

Inhibitor of Apoptosis Proteins in Eukaryotic Evolution and Development: A Model of Thematic Conservation

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The past decade and a half has witnessed the discovery of a large, evolutionarily conserved family of cellular genes bearing homology to the prototype baculovirus *Inhibitor of Apoptosis (IAP)*. The logical decision in the field to also refer to these cellular proteins as IAPs fails to do justice to this versatile group of factors that play a wide range of roles in eukaryotic development and homeostasis which include, but are not limited to, the regulation of programmed cell death. Here we describe the shared functional characteristics of several well-characterized IAPs whose defining motifs place them more in the category of multifunctional modular protein interaction domains.

IAPs: A Versatile, Evolutionarily Conserved Family of Intracellular Proteins

The prototype Inhibitor of Apoptosis (IAP) protein was reported in 1994 through a genetic screen designed to identify cytoprotective proteins encoded in genes from baculovirus, which primarily infect members of the *Lepidoptera* order (Birnbaum et al., 1994). Through an elegant complementation approach, an open reading frame was identified from *Cydia pomonella* granulosis virus, a baculovirus used agriculturally as a commercial pesticide to control fruit tree infestation with the Codling moth, *C. pomonella* and which, when expressed in *trans*, was able to protect insect cells from virus-induced cell death, thus enhancing viral replication and titer (Crook et al., 1993). It is probably fair to state that Lois Miller and colleagues could not have imagined the sweeping implications their original study identifying IAPs would have for a huge scope of disciplines including metazoan development, mitotic regulation, pathogenesis of neoplastic and immunoproliferative diseases, intracellular metal ion trafficking, and receptor-initiated cell signaling. At the same time, despite efforts to rename and reclassify this multifaceted family of factors, the term “IAP” has stuck, a fact that frequently causes confusion to those new to the field, primarily because not all IAPs inhibit apoptosis or cell death.

Two prominent structural features of the baculovirus IAPs were originally described: the baculovirus IAP repeat (BIR) and the RING finger domain. The BIR motif is a domain of approximately 65 residues, characterized by an invariant arrangement of conserved cysteines and histidines that adopt a classical zinc coordination configuration (Hinds et al., 1999; Miller, 1999). The BIR is the defining motif of the IAP family, and IAPs contain one, two, or three BIRs (Figure 1). Early findings suggested that the function of the BIRs was to prevent cell death through the direct binding and inhibition of caspases (Deveraux et al., 1998), the principal effector proteases of the apoptotic program. However, as will be described below, caspase inhibition is

just one property of a small subset of BIR domains contained in only a minority of IAP proteins.

The second structural motif described in the prototype IAPs is the RING finger domain. RINGs are a specialized subset of zinc-finger-like domains, which are found in a variety of proteins in addition to IAPs, including the c-CBL and PML proto-oncoproteins and the RAG-2 protein involved in immunoglobulin gene rearrangement (Joazeiro and Weissman, 2000). Not all IAPs contain RINGs, but in those that do, the RING is characteristically located at the extreme carboxyl terminus of the protein. Many recent studies on RING-containing proteins, including IAPs, have revealed their involvement in ubiquitination of substrate proteins by functioning as E3 ubiquitin ligases. In many situations ubiquitination catalyzes the proteasome-mediated degradation of target proteins.

Reports of the existence of cellular IAP-like proteins appeared in the literature only two or three years after the discovery of the baculovirus IAPs. The first of these was neuronal apoptosis inhibitory protein (NAIP), which was first identified as a candidate gene potentially disrupted in a class of neurodegenerative diseases known as spinal muscular atrophy (Roy et al., 1995). Shortly after the description of NAIP, numerous cellular IAPs were also identified in an evolutionarily diverse range of organisms, discovered in some cases through sequence homology with the baculovirus IAPs, and in others through biochemical and genetic screens (Hay et al., 1995; Duckett et al., 1996; Rothe et al., 1995; Uren et al., 1996; Ambrosini et al., 1997; Liston et al., 1996). While eight distinct human IAPs have been characterized (Figure 1), in this review we will focus primarily on the five most studied: X-linked IAP (XIAP), c-IAP1, c-IAP2, NAIP, and Survivin.

Caspase Inhibitory Properties of the IAPs

The widely expressed mammalian XIAP was identified by virtue of its sequence homology to the prototype baculoviral IAPs (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996).

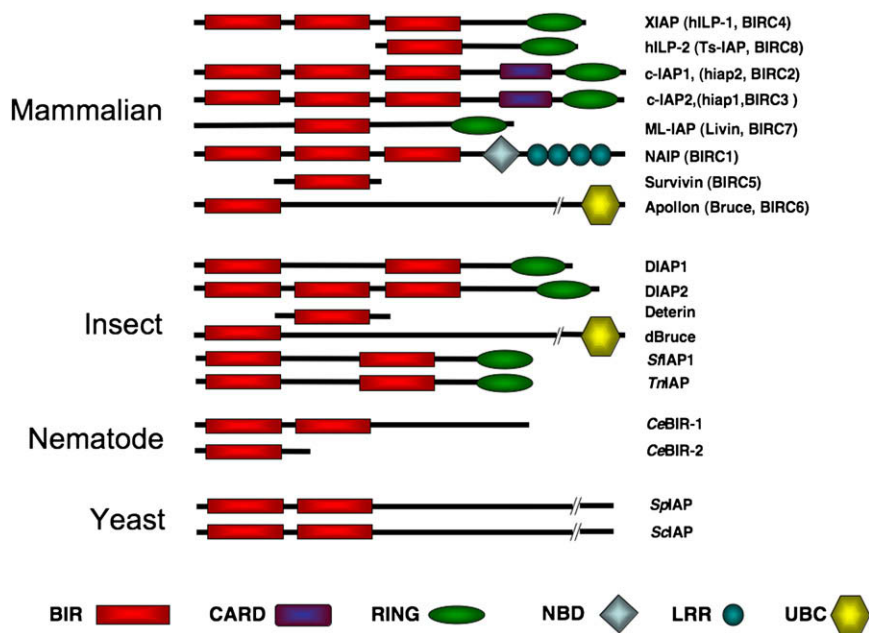


Figure 1. Domain Structure of the IAP Protein Family

The characteristic BIR domains are indicated by red rectangles, CARD domains by purple rectangles, RING domains by green ovals, NBD domains by diamonds, LRR domains by teal circles, and UBC domains (conserved domains found in E2 ubiquitin-conjugating enzymes) by yellow hexagons. Diap1, Diap2, Deterin, and dBruce are *Drosophila* IAPs, while SfIAP1 and TnIAP are lepidopteran IAPs. IAP, inhibitor of apoptosis; XIAP, X-linked IAP; BIRC, baculoviral IAP repeat containing; hILP, human IAP-like protein; Ts-IAP, testis-specific IAP; c-IAP, cellular IAP; ML-IAP, melanoma-IAP; NAIP, neuronal apoptosis inhibitory protein; DIAP, *Drosophila* IAP; SfIAP1, *Spodoptera frugiperda* IAP; TnIAP, *Trichoplusia ni* IAP; CeBIR-1,-2, *Caenorhabditis elegans* BIRC; SplAP, *Schizosaccharomyces pombe* IAP; SciAP, *Saccharomyces cerevisiae* IAP; BIR, baculoviral IAP repeat; CARD, caspase recruitment domain; NBD, nucleotide binding oligomerization domain; LRR, leucine rich repeat.

