Exp Oncol 2012
34, 3, 200–211

INVITED REVIEW

Experimental ONCOLOGY

THE INHIBITOR OF APOPTOSIS (IAP) PROTEINS ARE CRITICAL REGULATORS OF SIGNALING PATHWAYS AND TARGETS FOR ANTI-CANCER THERAPY

*M.C. de Almagro, D. Vucic**

Department of Early Discovery Biochemistry, Genentech, Inc., South San Francisco, CA, 94080, USA

Cell death regulation is vital for maintenance of homeostasis and proper development of multicellular organisms. Inhibitor of apoptosis (IAP) proteins are implicated in multiple ways in cell death regulation, ranging from inhibition of apoptosis and necrosis to the regulation of cell cycle and inflammation. Due to their prominent ability to control cell death and elevated expression in a variety of cancer cell types, IAP proteins are attractive targets for the development of novel anti-cancer treatments. The most widely used strategy for targeting IAP proteins is based on mimicking the natural IAP antagonist, SMAC/DIABLO. IAP antagonists are currently being tested in humans and they were designed for anti-cancer therapy but they could potentially also be considered for treatments of the immune system disorders. In this manuscript we will review the functional roles of IAP proteins, specifically of c-IAP1, c-IAP2, ML-IAP and XIAP, and evaluate IAP targeting strategies for disease treatments. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: IAP, BIR, TNF, apoptosis, ubiquitin, Smac, NF-kB, cancer, RING.**

The balance between cell death and survival is one of the main features of cellular homeostasis [1]. Cell death could be seen *a priori* as something negative but programmed mechanisms such as apoptosis or necroptosis have important roles in maintenance of desired cell number [2]. Apoptosis is critical in embryogenesis, where certain cells need to die in order to allow the formation of particular morphological features [3]. Programmed cell death is also beneficial for prevention of tumors or the spread of infectious diseases, as it enables elimination of damaged or infected cells, or cells that harbor too many mutations.

The inhibitors of apoptosis (IAP) proteins are a family of proteins that are involved in cell death, immunity, inflammation, cell cycle and migration [4]. The members of this protein family are characterized by the presence of one to three baculoviral IAP repeats (BIR) domains [5].

IAPs were first identified in 1993 in baculoviral genomes because of their ability to suppress the host-cell death response during viral infection [6, 7]. The first identified human IAP protein, NAIP, as well as X-chromosome-linked IAP (XIAP), were identified using homology searches for BIR domain containing proteins [8]. Cellular IAP proteins (c-IAPs) were first identified as components of the Tumor Necrosis Factor Receptor 2 (TNFR2) complex, which is mediated through binding to TRAF1 and TRAF2 [9].

The human IAP family is composed of eight proteins: NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), XIAP (BIRC4), survivin (BIRC5), Apollon/Bruce (BIRC6), ML-IAP (BIRC7 or livin) and ILP-2 (BIRC8). Among these IAP proteins, c-IAP1, c-IAP2, ML-IAP and XIAP are directly involved in apoptosis regulation [10], while other members of the family can regulate cell survival by other means such as cell cycle control or inflammation. In this review we focus on c-IAP1, c-IAP2, XIAP and ML-IAP, and their role in cell death and signaling pathways with emphasis on potential new anti-cancer treatments.

Received: June 27, 2012.

*Correspondence: E-mail: domagoj@gene.com

Abbreviations used: BIR – baculovirus IAP repeat; CARD – caspase recruitment domain; DD – death domain; IAP – inhibitor of apoptosis; IBM – IAP binding motif; NEMO – NF-κB essential modifier; NF-κB – nuclear factor kappa B; RING – really interesting new gene; RIP – receptor interacting protein; SMAC – second mitochondrial activator of caspases; TNF – tumor necrosis factor; TRAF – TNF receptor associated factor; UBA – ubiquitin associated.

IAP PROTEINS AND APOPTOTIC PATHWAYS

Apoptosis is a meticulously regulated cell death program that relies on caspases, a family of cysteine-dependent aspartic acid proteases [11]. Caspases are synthesized as inactive proteins or zymogens, but once they dimerize or are cleaved by another protease, usually another caspase in the caspase activation cascade, they become active enzymes [11]. Although caspases are well known because of their role in apoptosis, some caspases are involved in cytokine processing, such as IL-1β processing by caspase-1. Apoptotic pathways engage 2 types of caspases, the initiator (caspase-2, -8 and -9) and the effector caspases (caspase-3, -6 and -7) [12].

Apoptosis can be activated via an extrinsic or death-receptor-mediated pathway and an intrinsic or mitochondrial pathway (Fig. 1). The extrinsic apoptotic pathway is initiated by activation of death receptors of the TNF receptor superfamily by their respective ligands. This superfamily is composed of transmembrane proteins that share a conserved extracellular cysteine-rich domain. The death receptor members of the TNFR superfamily possess intracellular death domains (DD) and include: TNFR1, Fas (CD95, APO-1), and death receptors 3, 4 and 5 (DR3, DR4 and DR5) [13, 14]. The activation of these receptors leads to the formation of a receptor-associated complex

of Fas-associated DD (FADD) and caspase-8 and -10 that triggers caspase-8/-10 activation, and subsequent cleavage of caspase-3 and -7 for apoptosis initiation [15]. For TNFR1, the cytokine TNFα binds to the receptor and triggers its trimerization, which leads to the assembly of the receptor complex and initiation of signaling. The death domain of TNFR1 recruits TNF receptor associated death domain protein (TRADD), an adaptor molecule that allows binding of TRAF2 and c-IAP1 and c-IAP2 to the receptor complex [16]. The DD containing kinase RIP1 can bind TNFR1 through TRADD association, although the binding of RIP1 to the TNFR1 has also been observed in TRADD deficient cells [17, 18].

c-IAP1 and c-IAP2 regulate the extrinsic apoptotic pathway through their ubiquitin ligase activity [19]. c-IAP proteins are responsible for RIP1 ubiquitination, and in their absence RIP1 cannot be ubiquitinated [20, 21]. Nonubiquitinated RIP1 can form a cytosolic complex with the adaptor molecule FADD and caspases-8, leading to induction of apoptosis [20, 22]. In addition to c-IAP proteins, another major negative regulator of death-receptor-mediated cell death is cellular FLICE inhibitory protein long ($FLIP_L$), a protein similar to caspases-8 but with no catalytic activity [23].

The intrinsic pathway is initiated by cellular stress, growth serum withdrawal, DNA damage, radiation or other stress signals that are detected by Bcl-2-homology 3 only (BH3-only) proteins resulting in alterations of the outer mitochondrial membrane potential and permeability (see Fig. 1) [24]. This will cause the release of cytochrome *c* and second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO) from the mitochondria. Cytochrome *c* released to the cytosol binds apoptosis protease activating factor (Apaf1) and induces formation of the apoptosome, which leads to the activation of caspase-9 and later caspase-3 and -7. XIAP inhibits caspase-3, -7 and -9 directly by binding to them. Meanwhile, SMAC can bind XIAP through its N-terminal IAP-binding motif (IBM), AVPI, prevent its inhibition of caspase-3, -7 and -9, and thus remove the apoptosis blockade [10].

Apart from SMAC, there are additional factors that can inhibit XIAP in order to allow apoptosis. Another mitochondrial protein that inhibits XIAP is Omi/HtrA2. Similarly to SMAC, this serine protease has an IAP-binding motif with the sequence AVPS [25]. Omi/HtrA2 has a dual pro-apoptotic activity: on one hand it blocks XIAP binding to caspases, and on the other its protease activity cleaves XIAP rendering it unfunctional [25]. XIAP-associated factor 1 (XAF1) can also bind XIAP, c-IAP1/2 and other IAP proteins and interfere with their anti-apoptotic activity [26, 27].

Among the numerous proteins and protein complexes that control apoptotic pathways, IAP proteins play an important role. There are two predominant ways by which IAP proteins regulate apoptosis. c-IAP proteins do not bind caspases at a physiologically meaningful level [28]. Instead, they regulate caspase activation indirectly through their E3 ligase activity and modulation of TNF-mediated cell death as well as Toll signaling, innate immunity and NF-κB pathways [19, 29–31]. Anti-apoptotic activity of XIAP stems primarily from its direct binding to and inhibition of caspase-3, -7 and -9 [32, 33].

