work illustrating that cell cycle-arrested cells are more sensitive to death-inducing ligands, such as TNF and TRAIL (Darzynkiewicz et al., 1984; Lu et al., 2008a; Kuratnik et al., 2012; Jin et al., 2002; Ehrhardt et al., 2013), SM-induced cell cycle arrest

would increase the cell's susceptibility to TNF-mediated death, a known component of SM-killing. Therefore, cell cycle arrest may be another mechanistic facet of SM treatment-induced cell death.

While the data presented in this dissertation identified a novel role for the c-IAPs in the regulation of protein synthesis, the exact mechanism of how this occurs remains unresolved. Future studies may initially focus on the potential contributions of established regulators of protein synthesis and ribosome biogenesis, including mTOR, Myc, and Akt, during c-IAP degradation-mediated protein inhibition. Akt, for example, promotes protein synthesis and ribosome biogenesis through activation of mTORC1 (Hsieh et al., 2011; Park et al., 2009; Mendoza et al., 2011), while Myc affects protein synthesis through the increased the expression of both rRNA and genes encoding ribosomal proteins (van Riggelen et al., 2010; Poortinga et al., 2015; Lempiainen and Shore, 2009). Notably, some of these known regulators of protein synthesis appear to have relationships with the IAPs. For example, expression of c-IAP2 and XIAP was increased by phosphatidylinositol 3-kinase (PI3K) and Akt following endoplasmic reticulum stress (Hu et al., 2004), and RIP1, an important ubiquitination target of the c-IAPs, has been implicated in activation of the PI3K-Akt pathway (McNamara et al., 2013; Park et al., 2009). In this context, degradation of the c-IAPs by CD30 or SM treatment may alter the ubiquitination of RIP1, affecting its ability to regulate the PI3K-Akt pathway and having a downstream impact on protein synthesis. Additionally, overexpression of c-IAP1 has been shown to promote Myc activity through the ubiquitination and degradation of the Myc regulatory factor Mad1 (Xu et al., 2007), and

therefore, degradation of c-IAP1 could result in higher levels of Mad1 and result in the down-regulation of Myc-mediated ribosome biogenesis and protein synthesis.

In addition to identifying how the c-IAPs regulate ribosomal gene expression and protein synthesis, future work may also explore the downstream consequences of decreased ribosomal protein gene expression. Specifically, down-regulation of these genes has been shown to affect the activity of microRNAs and their ability to associate with their target mRNA (Janas et al., 2012). Notably, decreased expression of ribosomal proteins resulted in an increase in translation of specific mRNAs, potentially contributing to a pro-tumorigenic effect (Janas et al., 2012; van Riggelen et al., 2010). Since inhibition of protein synthesis was observed with decreased ribosomal gene expression following CD30 activation and SM treatment, this previous work on miRNAs appears to contradict the data presented in this dissertation and therefore warrants future investigation.

The data presented in Chapter II also implicated caspases in the regulation of the ribosomal gene expression, supporting the intriguing proposition that caspases have additional, non-apoptotic roles. While caspases are traditionally associated with the cell death signaling cascades, there is growing evidence of caspases having additional, non-apoptotic roles as regulators of signaling. For example, in humans, caspase-1, -4, -5, and -12 are considered inflammatory caspases and are involved in the innate immune response (Shalini et al., 2014). Caspase-1, more specifically, plays an important role in the processing and maturation of IL-1β during inflammation (Thornberry et al., 1992; Ghayur et al., 1997). Additionally, the apoptotic caspases have also been shown to possess apoptosis-independent functions. For example, caspase-3 plays a role in cell proliferation and differentiation, which may stem from its ability to regulate Akt

phosphorylation (Shalini et al., 2014; Fernando et al., 2002). Caspase-8, an important factor in apoptosis, has been implicated in the regulation of inflammation in a manner that is independent of apoptosis (Maelfait et al., 2008; Vince et al., 2012; Allam et al., 2014), and has been shown to participate in the differentiation of monocytes into macrophages through the regulation of NF-κB via cleavage of RIP1 (Rebe et al., 2007; Kang et al., 2004). For these reasons, it would be interesting to examine the role of caspases in the context of SM-induced signaling. While preliminary data not shown in this dissertation indicated that SM-induced activation of NF-κB was independent of caspase activity, regulation of ribosomal gene expression and protein synthesis was dependent on caspases, suggesting potential downstream or co-regulatory roles for caspases in this system. However, while caspases play a role in SM-mediated signaling, the requirement of caspase activity in CD30 signaling has not been explored. Additionally, it would be interesting to identify the genes that are dependent on caspase activity for expression. Bru-seq experiments could be performed following treatment with chemical caspase inhibitors, such as z-VAD-fmk, or RNAi-mediated suppression of caspase expression to identify these genes and further clarify the role of caspases following IAP antagonism, as well as their relationship to NF-κB signaling.