However, unlike the baculovirus IAPs, which are composed of two BIRs, XIAP is composed of three BIRs and a carboxy-terminal RING (Figure 2). XIAP exhibits antiapoptotic properties, and is the only mammalian IAP that directly inhibits the enzymatic activity of caspase-3, -7 and -9—three proteases that are central to the apoptotic program (Deveraux et al., 1997; Takahashi et al., 1998; Riedl et al., 2001; Shiozaki et al., 2003). A domain composed of the second BIR (BIR2) and a linker region immediately amino-terminal to BIR2 is necessary and sufficient for inhibition of caspase-3 and -7, while the most carboxy-terminal BIR (BIR3) inhibits caspase-9 (Deveraux et al., 1999). Both BIR domains use a two-site binding mechanism for potent caspase inhibition. One of these sites is a conserved surface groove found in most IAP BIR domains. To achieve potent caspase inhibition, the XIAP BIR domain binds and anchors the caspase IAP binding motif (IBM) that is generated following caspase activation (Figure 2). This binding functions as an exosite, thus strengthening inhibitor binding. Although this anchoring interaction is conserved, the mechanism of enzyme inhibition is not. The peptide strand flanking the amino terminus of XIAP BIR2 binds directly to the active site of caspase-3 and -7 (Figure 2, right) (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). Thus, tight inhibition of the executioner caspases requires two surfaces—a surface groove anchoring motif and an active site-directed inhibitory interaction. As opposed to targeting the enzyme active site directly, the functional inhibitory surface of XIAP BIR3 is a helix immediately following the BIR3 domain (Figure 2, left), which packs against the dimer interface of caspase-9, forcing the protease into an inactive conformation (Shiozaki et al., 2003).

Although initial reports suggested other mammalian IAPs could also directly inhibit the proteolytic activity of caspases, this has turned out not to be the case (reviewed in Eckelman et al., 2006). Comparing the primary sequence of other IAP BIR domains to the XIAP BIRs, it is clear why they do not directly inhibit caspase activity (Figure 2, alignments). Although they share

the BIR surface groove that mediates caspase binding, no other IAP (besides the unstable ILP2) contains all the critical residues required for the functional inhibitory interaction. Therefore it is likely that XIAP evolved these specialized caspase-binding flanking regions to specifically inhibit caspase activity, and that the more evolutionarily conserved surface groove distinguishes the BIR domain as a protein-protein binding module. This is analogous to the SH2 and LIM domains of cell-signaling proteins. Indeed, c-IAP1 and c-IAP2 can bind to mammalian caspases, yet the physiological consequence remains unclear (Eckelman and Salvesen, 2006; Tenev et al., 2004). It is possible that c-IAP1 and c-IAP2 may regulate caspases and other proteins by targeting them for ubiquitination, much like the mechanism utilized by Diap1 to regulate *Drosophila* caspases. This redundancy may explain why *Xiap*-deficient mice, while exhibiting differences from their wild-type counterparts, do not display a more catastrophic phenotype even if XIAP is the only IAP that directly inhibits caspases (Harlin et al., 2001). It also suggests that XIAP is likely to have other functions, as discussed below. From an evolutionary perspective, the ability of XIAP to regulate apoptosis by direct caspase inhibition may represent a relatively recent acquisition as the family has diversified structurally and functionally to play a wide variety of physiological roles. For this reason, IAPs are also referred to as BIR-domain-containing proteins (BIRPs), to denote the fact that they can play multiple roles within the cell.

Non-Caspase Inhibitory Mammalian IAPs

Like XIAP, two other mammalian IAP proteins, c-IAP1 and c-IAP2, have three BIR domains and a carboxy-terminal RING (Figure 1). In terms of homology and function, these two proteins are much more similar to each other than they are to XIAP. These cellular proteins were identified through interactions with the type-2 tumor necrosis factor receptor (TNFR2), perhaps foreshadowing a role for the IAP family as signaling modulators (Rothe et al., 1995). c-IAP1 and c-IAP2 were shown to associate with TNFR2 through interactions with the TNF receptor associated