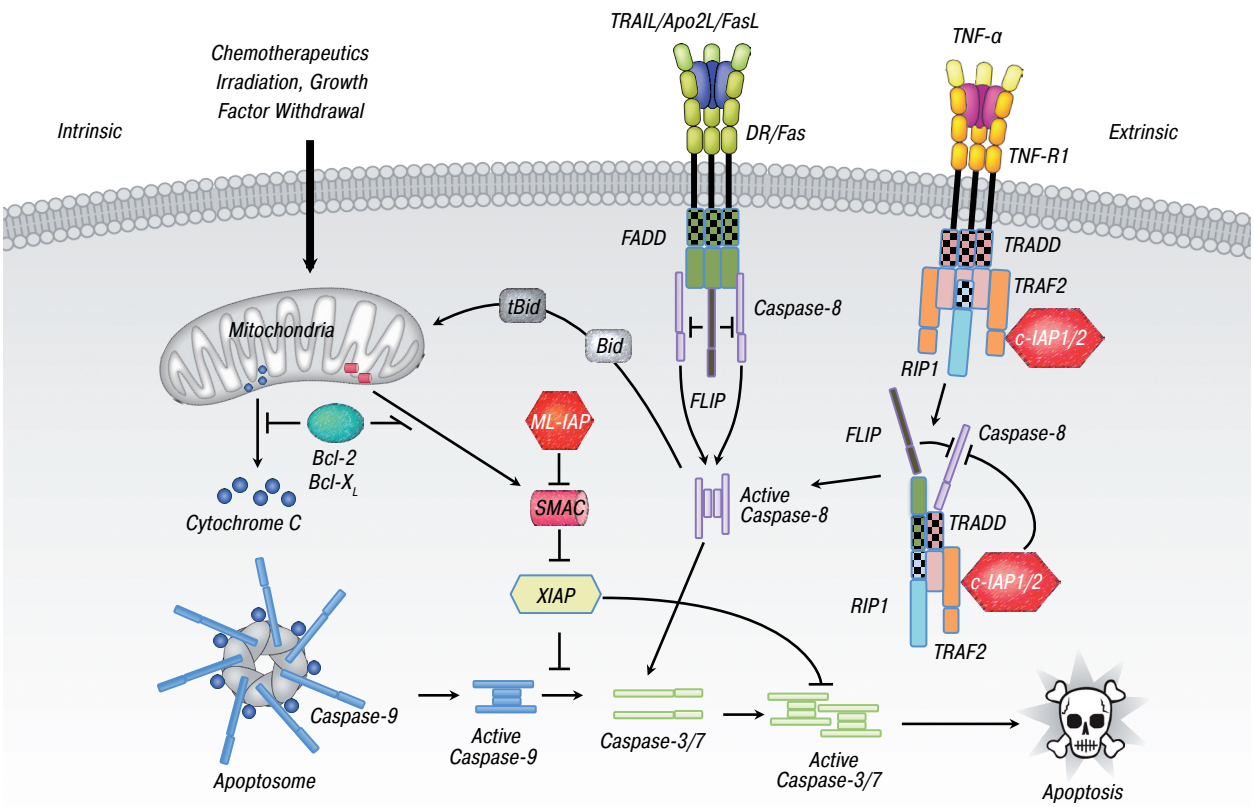

Fig. 1. The intrinsic and extrinsic apoptotic pathways

IAPS STRUCTURE AND FUNCTION

All IAP proteins contain at least one BIR domain, and several also possess a Ubiquitin Associated domain (UBA) domain and a Really Interesting New Gene (RING) domain (Fig. 2) [33]. c-IAP1, c-IAP2, NAIP and XIAP each contain three BIR domains, while ML-IAP, survivin, Bruce and hILP2 contain only one. Baculovirus IAP Repeats are zinc-binding domains of approximately 80 amino acids [5] that mediate protein–protein interactions and, in some cases, the binding and inhibition of caspases [34, 35]. The BIR domains coordinate a zinc ion through one histidine and three cysteine amino acid residues [36]. The N-terminal BIR domain (BIR1) of c-IAP1, c-IAP2 and XIAP is well conserved across species. However, even small sequence diversity allows binding to different binding partners: XIAP BIR1 binds TAB1 [37], while c-IAP1 and c-IAP2 BIR1 domains are necessary and sufficient for binding TRAF2 [38, 39]. The subtle differences in the N-terminal regions of the BIR1 domains provide c-IAP1 and c-IAP2 with the unique ability within the IAP family to interact with TRAF1 and TRAF2 [38–40].

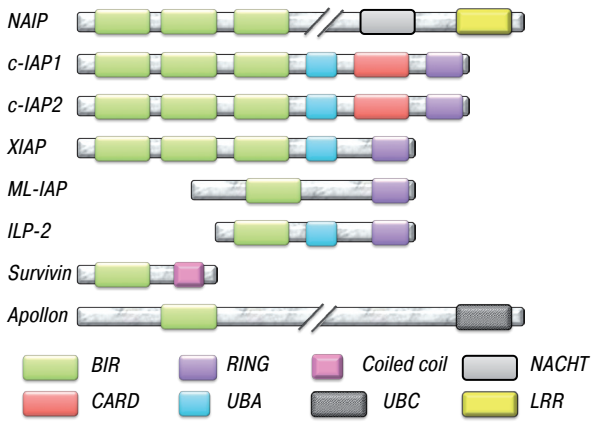

Fig. 2. Schematic representation of human IAP proteins

XIAP binds caspases through its BIR2 or BIR3 domains. The XIAP BIR2 domain and the linker region between the BIR1 and BIR2 domains are needed for the inhibition of activated caspas-3 and -7 [41–43]. This linker region of XIAP blocks the active site of those caspases, and XIAP BIR2 makes important contacts with the N-terminal regions of the small subunits of partially processed caspase-3 and -7 [41, 44]. The XIAP BIR3 domain targets and inhibits caspase-9 by binding caspase-9 monomer and preventing its dimerization and activation [45, 46]. Smac preferentially binds the BIR3 domain of c-IAPs and XIAP, although Smac can also associate with the BIR2 of XIAP [38, 47]. Smac, an endogenous IAP antagonist, is a dimer in solution, and it can bind the BIR2 and BIR3 domains of XIAP simultaneously to prevent XIAP from binding and inhibiting caspase-3, -7 and -9 [48, 49].

Apart from the BIR domains, another common feature among c-IAP1, c-IAP2, ML-IAP, hILP2 and XIAP is the possession of a C-terminal RING domain that provides these proteins with E3 ubiquitin ligase activity [50]. RING dimerization strongly potentiates the ubiquitin ligase activity [51]. IAP proteins have been shown to homodimerize and heterodimerize, thus allowing autoubiquitination and trans-ubiquitination within the family [52]. In addition, IAP proteins can promote ubiquitination of a number of their binding partners and other proteins that are present in the same signaling complexes. The most important feature of this 40 amino acid zinc-coordinating domain is the recruitment of E2 ubiquitin conjugating enzymes [50]. The RING domains of IAP proteins show a preference for the UbcH5 family of E2 enzymes although individual IAPs also have unique E2 partners [53]. The XIAP RING domain can mediate K48-linked polyubiquitination and affect the levels of caspase-3 and itself [54]. Furthermore, mice expressing a RING-deleted XIAP exhibit more apoptosis in the presence of TNFα or TRAIL than wild type animals [54]. Similarly, *Drosophila* DIAP1 can promote ubiquitination of fly caspases as well and thus inhibit apoptosis [55].

Another domain common to c-IAP1, c-IAP2, XIAP and hILP2 is the ubiquitin-associated (UBA) domain, a conserved domain located between the BIR and the RING domains [56, 57]. The IAP UBA domain enables IAP proteins to bind a variety of ubiquitin chains including K63, K48, K11 and linear chains as well as monoubiquitin [57]. c-IAP1 and c-IAP2 also have a Caspase Recruitment Domain (CARD) whose function is not yet completely understood.

IAP PROTEINS AND UBIQUITIN

Ubiquitination is one of the main post-translational protein modifications found in eukaryotic cells [58]. In addition to regulating efficient control of protein degradation and turnover, ubiquitination can modify the enzymatic activity of many important cellular regulators and alter cellular localization of substrate proteins. Ubiquitination involves covalent attachment of the 76 amino acid protein ubiquitin to a lysine of a target protein. This process is carried out by an E1 ubiquitin-activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase, with E3 enzymes providing the substrate specificity. There are two E1s, around 50 E2s, and over 600 E3s [59]. Two types of RING E3 ligases can be found: ones that form a multiprotein complex with substrate binding protein(s), and others where the E2 binding and the substrate binding domains are encoded by the same polypeptide [60]. IAP proteins belong to the later group as they use predominantly their BIR domains for substrate binding and the RING for the interaction with E2 enzymes [50]. Ubiquitinion can result in the transfer of a single ubiquitin molecule to target proteins — monoubiquitinion, or in the assembly of polyubiquitin chains. Since ubiquitin has seven lysines and an N-terminal methionine, eight different kinds of ubiquitin chains are possible: linear (through N-terminus), K6, K11, K27, K29, K33, K48, K63, although the most common and best studied are K48 and K63 linkage chains. In most cases K48 chains of more than four ubiquitins tag a protein for 26S proteasomal degradation [50], while K63-linked chains can provide a signal for functional

activity or change of cellular distribution. c-IAP proteins are capable of promoting the assembly of a wide range of different ubiquitin chains on their substrates and on themselves.