As previously discussed, the consequences of CD30 activation and SM treatment, while similar, possessed key differences, such as variances in kinetics and gene expression profiles. These differences may be due to additional pathways activated by CD30, unique genes regulated by the receptor, or a combination thereof. Conversely, SM treatment may also have unique effects that may contribute to the observed differences. One particularly noteworthy difference between the two stimuli is the mechanism of

canonical NF-κB activation by the receptor. CD30, like its close TNF receptor superfamily relatives, is thought to activate NF-κB through its TRAF binding domains (Duckett and Thompson, 1997; Vallabhapurapu et al., 2008; Zarnegar et al., 2008b). However, it has also been previously demonstrated that CD30 possesses the ability to activate canonical NF-κB in the absence of its TRAF binding domains (Buchan and Al-Shamkhani, 2012; Duckett et al., 1997), which would therefore be independent of c-IAP degradation. This raises the possibility that CD30 can activate multiple NF-κB pathways with undefined functions (Fig. 4.2). These pathways may not be replicated by SM treatment, as SM-induced canonical NF-κB appears to be due to c-IAP degradation and is most likely dependent on the accumulation of NIK, a protein normally associated with non-canonical NF-κB activation but also has a reported ability to activate canonical NF-κB (Malinin et al., 1997; Zarnegar et al., 2008a). These differences may explain the observable canonical NF-κB following prolonged CD30 stimulation that is absent after extended SM treatment, as well as the observed transcriptome profile disparities between CD30 activation and SM treatment.

To explore the possibility that CD30 can activate multiple NF-κB signaling pathways, genome-editing techniques, such as TALENs or CRISPR/Cas, could be used to generate CD30 mutants possessing truncated cytoplasmic tails. Using genome-editing techniques in an anaplastic large cell lymphoma cell line, such as Karpas 299, would generate a system wherein the endogenous, full-length CD30 would be absent. However, the downstream machinery for CD30 signaling would remain intact, which is an important consideration since not all cells appear to possess the ability to activate these pathways, as illustrated in Chapter III. Studying these new cell lines using the same

techniques discussed in Chapter II would provide valuable insight into the requirement of each domain of CD30 for the observed consequences of receptor activation, including as c-IAP degradation, TRAF degradation, MAPK activation, NF-κB activation, cell cycle arrest, protein synthesis, and cell death. Furthermore, performing Bru-seq experiments with the CD30 mutants may help identify exactly which aspects of receptor signaling SM treatment can mimic.