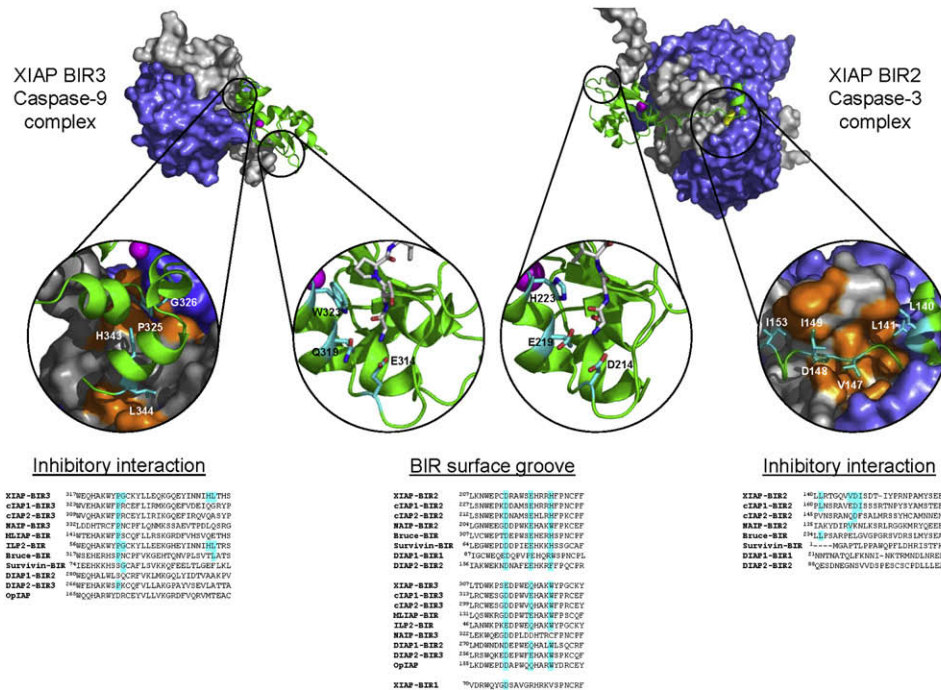


Figure 2. The Caspase-Binding Elements of IAPs

Structure of the complex between XIAP BIR3 and caspase-9 (left, PDB 1N99) and XIAP BIR2 with caspase-3 (right, PDB 1I30). The caspase is in surface representation (large subunit in blue, small subunit in gray). The XIAP BIR domain and flanking region are in green cartoon, with the coordinated zinc in pink. Caspase inhibition is achieved via two binding sites: an anchoring interaction with the BIR surface groove, and an “Inhibitory interaction.” (Insets) Critical XIAP residues that interact with the caspase are in cyan stick representation. Critical caspase residues that interact with XIAP are in orange surface representation. Primary sequence alignment reveals that the BIR surface groove is common to most IAP BIR domains, and there is overlapping binding specificity between IAP BIR domains. The BIR surface groove binds IAP-binding motif (IBM)-containing proteins including caspase-9 and -3 (shown here in inset; N terminus of caspase small subunit is in gray stick), caspase-7, SMAC/DIABLO, HtrA2/Omi, Grim, Rpr, Hid, and others. Alignment of primary sequence across the inhibitory interaction site demonstrates that XIAP is the only IAP that contains all the critical residues to confer direct inhibition of caspase catalytic activity. Although ILP2 also contains all the caspase-9 inhibitory elements, it is an unstable protein whose endogenous expression is yet to be demonstrated.

factors TRAF1 and TRAF2 (Figure 3). Despite their overall structural similarity, c-IAP1 and c-IAP2 exhibit very different functional properties from XIAP; in contrast to XIAP, the c-IAPs do not inhibit caspases (Eckelman and Salvesen, 2006), and conversely, XIAP does not bind to TRAFs (Roy et al., 1997; Duckett et al., 1998). TNF signaling through TRAFs leads to the activation of nuclear factor- κ B (NF- κ B), which then mediates inflammatory responses, cell proliferation, and cell survival by inducing transcription of proinflammatory and prosurvival genes (Pomerantz and Baltimore, 2002). While the role of c-IAP1 and c-IAP2 in TNF-mediated signaling was unclear, recent findings describe critical roles for the c-IAP proteins in TNF-mediated induction of NF- κ B (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Gaither et al., 2007; Wang et al., 2008). c-IAP1 negatively regulates NF- κ B-inducing kinase (NIK), preventing ubiquitination and subsequent processing of the p100 precursor form of NF- κ B to the active p52 form (Varfolomeev et al., 2007; Vince et al., 2007). Thus, the physiological role of c-IAP proteins appears to involve inhibition of the noncanonical NF- κ B pathway by TNF receptors.

Survivin is the smallest mammalian IAP, composed of a single BIR (Ambrosini et al., 1997). While its role in modulating caspase activity is complex and likely indirect, it plays a crucial role in embryonic development and in mitotic spindle formation, as discussed in more detail below (Li et al., 1998).

Clues from Invertebrate IAPs: A Division of Labor

IAPs were first identified in baculoviruses, and subsequent phylogenetic studies suggested that the baculoviral IAP genes arose by capture of a host gene early in the evolution of *Lepidoptera* (Hughes, 2002). Given the vast phylogenetic diversity of the *Lepidoptera* order, which includes moths, and its dissimilarity to the *Dipteran* order to which the *Drosophila* genus belongs, it was not at all obvious that orthologs of the baculovirus IAPs would be found in the fruit fly. Remarkably, however, the *Drosophila melanogaster* genome was found to encode four IAPs with diverse functions, although mechanisms of cell death regulation have been the most extensively studied (Figure 1). In this section, we discuss the insights gleaned into the IAPs from the well-characterized *Drosophila* model, and will subsequently expand these observations into a broader discussion of IAP function in other organisms.

Diap1 was originally identified as an allele of *thread* through a genetic screen designed to identify modulators of cell death (Hay et al., 1995). Diap1 contains two BIRs repeated in tandem and a RING domain at the extreme carboxyl terminus. The use of genetic and biochemical approaches to dissect Diap1 function has generated strong evidence for this IAP in regulating cell death. The RING domain of Diap1 promotes ubiquitylation of Diap1 itself and Dronc, the ortholog of mammalian caspase-9, resulting in inactivation, although whether caspase

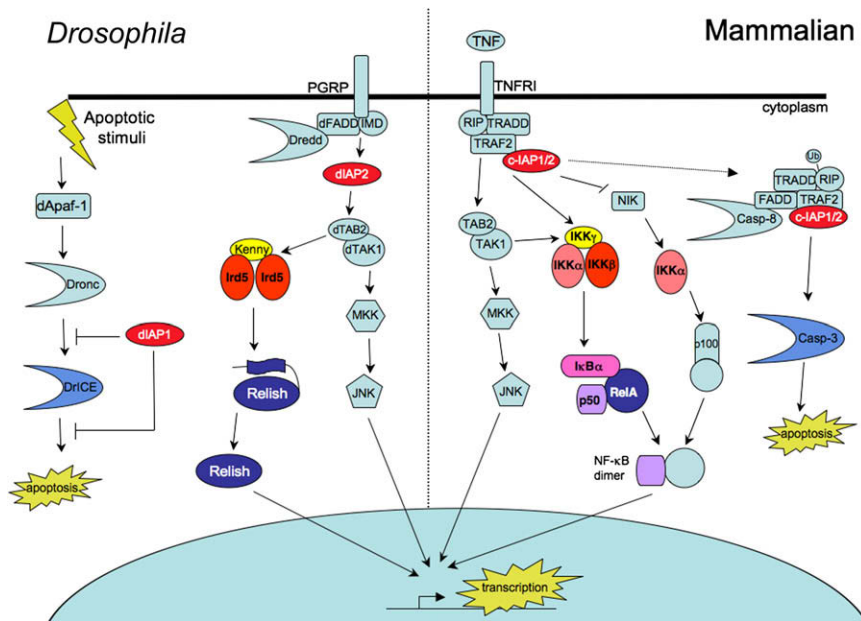


Figure 3. IAP-Dependent Regulation of Conserved *Drosophila* and Mammalian Signaling Pathways