The best-known substrates for the E3 ligase activity of c-IAP proteins are RIP1 and NIK. c-IAP1/2 mediated polyubiquitinatination of RIP1 is the critical signal needed for the activation of the canonical NF-κB signaling pathway [20, 21]. In the noncanonical NF-κB pathway c-IAP1 and c-IAP2 promote K48-linked polyubiquitination of NIK, which blocks noncanonical NF-κB activity [31, 47]. Apart from RIP1 and NIK, c-IAP1 and c-IAP2 promote ubiquitination of a variety of signaling molecules including TRAF2, TRAF3, Ask, as well as Smac, which may have direct implications for cellular survival [19, 50]. XIAP ubiquitination activity does not seem to be essential for its anti-apoptotic activity but it can still influence caspase stability and cellular survival [54].

REGULATION OF SIGNALING PATHWAYS BY IAP PROTEINS

NF-kB signaling pathways

The NF-κB family of transcription factors transduces the signal from a variety of stimuli leading to the transcription of a broad spectrum of genes involved in cell survival, immunity, and inflammation. There are five different types of NF-κB transcription factors: p50 or NF-κB1 (formed from a selective degradation of p105), p52 or NF-κB2 (generated from its p100 precursors), RELA or p65, RELB and c-REL [61]. Due to the high relevance of NF-κB pathways for cellular immunity and survival, they are tightly regulated by two crucial post-translational modifications, phosphorylation and ubiquitination. Two NF-κB signaling pathways, the canonical and the noncanonical, can be generally differentiated by the timing and signaling proteins that are involved in their activation (Fig. 3) [62].

Canonical NF-kB

The best-studied stimulus for the initiation of canonical NF-κB signaling is TNFα. Binding of TNFα to TNFR1 triggers the recruitment of the proximal receptor-associated complex consisting of TRADD, TRAF2, c-IAP1, c-IAP2 and RIP1 [16]. TRAF2 is one of seven TRAF proteins present in mammals. All TRAFs contain a TRAF domain and, except TRAF1, an N-terminal RING domain — although the RING domain is not functional in most of them. c-IAP1 and c-IAP2 bind TNF receptors through association with TRAF2. The BIR1 domain of c-IAP proteins binds the TRAF-N domain of TRAF2, and structural studies revealed that one c-IAP protein binds three TRAF2 molecules [40]. Ligand-stimulated aggregation of receptor complexes causes recruitment of multiple TRAF2 trimers, which in turn leads to c-IAP1 or c-IAP2 dimerization, activation of c-IAP E3 ligase activity and consequent ubiquitination of the kinase RIP1, themselves and other binding partners [40, 63].

RIP1 is a critical mediator of several signaling pathways because its posttranslational modifications can lead to the activation of NF-κB, apoptosis or necrosis. In the NF-κB pathway c-IAP1 and c-IAP2 mediate K63- and K11-linked polyubiquitination of RIP1 [53]. RIP1 ubiquitination allows the binding of the IκB kinase, IKK (IKKα/IKKβ/NF-κB essential modifier, NEMO) complex, the pro-survival kinase Transforming Growth Factor β-activated Kinase 1, TAK1-TAB

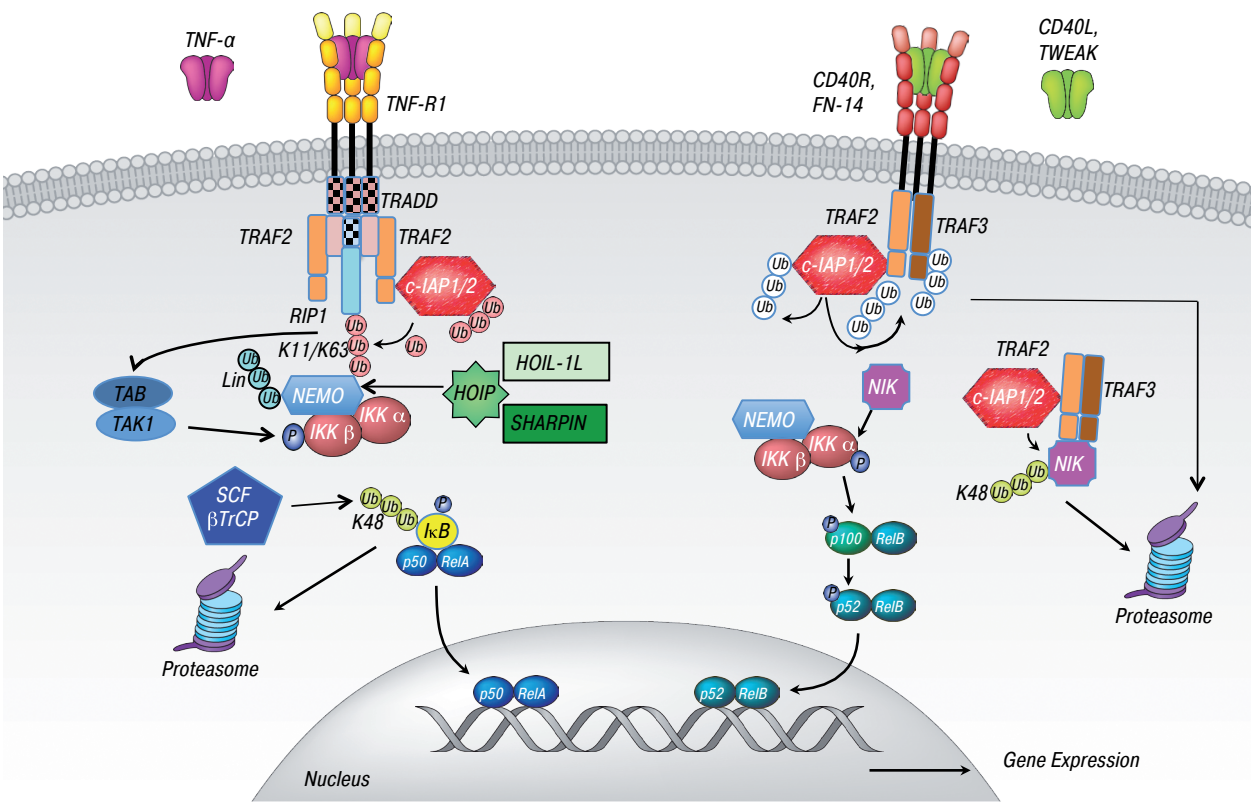

Fig. 3. Canonical and noncanonical NF-κB pathways

(TAK1/ TAK1-binding protein 2,TAB2/ TAK1-binding protein 3, TAB3) complex, and LUBAC, a complex containing two regulatory subunits: SHANK-associated RH domain interactor (SHARPIN) and heme-oxidized IRP2 ubiquitin ligase 1 homolog (HOIL-1L), and the catalytic subunit HOIL-1-interacting protein (HOIP) [64–68]. Interestingly, the kinase activity of RIP1 does not seem to be needed for the activation of the canonical NF-κB pathway [69]. Ubiquitinated RIP1 brings LUBAC and NEMO in proximity, which allows subsequent linear ubiquitination of NEMO by LUBAC [64, 65]. But the RIP1 relationship with c-IAP1/2 is not limited to NF-κB activation, because in the absence of c-IAP1 and c-IAP2 TNFα stimulation can lead to RIP1-mediated apoptosis or necroptosis.

NEMO, along with the other subunits of the IKK complex, IKKα and IKKβ, is recruited to the RIP1-assembled polyubiquitin chains in a TRAF2/c-IAP1/RIP1 dependent manner [70]. The ubiquitination of RIP1 brings the IKK complex into proximity of the TAK1 kinase complex, leading to phosphorylation and activation of IKKβ [71]. IKKβ phosphorylation triggers IκBα phosphorylation at two N-terminal serines [72], which is a signal for IκBα K48-linked ubiquitination by the F-box/WD-domain protein of the β-TrCP/Slimb family and subsequent proteasomal degradation [73]. In non-stimulated cells, IκBα sequesters the NF-κB transcription factors in the cytoplasm, but the degradation of IκBα frees p65 and RelA and enables them to translocate to the nucleus. The NF-κB subunits can act as homo or heterodimers [74]. Once in the nucleus, NF-κB transcription factors bind the promoters that harbor NF-κB recognition elements and induce their transcription [62]. Therefore, c-IAP proteins should be considered as critical positive regulators for the activation the NF-κB canonical pathway.