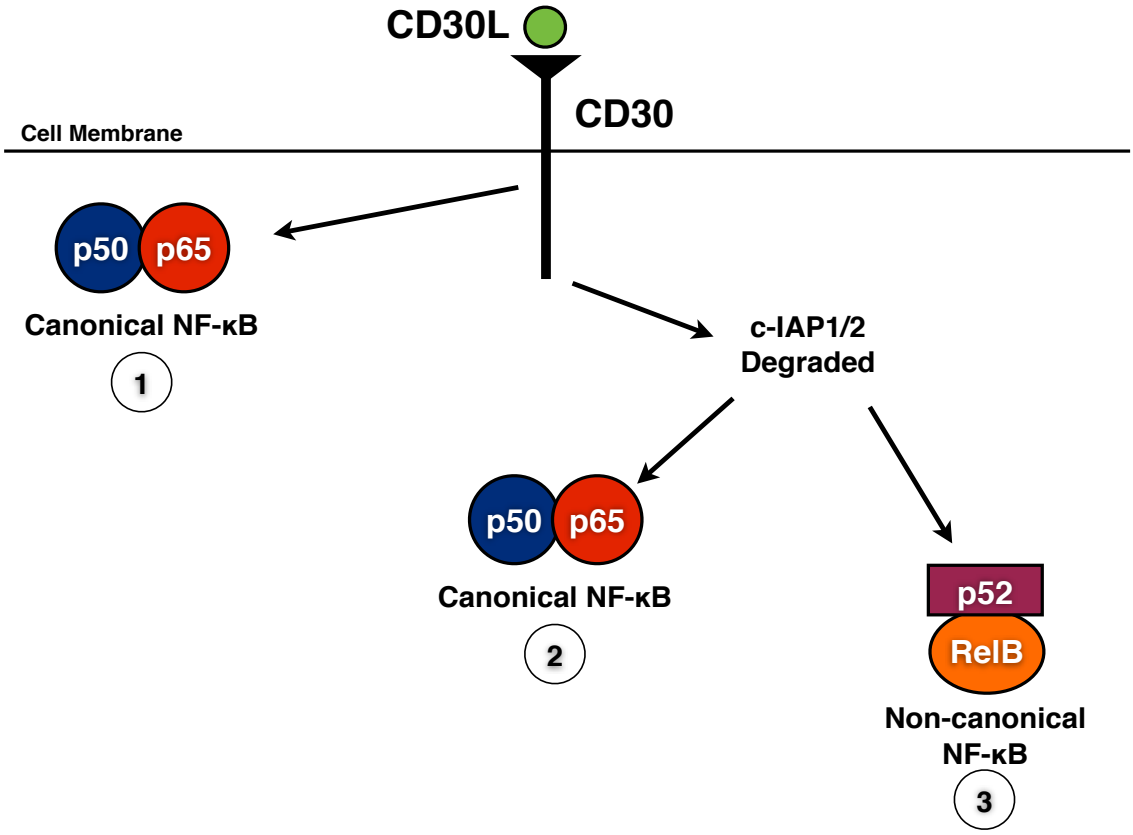


**Figure 4.2 Model of the three phases of CD30-mediated NF-κB activation.** Canonical NF-κB (1) is observed rapidly after receptor activation (~15 min). Activation of this canonical NF-κB is independent of the TRAF binding domains within the cytoplasmic tail of CD30 and is independent of c-IAP1/2 degradation. Occurring in parallel, the c-IAPs are degraded, mimicking the effects of SM treatment. This triggers the induction of a second canonical NF-κB (2), which is observed approximately 1 h after receptor activation. Lastly, non-canonical NF-κB (3) is observed approximately 3 h following initial stimulation.

Since the data suggest that CD30 activates multiple NF-κB pathways, future experiments may aim to characterize these NF-κB complexes and investigate their functions. CD30 appears to have the potential to activate at least two distinct canonical NF-κB complexes, one dependent on c-IAP degradation and one independent of c-IAP degradation (Fig. 4.2). While these NF-κB complexes may both be comprised of p65 and p50, these subunits may possess different modifications and downstream functions. In support of this idea, previous work has demonstrated that the subunits of NF-κB could be differentially modified by phosphorylation, methylation, and acetylation, and that these modifications affected the activity and gene targets of the NF-κB dimer (Viatour et al., 2005; Chen and Greene, 2003; Bhatt and Ghosh, 2014; Diamant and Dikstein, 2013; Lu et al., 2013; Chen et al., 2002). As an example, the canonical NF-κB subunits, p65 and p50, can be phosphorylated on multiple serine residues, resulting in enhanced DNA-binding and transactivation potential (Viatour et al., 2005). Identifying these modifications would be the first step in connecting each modification with specific gene signatures, as identified by Bru-seq or another sequencing technique. Additionally, the CD30 tail mutants discussed above would allow for the investigation into the necessity of each domain of CD30 for the modifications of NF-κB, helping to clarify the exact mechanism of CD30-mediated signaling. Furthermore, comparing the modifications of SM-induced NF-κB to the CD30 results, along with data from experiments using RNAi-mediated suppression of NIK, c-IAPs, and TRAFs, would help identify which factors and domains are needed for each modification of NF-κB. Once the outcomes of these experiments have been established, testing if the signaling from a truncated CD30 could be rescued by SM treatment would help assess the accuracy of the resulting model.