Inhibition of apoptosis in *Drosophila* cells by Diap1 occurs through its binding to the initiator and effector caspases Dronc and DrICE. Similarly, direct binding and inhibition of caspase-3 in mammalian cells is mediated by XIAP. Mammalian c-IAP1 and c-IAP2 can directly bind caspases but are poor caspase inhibitors, instead acting to regulate apoptosis by indirectly modulating caspase-8 activity. Binding of TNF to its receptor results in recruitment of TRADD, RIP, and TRAF2. The c-IAPs also participate in prosurvival signaling through TNFR by associating with TRAF2. c-IAP1 and c-IAP2 ubiquitinate RIP1, minimizing association with caspase-8 and preventing apoptosis. Additionally the association of RIP, TRAF2, and c-IAP1/2 leads to the activation of TAK and subsequent NF- κ B and JNK activation, resulting in enhanced transcription of prosurvival genes. c-IAP1 and c-IAP2 can also inhibit NIK and downstream processing of p100, thereby negatively regulating NF- κ B activation. Thus, the effects of c-IAP1- and c-IAP2-dependent signaling on NF- κ B are likely context dependent. A TNFR-like pathway regulates immune responses to microbial infection in *Drosophila*. Peptidoglycan from Gram-negative bacteria is recognized by peptidoglycan

recognition proteins (PGRP), which can activate the IMD signaling pathway. IMD is an insect homolog of mammalian RIP1. Genetic studies place IMD, dFADD, Dredd, and Diap2 upstream of or parallel to dTAK activation. dTAK activates both the JNK and Relish pathways analogously to TAK1 in mammalian cells, promoting induction of antimicrobial peptide genes.

degradation occurs *in vivo* is unclear (Wilson et al., 2002). During larval development, intrinsic death signaling initiated by Dronc appears to be constitutively activated at a low level, but cell death is minimized by physiological expression of Diap1 (Muro et al., 2002; Rodriguez et al., 2002). RNAi depletion of Diap1 in the *Drosophila* S2 cell line or a *diap1* null mutation in flies resulted in widespread caspase-dependent cell death in the absence of exogenous signals (Goyal et al., 2000; Meier et al., 2000; Wang et al., 1999). In contrast, *Drosophila* zygotes with a *Dronc* null mutation exhibit widespread defects in programmed cell death, resulting in markedly abnormal development (Chew et al., 2004; Xu et al., 2005). A tractable *in vivo* model of programmed cell death is the developing eye, which undergoes temporally and spatially restricted apoptosis during differentiation, regulated by the initiator caspase Dronc and the IAP antagonists Reaper (Rpr) and Head-involution defective (Hid), to produce the precise patterning of the ommatidia (Hay et al., 1995). Rpr and Hid were the first IAP antagonists identified, forming a novel protein family characterized by an N-terminal conserved region termed the IBM that now includes Grim and Sickie (Kornbluth and White, 2005). The *Drosophila* IBM-containing proteins promote cell death through multiple mechanisms, including competing with caspases for binding to Diap1 and suppressing Diap1 translation (Holley et al., 2002; Yoo et al., 2002). Overexpression of Dronc, Rpr, or Hid in the eye altered retinal structures, which caused aberrant eye phenotypes that could be rescued by coexpression of Diap1 (Hay et al., 1995; Meier et al., 2000). Notably, although overexpression of Diap2 in the eye suppressed programmed cell death, Diap2 mutant flies did not display the early lethality phenotype observed in *diap1* null animals (Huh et al., 2007; Leulier et al., 2006). These results suggest that conclusions about the physiological function of the IAPs may be more clearly drawn using loss-of-function approaches.

Diap1 binds Dronc through the BIR2 domain, which was required to inhibit Dronc-induced apoptosis in the developing eye (Chai et al., 2003; Wilson et al., 2002). In contrast, a Diap1 RING domain mutant protein still bound to Dronc, Rpr, and Hid, but failed to ubiquitinate Dronc or to ameliorate cell death caused by Dronc overexpression (Wilson et al., 2002). Levels of Diap1 itself are modulated by regulatory proteins, such as Rpr, which directs Diap1 autoubiquitination and degradation via recruitment of the ubiquitin conjugating enzyme UbcD1 (Ryoo et al., 2002). In addition, Diap1 degradation is promoted by the proapoptotic E2 ubiquitin conjugase-like protein Morgue; mutations in *morgue* suppress cell death in the *Drosophila* eye (Hays et al., 2002; Wing et al., 2002). Thus, the ratio of Diap1 to proapoptotic proteins in individual components of the eye such as Morgue and Dronc may serve as a biological rheostat for determining which cells undergo cell death. In this case, the rheostat could be sensitized to the levels of different proapoptotic versus antiapoptotic proteins, where the levels are controlled through a ubiquitination cascade mediated by Diap1 and other ubiquitin-modifying machinery. Consistent with the rheostat model, heterozygosity of *diap1* exacerbates the eye ablation phenotype caused by ectopic Dronc expression, while heterozygosity of *dronc* ameliorates cell death caused by overexpression of Rpr or Hid (Meier et al., 2000). Although the *Drosophila* genome encodes other IAP proteins, it is evident that Diap1 interacts uniquely with modulators of apoptosis to perform a nonredundant cytoprotective function during development. Notably, a recent report from Montell and colleagues has revealed a novel function for Diap1 in border cell migration in the fly ovary (Geisbrecht and Montell, 2004). A subset of follicle cells migrate to the border between the oocyte and surrounding nurse cells in a Rac-dependent manner, and are thus termed border cells. These cells require Diap1 for proper migration, but not

for protection from cell death. Overall, the wealth of data from these *Drosophila* studies unequivocally implicate Diap1 in protecting cells from programmed cell death, but also suggest additional roles for Diap1 in developmental contexts independent of cell survival.