Noncanonical NF-kB

One of the properties that differentiates the noncanonical from the canonical NF-κB pathway is the timing, since its activation is much slower than the canonical pathway due to a requirement for new protein synthesis. In addition to activating canonical NF-κB signaling, several TNF receptor family members, including FN14, LTβR and CD40, can stimulate the noncanonical NF-κB pathway.

In unstimulated cells c-IAP1/2, TRAF2 and TRAF3 constitutively associate with NIK, the key regulator of noncanonical NF-κB signaling [75, 76]. Through association with c-IAPs and NIK, TRAF2 and TRAF3, respectively, bring c-IAP proteins in proximity of NIK. c-IAP1 and c-IAP2 promote ubiquitination of NIK with K48 linkages and tag it for proteasomal degradation, thus preventing noncanonical NF-κB activation [77]. Activation of TNF superfamily receptors such as CD40, or the treatment with IAP antagonists, causes dimerization of c-IAP proteins, which in turn stimulates their autoubiquitination and proteasomal degradation [63]. Recruitment of c-IAPs, TRAF2 and TRAF3 to CD40 or TWEAK receptor complexes leads to their translocation to SDS soluble fractions, autoubiquitination and ultimately degradation [63, 77]. The loss of c-IAP proteins releases NIK from degradative ubiquitination, thus allowing its accumulation [47]. NIK phosphorylates IKKα dimers, leading to subsequent phosphorylation of p100 at its C-terminus, which triggers its ubiquitination [78]. Polyubiquitinated p100 is subject to partial proteasomal degradation from its C-terminus to yield a truncated fragment of p100, p52 [78]. The p52 fragment dimerizes with RelB and translocates to the nucleus where it binds the promoter regions of NF-κB dependent genes to activate their transcription. Therefore, c-IAP1 and c-IAP2 are negative regulators of noncanonical NF-κB signaling through their ability to suppress cellular NIK levels. After the c-IAP proteins are degraded or removed from their substrate NIK the noncanonical NF-κB pathway can be activated.

Regulation of other signaling pathways by IAP proteins

In addition to NF-κB regulation, IAP proteins play important role in other signaling pathways, such as Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), TGF-β, Myc, and PI3K/Akt [79–83]. The JNK and p38 pathways can be activated by the TNF family members leading to transcriptional activation of a series of genes through Fos and Jun [84].

The importance of c-IAP1 and c-IAP2 for the activation of MAPK signaling was shown in cells stimulated with different TNF ligands: TNFα, TL1A, TWEAK, or CD40L. Elimination of c-IAP1 and c-IAP2 resulting from IAP antagonist BV6 treatment drastically reduced the activation of MAPK p38 and JNK [63]. Furthermore, it was observed in B cells from c-IAP1 and c-IAP2 knockout mice that after CD40L stimulation, there was no phosphorylation of JNK, ERK or p38 [85]. Data from these reports suggest that the ubiquitin ligase activity of c-IAP proteins is critical not only for NF-κB pathways but also for efficient activation of MAPK signaling [63].

IAPS IN INFLAMMATION AND IMMUNITY

In addition to the involvement of IAP proteins in apoptosis, there are an increasing number of studies that link IAPs to innate immunity and inflammation. One of the reasons for this association is the ability of c-IAP proteins to regulate the expression of genes involved in innate immunity through the NF-κB and MAPK signaling pathways. However, they can also control cell death as a result of pathogen infections.

Innate immunity represents the defense mechanism against external pathogens. Immune cells can detect the pattern recognition receptors (PRRs) and initiate an immune response. There are different receptors for pathogen-associated molecular patterns (PAMPs): Toll-like receptors (TLRs) at the cellular membrane, and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) as well as DNA and RNA sensing receptors in the cytosol [29].

c-IAP1 and c-IAP2 are implicated in innate immunity because of their ability to ubiquitinate RIP2 and

mediate NOD signaling [29]. When NOD receptors are activated by bacterial peptidoglycans, they oligomerize and recruit c-IAP1/2, TRAF2 and RIP2. This allows c-IAP1/2 mediated K63-linked polyubiquitination of RIP2, which in turn recruits TAK1-TAB1/2/3 and IKK complexes and leads to MAPK and NF-κB activation and induction of cytokines and expression of proinflammatory genes [29, 86, 87].

XIAP has been speculated to have a role in transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) signaling, mainly through its ability to bind the kinase complex of TAK1 and TAB1 through its BIR1 domain [37, 88], although there was no proof that XIAP regulates these pathways in an endogenous setting. More recent data suggests that XIAP has a potentially instrumental role in immune response to intracellular bacteria, as cells lacking XIAP show deficiency in the activation of the NF-κB pathway following NOD2 receptor activation [89]. XIAP is proposed to exert its role by being the critical ubiquitin E3 ligase for RIP2, and the main instigator of LUBAC recruitment to the NOD2-associated complex [90].

c-IAP1/2 have been reported to promote TRAF3 K48-linked ubiquitination, but also nondegradative ubiquitination of TRAF3 and TRAF6, which is potentially important for type I IFN induction and antiviral response [91]. TRIF-dependent signals trigger K63-linked polyubiquitination of TRAF3 leading to IRF3 activation [59]. Knockdown of c-IAP1/2 resulted in the inhibition of virus-induced activation of IRF3, NF-κB, INF-β and the cytoplasmic dsRNA, retinoic acid-inducible gene (RIG-1) antiviral response [91].

IAP proteins can potentially regulate IL-1β production as well. IL-1β is a proinflammatory cytokine that is activated by caspase-1 cleavage and secreted in response to cell damage or pathogens [92]. However, in the presence of Smac mimetics, both caspase-1 and caspase-8 have been observed to process IL-1β in Toll-like receptor primed macrophages [92]. This IL-1β production in the absence of IAPs is dependent on RIP3, so it is believed that c-IAP1/2 and XIAP prevent RIP3 activation, thus inhibiting NLRP3-stimulated IL-1β cleavage [92].

XIAP has also been described as a protection factor against Sendai virus (SeV) infection [93]. SeV infection activates the kinases IKKε and TBK1, which can phosphorylate XIAP and trigger its K48-linked autoubiquitination and proteasomal degradation [93]. XIAP degradation will then allow apoptosis and avoid the spread of the virus.

THE ROLE OF C-IAP PROTEINS IN REGULATION OF RIP1

The regulation of the serine/threonine kinase RIP1 represents an important cellular checkpoint because the presence and post-translational modifications of RIP1 can be the determining factor between cell death and survival. RIP1 is a founding member of a family of kinases that is characterized by the presence of a kinase domain, an intermediate domain, and a death domain that allows RIP1 to interact with TRADD and FADD [94]. c-IAP proteins are main regulators of RIP1 through their ubiquitin ligase activity.

As described before, c-IAP1 and c-IAP2 mediated ubiquitination of RIP1 leads to canonical NF-κB pathway activation. But when c-IAP proteins are absent, like in the presence of IAP antagonists, RIP1 cannot be ubiquitinated, which precludes the binding of either TAB2/3-TAK1 or NEMO. This process can be also achieved in the presence of c-IAP1 when RIP1 gets deubiquitinated by the deubiquitinases CYLD (cylindromatosis) or A20. Nonubiquitinated RIP1 can dissociate from the TNFR1 complex and bind FADD and caspase-8 resulting in the initiation of apoptosis.

Apart from apoptosis, RIP1 can also trigger another type of cell death, necroptosis. Necroptosis is a programmed form of necrosis that relies on RIP1 and RIP3, and occurs when apoptosis pathways are blocked [95]. Necroptosis drives cell death in a caspase independent manner. The initial steps of necroptosis are similar to apoptosis, where TNFα binds to TNFR1 and the absence of c-IAPs prevents ubiquitination of RIP1. But during necroptosis RIP1 does not enter into a complex with FADD and caspase-8. In the absence of FADD or if caspase activity is inhibited, RIP1 will bind the kinase RIP3 leading to a series of auto and cross phosphorylation events culminating in the formation of the necrosome and activation of necrosis. RIP1 binds RIP3 through their RIP homotypic interaction motif (RHIM) [94].

The identity of molecules that mediate the necroptosis pathway downstream of RIP3 has remained an unsolved mystery for some time. However, a recent discovery has revealed mixed lineage kinase domain-like (MLKL) as the critical RIP3 target important for necroptosis [96, 97]. The Wang group proposed that phosphorylated RIP3 binds and phosphorylates MLKL [97]. In support of its crucial role in necroptosis, knock down of MLKL fully rescues cells from necrotic cell death [96]. Another RIP3-binding protein that has been described is PGAM5. PGAM5 is a phosphatase that provides a mitochondrial link to the RIP1–RIP3–MLKL complex and ensures efficient activation of necrosis [98].