While differences in signaling pathway activation may help explain the variances between CD30 activation and SM treatment, another potential contributing factor could be differences in downstream gene expression, as highlighted by the Bru-seq results in Chapter II. There were gene sets uniquely regulated by each of the stimuli, and these differences warrant future study since they may have contributed to the variances observed between the two stimuli. Furthermore, the Bru-seq results contain additional data that would be worthy of future investigation, including the observed down-regulation of gene sets related to cellular energetics and metabolism following both CD30 activation and SM treatment. While this preliminary data does not specify how this gene regulation affects the metabolic programming within the cell, it may have important biological implications. T cells, for example, exhibit metabolic reprogramming during their activation and differentiation (Palmer et al., 2015; Wang and Green, 2012), switching from primarily oxidative phosphorylation in their naïve state, to glycolysis upon activation (Macintyre et al., 2014; Verbist et al., 2012). Since CD30 has been associated with activated T cells (Del Prete et al., 1995; Nakamura et al., 1997; Croft, 2003), these data potentially suggest a specific role for the receptor during T cell activation. Additionally, as cancer cells generally rely on glycolysis for energy metabolism (Hsu and Sabatini, 2008; Jones and Thompson, 2009; Hanahan and Weinberg, 2011), future studies investigating the effects of SM treatment on cellular energetics may find that IAP antagonism alters or reprograms the metabolism of the cell, contributing to SM-induced cell death.

Since the c-IAPs are involved in NF-κB activation by other receptors, such as BAFF-R and CD40, future studies may explore if the results of this dissertation are more

widely applicable (Vallabhapurapu et al., 2008; Gardam et al., 2011). Other members of the TNF receptor superfamily have been shown to degrade the c-IAPs and subsequently activate NF-κB following ligand binding (Vallabhapurapu et al., 2008); yet the similarity of the downstream effects of NF-κB activation by these receptors is unclear. While these receptors are thought to activate NF-κB through the TRAF-binding domains of their cytoplasmic tails, the structure of these regions of the receptors differ (Mosialos et al., 1995; Lee et al., 1996; Boucher et al., 1997; Ishida et al., 1996; Lu et al., 2003), and it remains unclear how these structural differences affect the activation of NF-κB and downstream gene expression. Would CD40, for example, also affect protein synthesis in a manner similar to CD30? Comparing multiple receptors to the CD30 data presented in this dissertation would provide a stronger understanding of the functional consequences of NF-κB activation.

One comparison that was not explored in this dissertation was the mechanistic differences in IAP degradation by SM treatment and CD30 activation. Specifically, SM treatment does not degrade TRAF2, while CD30 activation does (Csomos et al., 2009; Wright et al., 2007), and the consequences of a pool of unbound TRAF2 remain unknown. Furthermore, SMs have been shown to interact with XIAP, a protein not associated with CD30 signaling (Vince et al., 2007; Varfolomeev et al., 2007; Sun et al., 2007; Lu et al., 2008b), and it is unclear how this interaction may affect the observed downstream consequences. Even if XIAP is not degraded, SM may still functionally inhibit it, potentially contributing to the observed differences between the two stimuli. Additionally, CD30-mediated degradation of the c-IAPs is dependent on the RING domain of TRAF2, but does not require the E3 ubiquitin ligase activity of c-IAP1

(Csomos et al., 2009), while SM-induced degradation of the c-IAPs, conversely, is dependent on the E3 ubiquitin ligase activity of c-IAP1 (Csomos et al., 2009), indicating that the mechanics of ubiquitination and degradation also differ between the two stimuli. However, the experiments that identified these differences were performed in HEK293 cells and did not account for c-IAP2, suggesting the potential need to repeat these experiments in Karpas 299 cells using genome-editing techniques to generate the appropriate TRAF and c-IAP mutants. This system would eliminate the wild type TRAF and c-IAP proteins and would provide a competent cellular system in which to investigate downstream signaling.