Diap2 and Deterin were both discovered by DNA sequence homology searches; Diap2 contains three BIRs and a carboxy-terminal RING and is important for innate immune function. In contrast to the clear role of Diap1 in regulating cell death during development, Diap2 null flies do not exhibit an embryonic phenotype, although they may exhibit increased sensitivity to some apoptotic stimuli (Huh et al., 2007; Leulier et al., 2006; Ribeiro et al., 2007). However, *diap2* was identified in several genetic screens as a candidate regulator of the invertebrate immune response, specifically the *immune deficiency (imd)* pathway (Gesellchen et al., 2005; Kleino et al., 2005; Leulier et al., 2006). Imd, an invertebrate homolog of the mammalian signaling adaptor TNF receptor interacting protein (RIP), controls a pathway required for induction of antimicrobial peptides in response to infection; *imd* mutant flies are peculiarly susceptible to infection by Gram-negative bacteria (Lemaitre et al., 1995) (Figure 3). Triggered by bacterial peptidoglycan, the Imd pathway activates the Dredd caspase, feeding into evolutionarily conserved signaling modules such as the MAP3K, dTAK, and *Drosophila* IKK complex (Cherry and Silverman, 2006). Imd-dependent signaling required the Diap2 RING domain, as well as dTAB2, a homolog of mammalian TAB1 that binds the BIR1 domain of XIAP (Huh et al., 2007; Lu et al., 2007). Epistasis studies placed dIAP2 parallel to dTAK1, which is upstream of both JNK and NF- κ B pathways (Gesellchen et al., 2005; Kleino et al., 2005). Although overexpression of Diap2 resulted in association with Rpr and Hid and inhibition of apoptosis, Diap2 null flies exhibited no apparent cell-death-related phenotypes in addition to the immune deficiency (Huh et al., 2007). Instead, Diap2 itself is regulated by increased expression of Rpr or Hid, indicating that Diap2 might be a node for integrating external signals through the Imd pathway with the internal state of cellular stress (Huh et al., 2007). In the immune signaling paradigm, Diap2 primarily serves as a signal transducer rather than a modulator of cell death.

Deterin and dBruce in *Drosophila*, and in other organisms as discussed in further mechanistic detail below, appear to be regulators of cytokinesis that are essential in specific cellular contexts. Deterin is a small, Survivin-like IAP that plays a key role in mitotic spindle formation and cell cycle progression (Jones et al., 2000). In contrast, dBruce is a giant (>500 kDa) protein, displaying a single BIR and a UBC-like E2 ubiquitin ligase domain, that is critical for sperm differentiation (Arama et al., 2003; Vernooy et al., 2002). Overall, the *Drosophila* model has provided important insights into the prosurvival function of IAPs such as Diap1, but has also pointed to an expanded model of IAP function where apoptosis is but one of many molecular programs controlled by these versatile signaling regulators.

Cell Cycle

Mitotic regulation by IAPs was first discovered in transformed cells, where Altieri and colleagues reported that expression of Survivin increased from G1 to S phase, and was highly expressed in G2/M (Li et al., 1998). Their data demonstrated that Survivin associated with microtubules, and disruption of the as-

sociation led to caspase-3 processing and mitotic catastrophe, providing a link between the integrity of the mitotic apparatus and cell death. Mammalian Survivin consists of a single BIR domain, and similar IAP-related sequences have also been characterized in lower invertebrates, specifically in the nematode *C. elegans*, whose genome encodes two BIR-containing proteins, and in both budding and fission yeast, each of which encode a single IAP-like protein (Fraser et al., 1999; Uren et al., 1999; Li et al., 2000). Comparisons with mammalian IAPs, coupled with the fact that yeast do not contain classic caspases with clear apoptotic substrates, suggest that BIR-containing proteins may have originally evolved to control nonapoptotic functions, such as cytokinesis and mitotic spindle formation.

Bir1p, the *Saccharomyces cerevisiae* protein homologous to Survivin, was found in a two-hybrid screen for proteins interacting with components of the yeast kinetochore, a structure that directs spindle fiber attachment to chromosomes to facilitate separation of sister chromatids during mitosis (Yoon and Carbon, 1999). Interestingly, a *bir1* null mutant strain of *S. cerevisiae* in the haploid state did not have noticeable growth or spindle morphology defects; however, over time Bir1p-deficient yeast cultures showed instability of a yeast minichromosome, indicating chromosome missegregation. The specific role of the BIR motifs in Bir1p-mediated chromosome segregation remains somewhat unclear, since overexpression of a truncated Bir1p lacking the BIR motifs in the *bir1*Δ mutant rescued stability of a reporter minichromosome. Combining the *bir1*Δ allele with mutations in the genes encoding kinetochore proteins, Cbf1p or Ctf19p, led to synthetic lethality, emphasizing the contribution of Bir1p to kinetochore function.

Further studies have revealed a wealth of mechanistic information about how Bir1p and its homologs direct chromosome segregation during cell division. Bir1p assembles in a chromosomal passenger protein complex (CPC) that includes INCENP, Aurora-B kinase, and Borealin (Ruchaud et al., 2007; Yoon and Carbon, 1999). The Aurora-B kinase performs the enzymatic activity of the CPC, phosphorylating Survivin and other targets such as histone H3; however, all four proteins of the CPC are essential for mitosis (Jeyaprakash et al., 2007). During prophase, the CPC accumulates on condensing chromosomes, and is then targeted to the inner centromeres in a Survivin-dependent manner. The association of Survivin with centromeres requires K63 ubiquitylation, and is dynamically regulated by opposing activities of UFD1 and the deubiquitinating enzyme hFAM on Survivin (Vong et al., 2005). Upon transition to metaphase, the complex migrates to the spindle midzone, mediating centromeric cohesion and attachment to spindle microtubules. The CPC appears to act as a sensor of mechanical tension between centromeres and microtubules of the spindle, leading to proper chromosome segregation (Fraser et al., 1999; Sandall et al., 2006; Speliotes et al., 2000). High fidelity in chromosome segregation is a critical element of basic cellular function, and thus it could be predicted that Survivin would be involved in many aspects of development.

Regulation of cell division by Survivin homologs has turned out to be highly relevant in multicellular organisms, which have facilitated analysis of Survivin-like proteins in complex developmental processes. The nematode, *Caenorhabditis elegans*, is a genetically tractable model system with which to dissect the

precisely regulated processes of cell division, lineage determination, and cell death during embryonic development. In fact, genetic screens in *C. elegans* identified key determinants of apoptosis that have formed the basis of the current paradigm of cell death regulation (Lettre and Hengartner, 2006). Genomic analysis of *C. elegans* identified only two BIR proteins, BIR-1 and BIR-2, leading to the hypothesis that these proteins might regulate cell death or division in the nematode (*C. elegans* Sequencing Consortium, 1998). Inducible overexpression of either BIR-1 or BIR-2 did not suppress caspase-mediated cell death in the anterior pharynx of L3–L4 larvae (Fraser et al., 1999). Inhibition of BIR-1 expression in *C. elegans* by RNAi did not affect apoptosis in adult germ line, but instead resulted in early lethality of the embryos and a failure to complete cytokinesis (Fraser et al., 1999). Expression of mammalian Survivin in the *bir1* RNAi-treated embryos partially suppressed the cytokinesis defect, increasing cellularity. These genetic experiments revealed an important role for BIR-domain proteins in directing the cell division machinery in more complex organisms, and demonstrated that mammalian Survivin participated in mitotic regulation. Since then, the physiological role of Survivin in mammalian development has been amply documented. Mice lacking *Survivin* exhibit embryonic lethality around embryonic day 4.5, characterized by grossly abnormal nuclear morphology and defects in cytokinesis (Uren et al., 2000). The multinucleate phenotype of *Survivin*-deficient animals suggests that regulation of chromosome separation and cytokinesis is the predominant mechanism by which Survivin promotes early embryonic development.