Finally, some viruses, like the murine cytomegalovirus, have found a way to block cell necrosis. Cytomegaloviruses encode a RHIM protein domain that allows binding to RIP3, thereby blocking its interaction with RIP1 and activation of necroptosis [99].

CANCER

Cancer cells have acquired survival capabilities that allow them to grow in suboptimal conditions and to escape from cell-death signals [100]. Mutations in the apoptotic pathways can cause tumor initiation, progression or metastasis, since resistance to cell death gives cancer cells a survival advantage and leads to resistances to anti-tumor treatments [101].

IAP proteins are often overexpressed in cancers, which makes them attractive as therapeutic targets [102]. In a study of the expression levels of different

IAPs across cancer cell lines, XIAP and c-IAP1 were expressed in almost all and c-IAP2 in more than half of the examined samples [102]. Interestingly, the mRNA levels of XIAP, c-IAP1 and c-IAP2 did not correlate with protein levels in the tumor lines, suggesting a post-transcriptional regulation [102].

The higher expression of IAP proteins might contribute to colon cancer and poor prognosis of colorectal cancer patients [103]. XIAP has been reported to be overexpressed in breast cancers, melanomas and clear-cell renal carcinoma [104, 105]. In contrast, in non-small cell lung cancer patients, XIAP potentially correlated with a good prognosis [106], and high levels of XIAP were observed to correlate with high sensitivity to the chemotherapeutic cytarabine as well as other nucleoside analogs [102]. c-IAP proteins have been implicated in resistance to treatment of cervical tumors and other types of cancer [102, 107]. In multiple myeloma patients that undergo drug resistance, the overexpression of survivin, c-IAP1, c-IAP2, and XIAP was associated with a poor prognosis [108]. In mucosa-associated lymphoid tissue (MALT) lymphoma, the most common subtype of lymphomas, a gene translocation gives rise to a fusion protein between c-IAP2 and the paracaspase MALT1 [109]. The fusion takes place between the BIR domains of c-IAP2 and various C-terminal portions of MALT1 [109]. This fusion protein has pro-oncogenic properties as it can constitutively activate the NF-κB pathway independently of the adaptors (TRAFs) and cleave its substrates in the absence of extracellular stimuli [39, 110].

ML-IAP, as its name indicates, is highly overexpressed in melanomas, but very rarely in other tissues [111]. In melanomas and in non-small cell lung cancer, ML-IAP overexpression has been observed to confer resistance to apoptosis [112]. ML-IAP overexpression in tumors has been related to poor prognosis [113]. In melanomas, ML-IAP expression has been linked to the pro-survival oncogene Microphthalmia-Associated Transcription Factor (MITF) [114]. Survivin is also almost exclusively expressed in tumor cells at higher levels [115]. In colorectal cancer, overexpression of survivin in the cytoplasm was associated with poor patient prognosis [116].

IAPs are involved in cancer due to their ability to inhibit apoptosis but also because they mediate pro-survival signals due to the activation of NF-κB and MAPK signaling pathways, contributing to tumor cell proliferation. Together with strong expression in cancer tissue, these properties suggest that IAP proteins are attractive targets for anti-tumor therapy.

TARGETING IAP PROTEINS

The two main strategies for targeting IAP proteins involve Smac-derived peptides and small-molecule antagonists, and antisense oligonucleotides [117]. The small-molecule IAP antagonists can be further divided into monovalent and bivalent IAP antagonists [118]. The monovalent antagonists emulate one Smac AVPI motif, while the bivalent antagonists comprise two AVPI motif mimetics connected by a chemical linker (Fig. 4) [119, 120]. The bivalent antagonists have the ability to bind simultaneously to XIAP BIR2 and BIR3 domains, leading to better activation of caspases [47, 121].

Mechanistic aspects of IAP antagonism

Work from several groups established that single-agent pro-apoptotic activity of Smac-mimicking IAP antagonists results from c-IAP1/2 antagonism and TNFα-dependent cell death [47, 119, 122–124]. Treatment with IAP antagonists causes c-IAP1 and c-IAP2 degradation within minutes. For a long time this rapid loss of c-IAP proteins was not well understood. However, recent biochemical and structural studies have solved this mystery and provided a molecular explanation. IAP antagonist binding to c-IAP1 cause a conformational change that opens the c-IAP1 structure and allows c-IAP RING domain dimerization (Fig. 5) [125]. RING mediated dimerization activates c-IAP1/2 E3 ligase activity, leading to autoubiquitination and subsequent proteasomal degradation [125, 126].

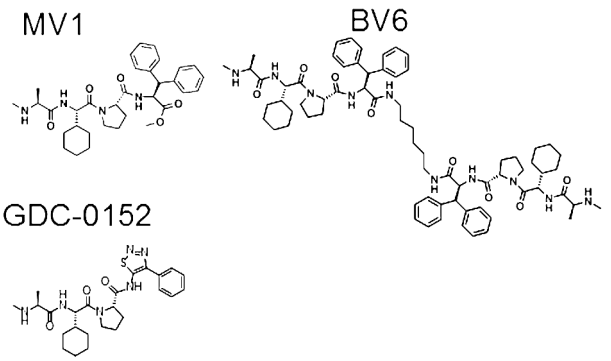

Fig. 4. Examples of monovalent (MV1 and GDC-0152) and bivalent IAP antagonists (BV6)

A concomitant consequence of heightened ubiquitin ligase activity of the c-IAP proteins is ubiquitination of RIP1, leading to canonical NF-κB activation. Proteasomal degradation of c-IAPs allows for stabilization of NIK and stimulation of the noncanonical NF-κB pathway [47, 120]. The activation of NF-κB as well as MAPK pathways induces the transcription and synthesis of TNFα, which, in an autocrine or paracrine fashion, can subsequently activate TNFR1 signaling [47]. However, in the absence of c-IAP1 and c-IAP2 the canonical NF-κB pathway cannot be activated and RIP1 cannot be ubiquitinated. Instead, RIP1 will form the apoptotic complex with FADD-caspase-8 to provoke cell death [47]. By now, it has been shown in numerous studies that single-agent IAP antagonist stimulated cell death is absolutely TNFα-dependent and most of the cell lines that are sensitive to this treatment constitutively secrete elevated levels of TNFα [33, 47, 122].

IAP antagonists predominantly induce apoptotic cell death, as is the case of Jurkat cells when treated with IAP antagonists in combination with TNFα. However, in the absence of FADD or caspase-8, the treatment with TNFα and IAP antagonists leads to activation of necrosis in Jurkat cells [127]. Similarly, treatment of HT29 cells with IAP antagonists and TNFα in the presence of caspase inhibitors strongly stimulates a necrotic response

[128]. Therefore, IAP antagonists can cause cell death even if the apoptotic pathways are blocked. Although many aspects of necroptosis are still poorly defined, it seems that the ability of IAP antagonists to stimulate necrotic cell death is limited to cells expressing RIP3, a critical mediator of this death pathway.

As stated earlier, cells that already secrete TNFα or can be induced to produce TNFα upon IAP antagonist treatment, can be efficiently killed by IAP antagonism [33, 47, 122]. Nevertheless, some resistant cell lines can be pushed over the death threshold by the addition of TNFα [33, 47, 122, 129]. These resistant cell lines might lack the capacity to produce TNFα. However, there is another group of resistant cell lines that cannot be killed by IAP antagonists even in the presence of exogenously added TNFα. The reason for this resistance is not clear but it might include increased levels of c-IAP2 and XIAP or low levels of critical mediators of TNF signaling.

In a few instances it has been reported that IAP antagonist treatment will boost c-IAP2 levels in resistant cancer cell lines [130]. c-IAP2 can functionally substitute for the absence of degraded c-IAP1 and block apoptosis induction by maintaining ubiquitination of RIP1 within the TNFR1 complex [130]. Upregulation of c-IAP2 can be a consequence of the activation of the NF-κB pathways by IAP antagonists, since c-IAP2 is a NF-κB inducible gene, or of c-IAP1 loss, given that c-IAP1 can promote ubiquitination of c-IAP2 [130]. It was suggested that c-IAP2 up regulation in some resistant cell lines could be due to modifications in other pathways that regulate c-IAP2 expression such as the phosphoinositide-3 kinase (PI3K) pathway [130].

In general, induction or presence of TNFα is not always sufficient for effective induction of cell death after IAP antagonist treatment. It has been speculated that in those cases antagonism of XIAP is incomplete leading to a lack of pro-apoptotic activity of IAP antagonists [121, 129, 131]. In agreement with those reports, a c-IAP-selective antagonist (CS3) can produce the same levels of NF-κB signaling pathway activation and cause c-IAP1 and c-IAP2 degradation as a pan-IAP antagonist (PS1) [132]. However, CS3 does not induce cell death nearly as efficiently as PS1 [132]. These results reinforce the idea that XIAP needs to be effectively antagonized to achieve significant apoptosis.