## Synthetic IAP Antagonism and NF-κB

As discussed in earlier chapters, the IAPs have been implicated in the progression of cancer, and elevated expression of IAPs has been associated with poor survival rates (Xiang et al., 2009; Hussain et al., 2010; Esposito et al., 2007; Krajewska et al., 2005; Imoto et al., 2002). For these reasons, research has focused on means to inhibit the IAPs as a potential therapeutic strategy against cancer, leading to the development of SMs, which bind and induce the autoubiquitination and subsequent degradation of the IAPs (Vince et al., 2007; Varfolomeev et al., 2007; Darding et al., 2011), resulting in cell death in certain cells or sensitization of cells to exogenous TNF-mediated cell death (Petersen et al., 2007; Tenev et al., 2011). While the ability to induce cell death is promising for an anti-cancer therapeutic, SMs have also been shown to induce both canonical and non-canonical NF-κB activation, events normally considered to be pro-survival (Beinke and Ley, 2004; Beg et al., 1995; Vince et al., 2007; Varfolomeev et al., 2007). In contrast to

the pro-survival function typically associated with NF-κB, previous work with SMs has indicated that SM-induced cell death is dependent on NF-κB signaling, thereby suggesting a potential multifaceted role for NF-κB following c-IAP antagonism (Vince et al., 2007; Varfolomeev et al., 2007). To investigate this notion, the aim of Chapter III was to characterize SM-induced NF-κB activation and explore its functional consequences in different cellular contexts, and the resulting data demonstrated that SM-induced NF-κB activation is cell type-specific. Specifically, while canonical NF-κB was observed in a limited number of cell types, activation of non-canonical NF-κB was restricted to Karpas 299 cells, even though p100 processing to p52, a widely used surrogate marker of non-canonical NF-κB activation, was observed in all treated cells (Fig. 4.3). Functionally, SM-induced canonical NF-κB was found to promote cell death, complementing previous work that described a death-promoting canonical NF-κB complex (Campbell et al., 2004; Wu and Miyamoto, 2008). In contrast, activation of non-canonical NF-κB was found to promote cell survival through the inhibition of death-promoting canonical NF-κB. Suppression of non-canonical NF-κB was found to result in prolonged DNA binding of canonical NF-κB in the nucleus, and, notably, the presence of this transcription factor inhibited gene expression and resulted in the continued down-regulation of multiple gene sets. Non-canonical NF-κB reversed these effects and resulted in increased gene expression and up-regulation of numerous gene sets, indicating that non-canonical NF-κB functions, in part, as a regulator of canonical NF-κB, thereby preventing further SM-induced cell death (Fig. 4.3).