The early lethality phenotype of animals lacking *Survivin* initially prevented analysis of the role of Survivin in other developmental processes. To determine if Survivin might contribute to differentiation and organ development, several groups employed a conditional knockout strategy, crossing animals with an allele of *Survivin* flanked by *loxP* sites with different *cre* recombinase transgenes. *Survivin* depletion in different contexts yielded phenotypes that implicated aberrant cell cycle regulation or decreased cell survival (to be discussed later in this review). Cell cycle defects were primarily associated with loss of Survivin in hematopoietic lineages. Survivin is expressed in hematopoietic stem cells and lymphocytes, as well as other adult cells types including vascular endothelial cells (Leung et al., 2007). *Cre*-mediated deletion of *Survivin* in adult animals resulted in lethality within 2 weeks, marked by anemia, reduction in the hematopoietic stem cell and progenitor compartments, and substantial loss of cellularity in the bone marrow and spleen (Leung et al., 2007). Immunophenotyping analysis of erythroid differentiation established that mature erythrocytes, which had exited the cell cycle, were unaffected in *Survivin*-depleted mice as compared to control animals, but the highly proliferative erythroid precursor populations were notably decreased. *Survivin*-deficient erythroblasts exhibited an altered cell cycle profile and polyploidy, with more cells in G1 phase and fewer cells in S phase than control erythroblasts. This study provided evidence that *in vivo*, Survivin is involved in hematopoietic proliferation and differentiation (Leung et al., 2007). Further evidence of the importance of cell cycle regulation by Survivin in hematopoietic cells came from studies in which Survivin was depleted specifically in the T cell lineage. Deletion of a floxed allele of *Survivin* in thymocytes, driven by an *Ick-cre* transgene, blocked early differentiation of

T cell precursors at the CD4⁺CD8⁻ stage (Okada et al., 2004). Cell cycle arrest and defects in spindle formation were observed in *Survivin*-deficient thymocytes, concomitant with increased cell death, which could not be rescued by the introduction of a *bcl-2* transgene. *Survivin* depletion at later stages of thymocyte development, mediated by a *CD4-cre* transgene, allowed thymocytes to differentiate past the CD4⁺CD8⁻ stage but caused a substantial decrease in peripheral T cell populations (Xing et al., 2004). In these *Survivin*-depleted mice, no increase in T lymphocyte apoptosis was observed, but the *Survivin*-deficient peripheral T cells were unable to proliferate in response to mitogenic signals, arresting in G2/M phase. Therefore, among the IAP proteins, Survivin uniquely plays a role in cell cycle regulation that is conserved from yeast to mammals and may represent the earliest function of the BIR domain.

More recently, studies have revealed that BRUCE (also termed Apollon or BIRC6), a large multidomain IAP present in most higher eukaryotes, but not in yeast, also has an essential function in the cell cycle: directing final resolution of the midbody channel that connects two dividing cells (Pohl and Jentsch, 2008). BRUCE is a noncanonical IAP, as the protein exhibits both E2 ubiquitin conjugating and E3 ubiquitin ligase activity toward targets like second mitochondrial activator of caspases (SMAC) (Bartke et al., 2004). However, targets of BRUCE appear primarily monoubiquitinated, suggesting that the physiological function of BRUCE may not be to directly target proteins for proteasomal degradation, but rather to modify their function. Interestingly, BRUCE is required for recruitment of membrane vesicles to the midbody ring, a circular phase-dense structure between the two daughter cells that acts as a platform for BRUCE-dependent ubiquitination (Pohl and Jentsch, 2008). BRUCE is essential for mammalian development, as BRUCE-deficient embryos exhibit growth retardation at embryonic day 14, apparently due to defects in maturation of the placenta (Hitz et al., 2005; Lotz et al., 2004; Ren et al., 2005). The three different laboratories constructed distinct *BRUCE* targeting alleles, and although all reported a similar embryonic lethality phenotype, the groups differed in their interpretation of whether the embryonic lethality in *BRUCE*-deficient mice was due to altered cell survival, defective cytokinesis, or both. Nevertheless, the available evidence strongly suggests that like Survivin, BRUCE can play a role in regulation of the cell cycle.

Signaling and Cell Survival

Proteins of the mammalian IAP family exhibit one of two predominant patterns of expression that may reflect differing roles in development. Survivin is expressed during embryogenesis and in many tumors, but to a lesser degree in normal adult animals, whereas XIAP, c-IAP1, c-IAP2, and NAIP are broadly expressed in adult tissues (Verhagen et al., 2001). Of the IAP proteins, data from diverse model organisms suggests that Diap1, c-IAP1, and Survivin are required early in development, participating in both apoptosis-independent and apoptosis-dependent processes. In vertebrates, IAPs also promote cell survival in organ and tissue development. Experimental vertebrate models with fewer IAP genes and excellent genetic tractability, such as *Danio rerio*, have provided insights into IAP function that may have been obscured by gene duplication and redundancy in mammals. A zebrafish strain containing a null mutation in the *ciap1* (*birc2*) gene was identified in a forward genetic screen for mutants