Anti-tumor activity and clinical applications of IAP antagonists

IAP antagonists such as BV6, GDC-0152 and SM-164 have demonstrated tumor-inhibiting activity in several *in vivo* xenograft models [33, 118, 129]. Importantly, these compounds did not show any significant toxicity or weight loss in mice and could produce lasting effects on tumor growth inhibition [33, 133]. In addition, IAP antagonists have not shown a marked sensitization to apoptosis in normal primary cells to date, nor have they affected highly proliferative tissues in mice [33, 133].

The IAP antagonist LBW242 was tested in human and murine neuroblastoma cells and it sensitized cells to apoptosis when it was given in combination with vincristine or doxorubicin [134]. It was reported that caspase-8 activation might be achieved in a TNFα independent manner through the formation of the ripoptosome, a complex specific to some cancer cell lines [135, 136]. A number of other chemotherapeutic drugs seem to have a synergistic effect when given in combination with Smac mimetics including gemcitabine, etoposide, cisplatin, 5-fluorouracil, vinorelbine, irinotecan and cytarabine [33]. IAP antagonists have also been reported to enhance not only chemotherapy but also radiotherapy effects in pancreatic cancer, prostate cancer and glioblastoma [33]. In gliobastoma, it was shown that NF-κB activation was crucial for sensitization of IAP antagonists to radiation [137]. IAP antagonists have also been successfully tested in combination with the death receptor ligand TRAIL/Apo2L and death receptor agonistic antibodies in numerous studies. In most instances, the treatment with IAP antagonists was able to render cells sensitive to TRAIL-induced cell death [138], and in IAP antagonist resistant cells this combination did not rely on TNF signaling, but rather on the antagonism of the caspase inhibitor XIAP [121].

Based on the positive results from pre-clinical studies, several IAP antagonists have entered phase I clinical trials [33, 118]. Bivalent IAP antagonists are more efficacious in tumor growth inhibition compared to monovalent antagonists, probably because of their ability to more effectively block XIAP-mediated inhibition of caspases [33, 121]. The first IAP antagonist to enter human clinical trials was compound GDC-0152, a potent inhibitor of c-IAP1/2, XIAP and ML-IAP [133]. Administration of GDC-0152 in mice that had been implanted with a xenograft of a sensitive breast cancer cell line showed a significant reduction of the tumor growth [133]. GDC-0152 showed linear pharmacokinetics over a wide range of doses in humans without any signs of significant toxicity [133]. Similarly, clinical trials with other IAP antagonists, LCL161, HGS1029 and TL32711, reported no dose-limiting

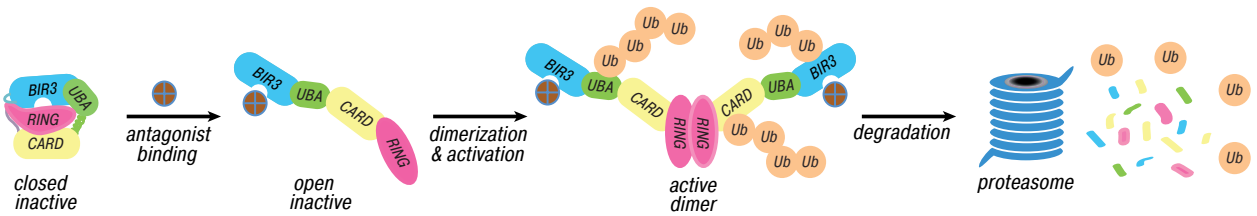

Fig. 5. IAP antagonists trigger a conformational change in c-IAP1 prompting RING dimerization and activation of E3 ligase activity

toxicity, target antagonism and dose proportional pharmacokinetics [33]. These and other ongoing and future clinical trials will examine the safety and the efficacy of IAP antagonists for the treatment of human malignancies in hopes of bringing new anti-tumor agents to cancer patients.

ACKNOWLEDGMENTS

The authors thank Wayne Fairbrother and Eugene Varfolomeev for critical reading of the manuscript. Both authors are employees of Genentech, Inc.

REFERENCES

1. **Hanahan D, Weinberg RA.** Hallmarks of cancer: the next generation. Cell 2011; **144**: 646–74.

2. **Galluzzi L, Vitale I, Abrams JM, *et al.*** Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 2012; **19**: 107–20.

3. **Steller H.** Mechanisms and genes of cellular suicide. Science 1995; **267**: 1445–9.

4. **Lopez J, Meier P.** To fight or die — inhibitor of apoptosis proteins at the crossroad of innate immunity and death. Curr Opin Cell Biol 2010; **22**: 872–81.

5. **Miller LK.** An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol 1999; **9**: 323–8.

6. **Crook NE, Clem RJ, Miller LK.** An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol 1993; **67**: 2168–74.

7. **Birnbaum MJ, Clem RJ, Miller LK.** An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J Virol 1994; **68**: 2521–8.

8. **Liston P, Roy N, Tamai K, *et al.*** Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996; **379**: 349–53.

9. **Rothe M, Pan MG, Henzel WJ, *et al.*** The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell 1995; **83**: 1243–52.

10. **Vaux DL, Silke J.** Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun 2003; **304**: 499–504.

11. **Thornberry NA, Lazebnik Y.** Caspases: enemies within. Science 1998; **281**: 1312–6.

12. **Chang HY, Yang X.** Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol Biol Rev 2000; **64**: 821–46.

13. **Ashkenazi A, Dixit VM.** Death receptors: signaling and modulation. Science 1998; **281**: 1305–8.

14. **Bodmer JL, Schneider P, Tschopp J.** The molecular architecture of the TNF superfamily. Trends Biochem Sci 2002; **27**: 19–26.

15. **Ashkenazi A.** Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev 2008; **19**: 325–31.

16. **Park YC, Ye H, Hsia C, *et al.*** A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. Cell 2000; **101**: 777–87.

17. **Stutz F, Neville M, Rosbash M.** Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. Cell 1995; **82**: 495–506.

18. **Wong WW, Gentle IE, Nachbur U, *et al.*** RIPK1 is not essential for TNFR1-induced activation of NF-kappaB. Cell Death Differ 2010; **17**: 482–7.

19. **Vucic D, Dixit VM, Wertz IE.** Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol 2011; **12**: 439–52.

20. **Bertrand MJ, Milutinovic S, Dickson KM, *et al.*** cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell 2008; **30**: 689–700.

21. **Varfolomeev E, Goncharov T, Fedorova AV, *et al.*** c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor alpha (TNFα)-induced NF-kB Activation. J Biol Chem 2008; **283**: 24295–9.

22. **Micheau O, Tschopp J.** Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 2003; **114**: 181–90.

23. **Wilson NS, Dixit V, Ashkenazi A.** Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009; **10**: 348–55.

24. **Fulda S, Debatin KM.** Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006; **25**: 4798–811.

25. **Hunter AM, LaCasse EC, Korneluk RG.** The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 2007; **12**: 1543–68.

26. **Liston P, Fong WG, Kelly NL, *et al.*** Identification of XAF1 as an antagonist of XIAP anti-caspase activity. Nat Cell Biol 2001; **3**: 128–33.

27. **Arora V, Cheung HH, Plenchette S, *et al.*** Degradation of survivin by the X-linked inhibitor of apoptosis (XIAP)-XAF1 complex. J Biol Chem 2007; **282**: 26202–9.

28. **Eckelman BP, Salvesen GS.** The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. J Biol Chem 2006; **281**: 3254–60.

29. **Bertrand MJ, Doiron K, Labbe K, *et al.*** Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. Immunity 2009; **30**: 789–801.

30. **Silke J, Brink R.** Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. Cell Death Differ 2010; **17**: 35–45.

31. **Varfolomeev E, Vucic D.** (Un)expected roles of c-IAPs in apoptotic and NF-kB signaling pathways. Cell Cycle 2008; **7**: 1511–21.

32. **Deveraux QL, Takahashi R, Salvesen GS, Reed JC.** X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997; **388**: 300–4.

33. **Fulda S, Vucic D.** Targeting IAP proteins for therapeutic intervention in cancer. Nat Rev Drug Discov 2012; **11**: 109–24.

34. **Deveraux QL, Leo E, Stennicke HR, *et al.*** Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J 1999; **18**: 5242–51.

35. **Yang YL, Li XM.** The IAP family: endogenous caspase inhibitors with multiple biological activities. Cell Res 2000; **10**: 169–77.

36. **Hinds MG, Norton RS, Vaux DL, Day CL.** Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nat Struct Biol 1999; **6**: 648–51.