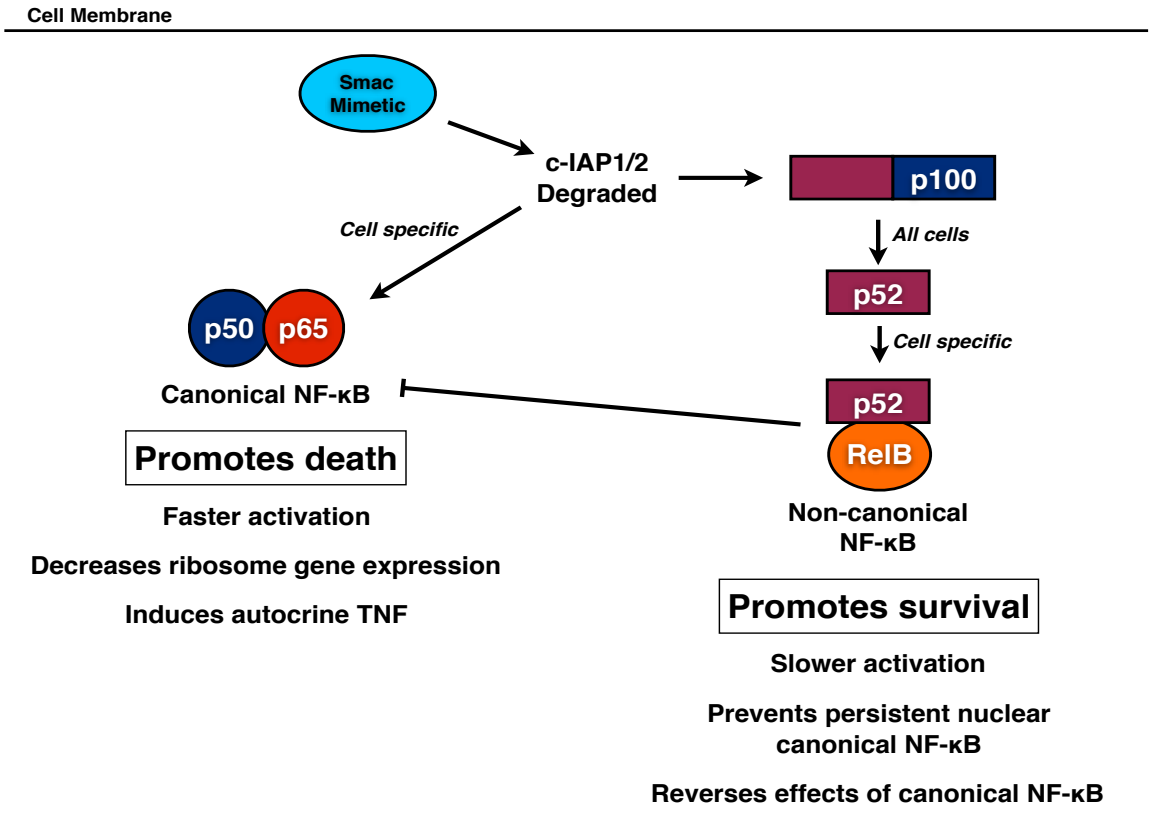


**Figure 4.3 Model of canonical and non-canonical NF-κB activation following SM treatment.**
SM-induced degradation of the c-IAPs initially results in a cell type-specific activation of canonical NF-κB. This NF-κB complex promotes death through the production of autocrine TNF, which results in TNF-R1-mediated cell death. Furthermore, SM-induced canonical NF-κB may be involved in the down-regulation of ribosomal gene expression, sensitizing the cell to TNF-mediated cell death. Additionally, c-IAP degradation results in the processing of p100 to p52, one subunit of the active non-canonical NF-κB dimer. This event was observed in every cell line treated with SM. Observation of nuclear, DNA-binding non-canonical NF-κB, however, was cell type-specific. This NF-κB dimer promoted cell survival and regulated canonical NF-κB. Non-canonical NF-κB was found to reverse the effects of canonical NF-κB and induce the expression of numerous genes, including inhibitors of canonical NF-κB.

While much of the data presented in Chapter III relates to the consequences of SM-induced NF-κB activation, one notable finding suggested that SM treatment might have additional effects, independent of NF-κB signaling. Gene expression in HEK293 cells was affected by SM treatment, even though these cells did not activate NF-κB.

While this may have been a cell type-specific event, these data suggest that SM treatment results in additional undefined consequences. Future studies may investigate if these effects occurred in other cells, potentially through monitoring gene expression following NF-κB inhibition and SM treatment. Observation of gene expression under these conditions would indicate that SM also regulates gene expression through NF-κB-independent means. Additionally, the observed gene expression patterns in HEK293 cells, as well as the other findings presented in Chapter III, may have been due to potential off target effects of the SM, rather than c-IAP degradation. Comparing the effects of multiple SMs, both monovalent and bivalent forms, could test this idea, as could RNAi-mediated suppression of c-IAP expression and ectopic expression of Ub-Smac, an engineered form of Smac that bypasses the mitochondria and is present in the cytoplasm in the absence of apoptotic stimuli (Hunter et al., 2003). Common consequences that were observed under these conditions would more clearly define the role of IAP antagonism in signaling.

While the focus of this dissertation was on the c-IAPs, SM treatment has been shown to also target XIAP for degradation (Darding et al., 2011; Lu et al., 2008b; Vince et al., 2007; Varfolomeev et al., 2007), even though XIAP was not degraded under the conditions used in studies presented in this dissertation. Even though XIAP was not degraded, the possibility that SM treatment still results in the functional inhibition of XIAP cannot be excluded. Future studies that aim to better characterize the role of XIAP in this context may use immunoprecipitation to monitor the association of XIAP with caspases before and after treatment with SM-164. Parallel experiments may use biotinylated forms of SMs, which currently exist, to see if the SM interacts with XIAP in

the conditions used in these studies. Additionally, there have also been recent reports of SMs that specifically target c-IAP1/2 (Sun et al., 2014), and the use of these compounds would provide insight into the necessity of XIAP in the signaling pathways described in Chapters II and III. While these experiments would help separate the functions of XIAP and the c-IAPs, it may also be useful to discern the individual roles of c-IAP1 and c-IAP2. While the c-IAPs are often considered redundant, there is evidence that they possess non-redundant roles, as well (Darding et al., 2011). Furthermore, the two c-IAPs appear to have varying expression following certain stimuli, with c-IAP2 being highly expressed following NF-κB activation while c-IAP1 is not, and this disparity may underlie functional differences between the two proteins. Additionally, while c-IAP1 appears to be expressed in the cell lines tested in Chapter III, the expression of c-IAP2 varied between the cell lines, suggesting differential regulation and, perhaps, functional importance. Future experiments that target the individual c-IAPs with RNAi would help answer these questions, though the results from experiments using the Smac mimetic, Birinapant, in Chapter II suggest that efficient degradation of both c-IAPs is necessary for SM-induced NF-κB activation.