with abnormal vascular phenotypes (Santoro et al., 2007). The mutation was originally christened *tomato* (*tom*) due to the observed vascular hemorrhage, blood pooling, and vascular regression that occurred between 54 and 60 hr postfertilization (hpf). *ciap1* was expressed in the vasculature as early as 48 hpf, supporting a role for c-IAP1 in preventing apoptosis and regression of the vascular endothelium. *ciap1* mutants in either the BIR1 domain, which interacts with the TNFR adaptor TRAF2, or the RING domain failed to rescue the *tom* mutant phenotype. Activation of NF- κ B, which can occur through TNFR, was also required for vascular integrity in the zebrafish, as pharmacological inhibition of NF- κ B or the upstream IKK complex resulted in hemorrhage and vascular instability, mimicking some aspects of the *tom* phenotype. Overexpression of the IKK kinase NEMO rescued apoptosis of the vascular endothelium in the *tom* mutants, emphasizing a signaling function of c-IAP1 in promoting vascular homeostasis through the NF- κ B pathway. TNFR signaling regulates many aspects of cell physiology, including differentiation, apoptosis, and survival, and in mammalian cells involves both c-IAP1 and c-IAP2 (Samuel et al., 2006; Wang et al., 2008) (Figure 3). No evidence has been reported for a defect in vascular integrity or any other developmental process in mice deficient in c-IAP1 or c-IAP2, although the high degree of amino acid conservation between the two proteins suggests the possibility of at least partial redundancy. The close proximity of the *ciap1* (*birc2*) and *ciap2/birc3* genes in mammalian genomes, likely arising from a gene duplication (Rajcan-Separovic et al., 1996), has thus far prevented the generation of a double mutant to determine if these IAPs have an important and overlapping function in mammalian embryogenesis. In further support of a role for IAPs as key regulators of vasculogenesis, morpholinos targeting Survivin-1 injected into one- to four-cell zebrafish embryos increased apoptosis in the brain and neural tube, as well as in axial vasculature, resulting in perturbations in angiogenesis (Ma et al., 2007). Moreover, *tie2*-cre-mediated deletion of a conditional *Survivin* allele in the endothelial lineage caused lethality in mice at embryonic day 9.5, characterized by peripheral hemorrhaging, abnormal heart development, and endothelial cell defects (Zwerts et al., 2007). Since c-IAP1 and Survivin differ substantially in their domain structure and contain distinct BIR subtypes, these IAPs may act through multiple mechanisms to modulate survival of the vascular endothelium and vessel homeostasis. Overall, regulation of endothelial cell survival, particularly during vascular development, appears to be a critical function of IAPs in embryogenesis.

Despite the strong developmental phenotypes associated with defects in vertebrate IAPs, relatively little evidence connects these phenotypes to direct or indirect inhibition of caspase-dependent cell death, as has been described in *Drosophila*. One study reported that specific deletion of *Survivin* in murine neural progenitor cells at embryonic day 10.5 resulted in increased multifocal apoptosis of neuronal precursors and dramatically altered brain size and architecture (Jiang et al., 2005). Neuronal apoptosis in the brains of these *Survivin*-depleted mice was associated with an increase in caspase-3 and -9 activity, which did not appear to be accompanied by cell cycle arrest. However, the activation of caspases might be an indirect consequence of *Survivin* loss, as a more direct role for *Survivin* in inhibition of apoptosis has not been clearly demonstrated.

Of all the IAP proteins, XIAP has the strongest case for being a bona fide caspase inhibitor. Since XIAP has also been shown to modulate signaling through the TGF- β receptor and the NF- κ B pathway, both of which are implicated in embryonic development, it is therefore somewhat surprising that no striking developmental phenotype was found in *Xiap*-deficient mice until recently, although XIAP protein is detected in many adult tissues including spleen, liver, kidney, and mammary gland (Harlin et al., 2001; Olayioye et al., 2005). During gestation, XIAP expression in the mammary glands increased, peaking at embryonic day 18, and at late stages of pregnancy, lobuloalveolar development was delayed in *xiap*^{-/-} animals (Olayioye et al., 2005). No evidence of apoptosis in the mammary gland of *Xiap*-deficient pregnant mice was found, and lactation occurred normally. However, XIAP could be demonstrated to play a physiological role in adult mammals, directing copper homeostasis by regulating COMMD proteins and contributing to antimicrobial immunity (discussed below) by mechanisms that are not yet well defined, but appear to involve signal transduction rather than protection from apoptosis (Bauler et al., 2008; Mufti et al., 2006; Rigaud et al., 2006). Much of our mechanistic understanding of IAP-dependent regulation of apoptosis has been inferred from studies in cell culture, and interrogation of IAP function using animal models supports a cytoprotective role for IAP proteins in embryogenesis. However, it is more difficult to interpret the precise mechanisms by which individual IAPs promote cell survival in development. Although increased cell death was observed in many cases where IAP expression or function was perturbed, cell death may have been an indirect result of dysfunctional signaling or mitotic regulation, as opposed to a direct result of lack of inhibition of the cell death machinery. Overall, data generated using multiple approaches and animal models point to the IAP family as powerfully diverse modulators of cellular function, employing cell division, signaling, and cytoprotective regulatory mechanisms to direct development.

Innate Immunity

An emerging body of work in *Drosophila* has suggested that IAP proteins may serve an underappreciated role in modulating innate immunity to infection. As described above, *Diap2* potentiates the *lmd* signaling cascade responsible for upregulating antimicrobial peptides upon infection (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Leulier et al., 2006). Innate immune signaling pathways are well conserved from humans to *Drosophila*, leading to the hypothesis that mammalian IAP proteins also regulate innate immunity (Hoffmann and Reichhart, 2002).

Mammalian orthologs of proteins of the *lmd* pathway, such as FADD and RIP, are found as part of the TNFR signaling module and have been implicated in immune defense against intracellular pathogens (Balachandran et al., 2004; Chin et al., 2002; Kobayashi et al., 2002) (Figure 3). However, the role of IAPs in mammalian immunity is still incompletely understood. The neuronal apoptosis inhibitor protein NAIP5, which contains a leucine rich repeat (LRR) domain not characteristic of the IAP family, nucleates the assembly and activation of a caspase-1-activating, IL-1 β -processing protein complex termed the inflammasome during infection of murine macrophages by the intracellular bacterial pathogen *Legionella pneumophila* (Coers et al., 2007; Lamkanfi et al., 2007; Molofsky et al., 2006; Ren et al., 2006;

Wright et al., 2003; Zamboni et al., 2006) (Figure 1). While it is yet unclear whether NAIP5 is itself an innate immune sensor, coordinated signaling by NAIP5 and another LRR-containing protein, IPAF, is required for detection of bacterial flagellin within the host cytosol (Coers et al., 2007). Mice with mutations in the *Naip5/Birc1e* locus fail to restrict *L. pneumophila* infection in mice (Diez et al., 2003; Wright et al., 2003). Caspase-1 activation and IL-1 β production are characteristic of an inflammatory cell death termed pyroptosis that shares some aspects of apoptosis, but is more commonly observed in the context of microbial infection (Fink and Cookson, 2005). In the context of *L. pneumophila* infection, NAIP5 appears to promote cell death by activating caspase-1 rather than acting to promote survival as we have described for other IAP family members. However, depending on the strength of the inflammatory stimulus, infected macrophages can either upregulate autophagy, emerging as an important innate immune defense, or drive down an inflammatory cell death pathway (Swanson and Molofsky, 2005). These data strongly support a role for NAIP5 in innate immune signaling, and have led to the hypothesis that NAIP5 may act as a rheostat that determines cellular responses based on the signal strength and context. Based on its domain structure, NAIP5 may also be considered part of the Nod-like receptors (NLR), a family of cytosolic proteins that plays an important role in sensing both self and nonself (i.e., microbial) danger signals (Kanneganti et al., 2007). Thus, the hypothesis that regulation of innate immune signaling is a common attribute of many of the mammalian IAP proteins remains to be fully investigated.