37. **Lu M, Lin SC, Huang Y, *et al.*** XIAP induces NF-kappaB activation via the BIR1/TAB1 interaction and BIR1 dimerization. Mol Cell 2007; **26**: 689–702.

38. **Samuel T, Welsh K, Lober T, *et al.*** Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem 2006; **281**: 1080–90.

39. **Varfolomeev E, Wayson SM, Dixit VM, *et al.*** The inhibitor of apoptosis protein fusion c-IAP2.MALT1 stimulates

NF-kB activation independently of TRAF1 AND TRAF2. J Biol Chem 2006; **281**: 29022–9.

40. **Zheng C, Kabaleeswaran V, Wang Y, *et al.*** Crystal structures of the TRAF2: cIAP2 and the TRAF1: TRAF2: cIAP2 complexes: affinity, specificity, and regulation. Mol Cell 2010; **38**: 101–13.

41. **Riedl SJ, Renatus M, Schwarzenbacher R, *et al.*** Structural basis for the inhibition of caspase-3 by XIAP. Cell 2001; **104**: 791–800.

42. **Suzuki Y, Nakabayashi Y, Nakata K, *et al.*** X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and -7 in distinct modes. J Biol Chem 2001; **276**: 27058–63.

43. **Huang Y, Park YC, Rich RL, *et al.*** Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell 2001; **104**: 781–90.

44. **Scott FL, Denault JB, Riedl SJ, *et al.*** XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. EMBO J 2005; **24**: 645–55.

45. **Shiozaki EN, Chai J, Rigotti DJ, *et al.*** Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell 2003; **11**: 519–27.

46. **Srinivasula SM, Hegde R, Saleh A, *et al.*** A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature 2001; **410**: 112–6.

47. **Varfolomeev E, Blankenship JW, Wayson SM, *et al.*** IAP antagonists induce autoubiquitination of c-IAPs, NF-kB activation, and TNFα-dependent apoptosis. Cell 2007; **131**: 669–81.

48. **Du C, Fang M, Li Y, *et al.*** Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell 2000; **102**: 33–42.

49. **Huang Y, Rich RL, Myszka DG, Wu H.** Requirement of both the second and third BIR domains for the relief of X-linked inhibitor of apoptosis protein (XIAP)-mediated caspase inhibition by Smac. J Biol Chem 2003; **278**: 49517–22.

50. **Vaux DL, Silke J.** IAPs, RINGs and ubiquitylation. Nat Rev Mol Cell Biol 2005; **6**: 287–97.

51. **Silke J, Kratina T, Chu D, *et al.*** Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance. Proc Natl Acad Sci USA 2005; **102**: 16182–7.

52. **Mace PD, Shirley S, Day CL.** Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. Cell Death Differ 2010; **17**: 46–53.

53. **Dynek JN, Goncharov T, Dueber EC, *et al.*** c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J 2010; **29**: 4198–209.

54. **Schile AJ, Garcia-Fernandez M, Steller H.** Regulation of apoptosis by XIAP ubiquitin-ligase activity. Genes Dev 2008; **22**: 2256–66.

55. **Wilson R, Goyal L, Ditzel M, *et al.*** The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol 2002; **4**: 445–50.

56. **Blankenship JW, Varfolomeev E, Goncharov T, *et al.*** Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2(1). Biochem J 2009; **417**: 149–60.

57. **Gyrd-Hansen M, Darding M, Miasari M, *et al.*** IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. Nat Cell Biol 2008; **10**: 1309–17.

58. **Hershko A, Ciechanover A.** The ubiquitin system. Annu Rev Biochem 1998; **67**: 425–79.

59. **Malynn BA, Ma A.** Ubiquitin makes its mark on immune regulation. Immunity 2010; **33**: 843–52.

60. **Deshaies RJ, Joazeiro CA.** RING domain E3 ubiquitin ligases. Annu Rev Biochem 2009; **78**: 399–434.

61. **Rothwarf DM, Karin M.** The NF-kappaB activation pathway: a paradigm in information transfer from membrane to nucleus. Sci STKE 1999; **1999**: RE1.

62. **Scheidereit C.** IκB kinase complexes: gateways to NF-κB activation and transcription. Oncogene 2006; **25**: 6685–705.

63. **Varfolomeev E, Goncharov T, Maecker H, *et al.*** Cellular Inhibitors of apoptosis are global regulators of NF-kappaB and MAPK activation by members of the TNF family of receptors. Sci Signal 2012; **5**: ra22.

64. **Ea CK, Deng L, Xia ZP, *et al.*** Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 2006; **22**: 245–57.

65. **Tokunaga F, Nakagawa T, Nakahara M, *et al.*** SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex. Nature 2011; **471**: 633–6.

66. **Vanlangenakker N, Vanden Berghe T, Bogaert P, *et al.*** cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 2011; **18**: 656–65.

67. **Gerlach B, Cordier SM, Schmukle AC, *et al.*** Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 2011; **471**: 591–6.

68. **Ikeda F, Deribe YL, Skanland SS, *et al.*** SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. Nature 2011; **471**: 637–41.

69. **Ting AT, Pimentel-Muinos FX, Seed B.** RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J 1996; **15**: 6189–96.

70. **Devin A, Lin Y, Yamaoka S, *et al.*** The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol Cell Biol 2001; **21**: 3986–94.

71. **Wang C, Deng L, Hong M, *et al.*** TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 2001; **412**: 346–51.

72. **DiDonato J, Mercurio F, Rosette C, *et al.*** Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol 1996; **16**: 1295–304.

73. **Yaron A, Hatzubai A, Davis M, *et al.*** Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. Nature 1998; **396**: 590–4.

74. **Basak S, Hoffmann A.** Crosstalk via the NF-kappaB signaling system. Cytokine Growth Factor Rev 2008; **19**: 187–97.

75. **He JQ, Saha SK, Kang JR, *et al.*** Specificity of TRAF3 in its negative regulation of the noncanonical NF-kappa B pathway. J Biol Chem 2007; **282**: 3688–94.

76. **Malinin NL, Boldin MP, Kovalenko AV, Wallach D.** MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 1997; **385**: 540–4.

77. **Vallabhapurapu S, Matsuzawa A, Zhang W, *et al.*** Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat Immunol 2008; **9**: 1364–70.

78. **Xiao G, Harhaj EW, Sun SC.** NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol Cell 2001; **7**: 401–9.

79. **Asselin E, Mills GB, Tsang BK.** XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 2001; **61**: 1862–8.

80. **Birkey Reffey S, Wurthner JU, Parks WT, *et al.*** X-linked inhibitor of apoptosis protein functions as a cofactor in transforming growth factor-beta signaling. J Biol Chem 2001; **276**: 26542–9.

81. **Sanna MG, da Silva Correia J, Ducrey O, *et al.*** IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. Mol Cell Biol 2002; **22**: 1754–66.

82. **Zhao Y, Conze DB, Hanover JA, Ashwell JD.** Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem 2007; **282**: 7777–82.

83. **Xu L, Zhu J, Hu X, *et al.*** c-IAP1 cooperates with Myc by acting as a ubiquitin ligase for Mad1. Mol Cell 2007; **28**: 914–22.

84. **Karin M, Gallagher E.** TNFR signaling: ubiquitin-conjugated TRAFfic signals control stop-and-go for MAPK signaling complexes. Immunol Rev 2009; **228**: 225–40.

85. **Gardam S, Turner VM, Anderton H, *et al.*** Deletion of cIAP1 and cIAP2 in murine B lymphocytes constitutively activates cell survival pathways and inactivates the germinal center response. Blood 2011; **117**: 4041–51.

86. **Park JH, Kim YG, Shaw M, *et al.*** Nod1/RICK and TLR signaling regulate chemokine and antimicrobial innate immune responses in mesothelial cells. J Immunol 2007; **179**: 514–21.

87. **Hasegawa M, Fujimoto Y, Lucas PC, *et al.*** A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J 2008; **27**: 373–83.

88. **Hofer-Warbinek R, Schmid JA, Stehlik C, *et al.*** Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. J Biol Chem 2000; **275**: 22064–8.

89. **Krieg A, Correa RG, Garrison JB, *et al.*** XIAP mediates NOD signaling via interaction with RIP2. Proc Natl Acad Sci USA 2009; **106**: 14524–9.

90. **Damgaard RB, Nachbur U, Yabal M, *et al.*** The Ubiquitin Ligase XIAP Recruits LUBAC for NOD2 Signaling in Inflammation and Innate Immunity. Mol Cell 2012; **46**: 746-58.

91. **Mao AP, Li S, Zhong B, *et al.*** Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of interferon-beta (IFN-beta) and cellular antiviral response. J Biol Chem 2010; **285**: 9470–6.

92. **Vince JE, Wong WW, Gentle I, *et al.*** Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. Immunity 2012; **36**: 215–27.