Physiologically, IAP antagonism can occur during the cell death process when IAP binding proteins, such as Smac, are released from the mitochondria. However, as proteins typically associated with cell death, such as the IAPs and caspases, are implicated in signaling pathways other than apoptosis, it is possible that Smac also has non-apoptotic roles. Could Smac be a regulator of IAP and caspase activity in the absence of a death stimulus? In support of this idea, there have been reports of selective release of Smac from the mitochondria under certain conditions (Kandasamy et al., 2003;

Csomos et al., 2009). Furthermore, data not presented in this dissertation, along with previous work, have demonstrated that suppression of Smac expression by siRNA resulted in decreased baseline gene expression, suggesting that Smac may have non-apoptotic, housekeeping roles within a cell (Csomos et al., 2009).

While the results presented in Chapter III compared the effects of SM treatment in multiple cells, the limitation of the data being based on established cell lines remains. Since primary cells were not used in these studies, it is unclear if the c-IAPs play the same role in both cancer cells and healthy cells, leading to the question of whether primary samples from cancer patients and healthy controls would behave similarly. Future experiments that explore this question could compare healthy T cells to the Karpas 299 data presented in this dissertation, specifically focusing on both CD30 and SM-mediated c-IAP degradation, NF-κB activation, p100 processing, and cell death. If these events are also observed in the healthy T cells, additional assays investigating gene expression and protein synthesis may be warranted. Eventually, performing these assays in primary cells from cancer patients would help better convey the clinical relevance of the findings of this dissertation.

Since cancer cells proliferate rapidly, future studies may investigate and compare the effect of SM treatment in quiescent cells and rapidly proliferating cells, potentially monitoring cell cycle progression, which may be important in the context of c-IAP degradation since CD30 activation was found to induce cell cycle arrest (Buchan and Al-Shamkhani, 2012; Wright et al., 2007), though it is unclear if this directly resulted form IAP degradation. Additionally, in certain cells, such as HeLa and THP1, c-IAP1 was found to localize to the nucleus and was implicated in the regulation of cell cycle

progression (Xu et al., 2007; Samuel et al., 2005; Cartier et al., 2011; Plenchette et al., 2004), suggesting that SM-induced c-IAP degradation could induce cell cycle arrest. If SM treatment does lead to cell cycle arrest, this may mean that SMs are more effective in rapidly dividing cells, such as cancer cells, and may have a more limited effect in slow growing cells, potentially differentially targeting cancer cells over surrounding tissue. In addition to comparing the effects of SM treatment in healthy cells with cancer cells, quiescent T cells could be compared to activated and proliferating T cells. Collectively, these experiments would provide a better characterization of the optimal targets of SM treatment as well as how these compounds could affect other tissues in patients.

Another unresolved aspect of the work presented in this dissertation is the mechanistic basis for the observed variances in NF-κB activation following SM treatment in the different cell lines. The absence of NF-κB activation may be explained by the lack of functional upstream factors, either through differential expression or mutations. Mutations in NIK, for example, have been described, and these mutations result in defective canonical and non-canonical NF-κB signaling (Willmann et al., 2014). Future experiments may initially compare the different cell lines in more detail, examining, for example, the status of IκBα and the IKK complex, degradation of NIK, localization of the NF-κB dimers, and modifications of the NF-κB subunits. Once potential differences are identified, it would be interesting to determine if a correlation between functional NF-κB signaling and expression of TRAF-binding TNF receptor superfamily members exists, as these have restricted expression and induce activation of both NF-κB pathways. Future studies may also choose to investigate the function of p52 since SM treatment induced the processing of p100 to p52 in all the tested cell lines, yet DNA-binding non-canonical

NF-κB was not observed in the majority of the cells. Since the processing event occurred in all cells, it may imply that p52 has other roles, independent of its participation in the non-canonical NF-κB dimer. Investigating the functional consequences of the pool of p52 may identify additional functions and help us further understand the dynamics of NF-κB signaling.