Consistent with the idea that IAPs play an important role in immunomodulation, mutations in XIAP were found in a cohort of patients with X-linked lymphoproliferative syndrome (XLP), a primary immunodeficiency characterized by lymphoproliferation in response to infection by Epstein-Barr virus (Rigaud et al., 2006). XIAP-deficient XLP patients have fewer natural killer T (NKT) cells, and T lymphocytes derived from these patients were more susceptible to apoptosis upon T cell receptor signaling. However, mice lacking XIAP have similar numbers of NKT cells compared to wild-type mice (Bauler et al., 2008; Rigaud et al., 2006). Thus, XIAP may have a role in promoting NKT cell development or survival in humans that is not reflected in the murine model. However, we have recently found that XIAP is required in mice for innate immunity to infection by the intracellular bacterial pathogen *Listeria monocytogenes* (Bauler et al., 2008). XIAP potentiated JNK activation in response to the presence of bacteria in the cytosol, leading to amplification of proinflammatory cytokine production. Lastly, XIAP was necessary for integration of external Toll-like receptor and cytosolic NLR signaling to produce synergistic cytokine output. Notably, there are clear parallels in the molecular players involved in the mammalian NLR signaling pathway and the *Drosophila* Imd pathway, both of which sense bacterial peptidoglycan, which emphasizes a critical and conserved contribution of IAPs to immune signaling (Cherry and Silverman, 2006; Girardin et al., 2002).

Although there is yet relatively little published evidence for innate immune regulation by the c-IAPs, c-IAP2-deficient mice were more resistant to lipopolysaccharide (LPS)-induced sepsis, a syndrome mediated by proinflammatory cytokines (Conte et al., 2006). Wild-type macrophages upregulate expression of c-IAP2 upon treatment with LPS and were protected from apo-

ptosis in this model of septic shock, while c-IAP2-deficient macrophage populations were reported to exhibit increased cell death. Thus, c-IAP2 appears to contribute to macrophage survival in a proinflammatory model of innate immune signaling, but the mechanism by which c-IAP2 prevents macrophage cell death is still unclear, and may be an indirect effect of cytokine regulation. A recent study demonstrated that c-IAP1 and c-IAP2 influence cancer cell survival through K48 and K63 ubiquitin modification of RIP1, reducing formation of a proapoptotic RIP1/caspase-8 complex (Bertrand et al., 2008). Kinases of the RIP family modulate innate immune signaling in *Drosophila* (IMD) and mammalian cells (RIP2/RICK), inducing antimicrobial peptides or proinflammatory cytokines upon microbial infection (Figure 3) (Girardin et al., 2002). It is intriguing to speculate that the c-IAPs may also ubiquitinate RIP2 in mammalian cells, enhancing NF- κ B-dependent proinflammatory cytokine responses. Taken together, these data provide some evidence to suggest that immunomodulation is an important function of the IAP family. Further studies will be required to define both antiapoptotic and apoptosis-independent signaling mechanisms by which individual IAPs modulate immunity.

IAP Antagonists

As discussed above, the prototype IAP antagonist proteins were identified in *Drosophila* through an analysis of the H99 locus, which encoded Rpr, Hid, and Grim (Chen et al., 1996; Grether et al., 1995; White et al., 1994). Additionally, two elegant biochemical studies led to the independent identification of a mammalian IAP binding protein designated SMAC (DIABLO in the mouse) (Du et al., 2000; Verhagen et al., 2000). SMAC/DIABLO is a nuclear-encoded, mitochondrially localized protein that is released into the cytosol following an apoptotic trigger. Structural studies have shown that the amino-terminal four residues of the mature SMAC/DIABLO protein, the so-called IBM, are necessary and sufficient for binding to XIAP, an event which can lead to the competitive displacement of XIAP from bound caspases and so augment intracellular caspase activation (Liu et al., 2000; Wu et al., 2000). Thus, SMAC/DIABLO is a proapoptotic molecule that can function to neutralize the cytoprotective effects of XIAP (Vaux and Silke, 2003). Interestingly, SMAC/DIABLO also exhibits a high affinity for other IAP family members that do not inhibit caspases (Yang and Du, 2004). The consequences of these interactions are less clear, but a dynamic association appears to exist between SMAC/DIABLO and the IAPs that can trigger the autoubiquitination of the IAP in question, or conversely, the ubiquitination of SMAC/DIABLO itself; however, the kinetic details of these events are currently not well understood. Recent work has focused on the exploitation of this interaction by developing small-molecule synthetic compounds that mimic the IBM, and these may have great promise and therapeutic potential for the treatment of neoplastic and proliferative disease (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Gaither et al., 2007).

Concluding Remarks

The exponential increase in our understanding of the biology of the IAPs has illuminated their physiological roles in a wide variety of cellular processes including development, intracellular signaling, mitosis, and immunological responses. Recent studies are removing the misconception that the primary function of the

IAPs is to suppress apoptosis; indeed, the ability of XIAP to directly inhibit caspases appears to be a unique function restricted to that IAP, and others appear to participate largely in cellular processes that involve ubiquitination. Since the identification of IAP antagonists including Rpr in *Drosophila*, followed by the discovery of SMAC/DIABLO in mammals, numerous IAP-interacting proteins have been identified (Verhagen et al., 2007), many of which interact by virtue of their IBM, strongly suggesting that the interactions between IBM-containing proteins and IAPs evolved prior to XIAP's ability to inhibit caspases (Wing et al., 2001; Wright and Clem, 2001; Wu et al., 2001). Thus the IAP-neutralizing properties of IBM-containing proteins are likely to have a wide range of functions encompassing, but certainly not limited to, the regulation of cell death. The fact that IBM-bearing proteins are so prolific in number suggests a fine specificity for signaling under physiological conditions that has yet to be realized. Overall, the IAPs are emerging as a family of signal modulators that may act as hubs to integrate and translate molecular information into the appropriate biological currency of death, inflammation, or differentiation.

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