93. **Nakhaei P, Sun Q, Solis M, *et al.*** IkappaB kinase epsilon-dependent phosphorylation and degradation of X-linked inhibitor of apoptosis sensitizes cells to virus-induced apoptosis. J Virol 2012; **86**: 726–37.

94. **Meylan E, Tschopp J.** The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 2005; **30**: 151–9.

95. **Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G.** Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010; **11**: 700–14.

96. **Zhao J, Jitkaew S, Cai Z, *et al.*** Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci USA 2012; **109**: 5322–7.

97. **Sun L, Wang H, Wang Z, *et al.*** Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 2012; **148**: 213–27.

98. **Wang Z, Jiang H, Chen S, *et al.*** The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 2012; **148**: 228–43.

99. **Upton JW, Kaiser WJ, Mocarski ES.** Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 2010; **7**: 302–13.

100. **Naniche N, Sau D, Pasinelli P.** *In vivo* and *in vitro* determination of cell death markers in neurons. Methods Mol Biol 2011; **793**: 9–21.

101. **Lowe SW, Lin AW.** Apoptosis in cancer. Carcinogenesis 2000; **21**: 485–95.

102. **Tamm I, Kornblau SM, Segall H, *et al.*** Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res 2000; **6**: 1796–803.

103. **Miura K, Fujibuchi W, Ishida K, *et al.*** Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today 2011; **41**: 175–82.

104. **Jaffer S, Orta L, Sunkara S, *et al.*** Immunohistochemical detection of antiapoptotic protein X-linked inhibitor of apoptosis in mammary carcinoma. Hum Pathol 2007; **38**: 864–70.

105. **Kluger HM, McCarthy MM, Alvero AB, *et al.*** The X-linked inhibitor of apoptosis protein (XIAP) is up-regulated in metastatic melanoma, and XIAP cleavage by Phenoxodiol is associated with Carboplatin sensitization. J Transl Med 2007; **5**: 6.

106. **Ferreira CG, van der Valk P, Span SW, *et al.*** Expression of X-linked inhibitor of apoptosis as a novel prognostic marker in radically resected non-small cell lung cancer patients. Clin Cancer Res 2001; **7**: 2468–74.

107. **Imoto I, Tsuda H, Hirasawa A, *et al.*** Expression of cIAP1, a target for 11q22 amplification, correlates with resistance of cervical cancers to radiotherapy. Cancer Res 2002; **62**: 4860–6.

108. **Nakagawa Y, Abe S, Kurata M, *et al.*** IAP family protein expression correlates with poor outcome of multiple myeloma patients in association with chemotherapy-induced overexpression of multidrug resistance genes. Am J Hematol 2006; **81**: 824–31.

109. **Dierlamm J, Baens M, Wlodarska I, *et al.*** The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood 1999; **93**: 3601–9.

110. **Vucic D, Dixit VM.** Masking MALT1: the paracaspase's potential for cancer therapy. J Exp Med 2009; **206**: 2309–12.

111. **Gong J, Chen N, Zhou Q, *et al.*** Melanoma inhibitor of apoptosis protein is expressed differentially in melanoma and melanocytic naevus, but similarly in primary and metastatic melanomas. J Clin Pathol 2005; **58**: 1081–5.

112. **Crnkovic-Mertens I, Muley T, Meister M, *et al.*** The anti-apoptotic livin gene is an important determinant for the apoptotic resistance of non-small cell lung cancer cells. Lung Cancer 2006; **54**: 135–42.

113. **Zhou J, Yuen NK, Zhan Q, *et al.*** Immunity to the melanoma inhibitor of apoptosis protein (ML-IAP; livin) in patients with malignant melanoma. Cancer Immunol Immunother 2012; **61**: 655–65.

114. **Dynek JN, Chan SM, Liu J, *et al.*** Microphthalmia-associated transcription factor is a critical transcriptional regulator of melanoma inhibitor of apoptosis in melanomas. Cancer Res 2008; **68**: 3124–32.

115. **Velculescu VE, Madden SL, Zhang L, *et al.*** Analysis of human transcriptomes. Nat Genet 1999; **23**: 387–8.

116. **Qi G, Tuncel H, Aoki E, *et al.*** Intracellular localization of survivin determines biological behavior in colorectal cancer. Oncol Rep 2009; **22**: 557–62.

117. **Chen DJ, Huerta S.** Smac mimetics as new cancer therapeutics. Anticancer Drugs 2009; **20**: 646–58.

118. **Flygare JA, Fairbrother WJ.** Small-molecule pan-IAP antagonists: a patent review. Expert Opin Ther Pat 2010; **20**: 251–67.

119. **Zobel K, Wang L, Varfolomeev E, *et al.*** Design, synthesis, and biological activity of a potent Smac mimetic that sensitizes cancer cells to apoptosis by antagonizing IAPs. ACS Chem Biol 2006; **1**: 525–33.

120. **Sun H, Nikolovska-Coleska Z, Lu J, *et al.*** Design, synthesis, and characterization of a potent, nonpeptide, cell-permeable, bivalent Smac mimetic that concurrently targets both the BIR2 and BIR3 domains in XIAP. J Am Chem Soc 2007; **129**: 15279–94.

121. **Varfolomeev E, Alicke B, Elliott JM, *et al.*** X Chromosome-linked inhibitor of apoptosis regulates cell death induction by proapoptotic receptor agonists. J Biol Chem 2009; **284**: 34553–60.

122. **Vince JE, Wong WW, Khan N, *et al.*** IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007; **131**: 682–93.

123. **Petersen SL, Wang L, Yalcin-Chin A, *et al.*** Autocrine TNFalpha signaling renders human cancer cells susceptible to smac-mimetic-induced apoptosis. Cancer Cell 2007; **12**: 445–56.

124. **Gaither A, Porter D, Yao Y, *et al.*** A Smac mimetic rescue screen reveals roles for inhibitor of apoptosis proteins in tumor necrosis factor-alpha signaling. Cancer Res 2007; **67**: 11493–8.

125. **Dueber EC, Schoeffler AJ, Lingel A, *et al.*** Antagonists induce a conformational change in cIAP1 that promotes autoubiquitination. Science 2011; **334**: 376–80.

126. **Feltham R, Bettjeman B, Budhidarmo R, *et al.*** Smac mimetics activate the E3 ligase activity of cIAP1 protein by promoting RING domain dimerization. J Biol Chem 2011; **286**: 17015–28.

127. **Laukens B, Jennewein C, Schenk B, *et al.*** Smac mimetic bypasses apoptosis resistance in FADD- or caspase-8-deficient cells by priming for tumor necrosis factor alpha-induced necroptosis. Neoplasia 2011; **13**: 971–9.

128. **He S, Wang L, Miao L, *et al.*** Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009; **137**: 1100–11.

129. **Lu J, Bai L, Sun H, Nikolovska-Coleska Z, *et al.*** SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. Cancer Res 2008; **68**: 9384–93.

130. **Petersen SL, Peyton M, Minna JD, Wang X.** Overcoming cancer cell resistance to Smac mimetic induced apoptosis by modulating cIAP-2 expression. Proc Natl Acad Sci USA 2010; **107**: 11936–41.

131. **Jost PJ, Grabow S, Gray D, *et al.*** XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 2009; **460**: 1035–9.

132. **Ndubaku C, Varfolomeev E, Wang L, *et al.*** Antagonism of c-IAP and XIAP proteins is required for efficient induction of cell death by small-molecule IAP antagonists. ACS Chem Biol 2009; **4**: 557–66.

133. **Flygare JA, Beresini M, Budha N, *et al.*** Discovery of a potent small-molecule antagonist of inhibitor of apoptosis (IAP) proteins and clinical candidate for the treatment of cancer (GDC-0152). J Med Chem 2012; **55**: 4101–13.

134. **Eschenburg G, Eggert A, Schramm A, *et al.*** Smac mimetic LBW242 sensitizes XIAP-overexpressing neuroblastoma cells for TNF-alpha-independent apoptosis. Cancer Res 2012; **72**: 2645–56.

135. **Feoktistova M, Geserick P, Kellert B, *et al.*** cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 2011; **43**: 449–63.

136. **Tenev T, Bianchi K, Darding M, *et al.*** The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 2011; **43**: 432–48.

137. **Berger R, Jennewein C, Marschall V, *et al.*** NF-kappaB is required for Smac mimetic-mediated sensitization of glioblastoma cells for gamma-irradiation-induced apoptosis. Mol Cancer Ther 2011; **10**: 1867–75.

138. **Dai Y, Liu M, Tang W, *et al.*** A Smac-mimetic sensitizes prostate cancer cells to TRAIL-induced apoptosis via modulating both IAPs and NF-kappaB. BMC Cancer 2009; **9**: 392.