With the work presented in Chapter III, a role for RelB in the regulation of canonical NF-κB was illustrated. However, as this experiment was only performed using SM treatment, it is unclear how this would impact CD30 signaling. In Chapter II, the kinetics of NF-κB activation following CD30 stimulation were found to differ from the outcome of SM treatment. While a gradual shift from canonical NF-κB to non-canonical NF-κB was observed following SM treatment, CD30 stimulation resulted in a consistent nuclear presence of canonical NF-κB. The observable canonical NF-κB following prolonged stimulation with CD30L is most likely a mixture of differentially activated canonical NF-κB, specifically c-IAP degradation-dependent and -independent forms that may possess divergent modifications and functionality, and this may represent a distinct outcome compared to SM treatment, which appears to activate a single, c-IAP degradation-dependent canonical NF-κB signaling pathway. Due to the technical limitations of EMSAs, it is unclear if the observed canonical NF-κB band is comprised of more than one form of canonical NF-κB, i.e. c-IAP degradation-dependent and -independent canonical NF-κB. Therefore, if the experiments proposed above could identify multiple, differentially modified canonical NF-κB dimers following the various treatment conditions, it would be interesting to further investigate the role of RelB in this system. Would RelB preferentially regulate a specifically modified form of canonical

NF-κB? Would the ratio of the modified forms of canonical NF-κB be altered following suppression of RelB expression? Suppression of RelB expression by RNAi techniques in Karpas 299 cells followed by stimulation with CD30L and monitoring of NF-κB activation and gene expression would help answer these questions and provide valuable information into how the transcriptional outcomes following prolonged CD30 activation were affected by RelB and non-canonical NF-κB.

As this work is expanded beyond the Karpas 299 cellular system, studying both canonical and non-canonical NF-κB signaling cascades in other cellular contexts may be important as both pathways are activated throughout the development of the immune system and during its response to antigenic challenge (Oeckinghaus et al., 2011; Vallabhapurapu and Karin, 2009). As the work presented in Chapter III helped define a novel functional role for non-canonical NF-κB, it may be important to examine if non-canonical NF-κB regulates canonical NF-κB in these other cellular contexts, as well as in certain cancers. More specifically, both NF-κB pathways are activated in multiple myeloma and MALT lymphoma (Rosebeck et al., 2014; Rosebeck et al., 2011; Annunziata et al., 2007), and therefore if non-canonical NF-κB confers a pro-survival phenotype in these cells, blocking activation of non-canonical NF-κB may be needed for effective anti-cancer therapies.

As described above, NF-κB can be frequently modified, affecting its activity and gene targets (Viatour et al., 2005; Chen and Greene, 2003; Bhatt and Ghosh, 2014; Diamant and Dikstein, 2013; Lu et al., 2013; Chen et al., 2002). While the modifications of SM-induced NF-κB are presently unknown, future experiments may aim to investigate potential cell type-specificity of these modifications. Is SM-induced canonical NF-κB the

same in different cellular contexts? If so, it would imply that these cells have the proper machinery to perform the modifications, leading to an active NF-κB dimer, highlighting potential factors that may be absent in other cell lines. If the observed canonical NF-κB dimers were to be modified differently, how would this impact their function? The answer to this question would provide additional information on the mechanism of SMs and help more effectively guide their use in the clinic.

## Closing Remarks

In conclusion, the work presented in this dissertation examined the roles of the c-IAPs in intracellular signaling, identifying novel aspects of c-IAP-dependent signaling through the comparison of the functional consequences of physiological and synthetic c-IAP antagonism. The findings presented in this dissertation established a new role for the c-IAPs in the regulation of protein synthesis and also demonstrated that the downstream effects of IAP antagonism were partially stimulus-dependent. Furthermore, the data presented in this dissertation described the cell type-specific nature of SM-induced NF-κB activation and identified regulatory crosstalk between canonical and non-canonical NF-κB. Collectively, the data from Chapters II and III lay a solid foundation for future studies, and contributes to our understanding of the multifaceted roles of c-IAP1/2 in cellular signaling and provides valuable insight into the mechanism of synthetic IAP antagonists, furthering our understanding of their therapeutic potential.

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