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Regulation of Cell Death by IAPs and their Antagonists

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Abstract

Inhibitors of Apoptosis (IAP) family of genes encode BIR domain containing proteins with anti-apoptotic function. These proteins also contain RING or UBC domains and act by binding to major pro-apoptotic factors and ubiquitylating them. High levels of IAPs inhibit caspase-mediated apoptosis. For these cells to undergo apoptosis, IAP function must be neutralized by IAP-antagonists. Mammalian IAP knockouts do not exhibit obvious developmental phenotypes, but the cells are more sensitized to apoptosis in response to injury. Loss of the mammalian IAP-antagonist ARTS results in deficient stem cell apoptosis. In addition to the anti-apoptotic properties, IAPs regulate the innate immune response, and the loss of IAP function in humans is associated with immunodeficiency. The roles of IAPs in *Drosophila* apoptosis regulation is more apparent, where the loss of IAP1, or the expression of IAP antagonists in *Drosophila* cells, is sufficient to trigger apoptosis. In this organism, apoptosis as a fate is conferred by the transcriptional induction of the IAP antagonists. Many signaling pathways often converge on shared enhancer regions of IAP-antagonists. Cell death sensitivity is further regulated by post-transcriptional mechanisms, including those regulated by kinases, miRNAs and ubiquitin ligases. These mechanisms are employed to eliminate damaged or virus-infected cells, limit neuroblast (neural stem cell) numbers, generate neuronal diversity and sculpt tissue morphogenesis.

Keywords

Apoptosis; IAP-antagonist; REAPER; HID; ARTS; SMAC; DIAP1; XIAP; c-IAP1; BRUCE

Introduction

Apoptosis is one of the best-understood forms of cell death that is regulated through a combination of positive and negative factors. Among the negative regulators are anti-apoptotic proteins that share the Baculovirus IAP-Repeat (BIR) domains, which are now widely referred to as Inhibitors of Apoptosis (IAP) family of proteins.

The name of the BIR domain originates from the shared sequence of viral inhibitors of apoptosis found in the functional homologs of baculovirus p35, Cp-IAP and Op-IAP (Birnbaum, 1994; Clem, 1991, 1994; Crook, 1993). The family now includes X-linked IAP (XIAP), c-IAP1 and 2, *Drosophila* IAP1 and 2 (DIAP1 and 2), and BRUCE (BIR domain

containing Ubiquitin Conjugating Enzyme) (Figure 1). Not all BIR domain-containing proteins regulate cell death, and certain BIR domain proteins are dedicated to the regulation of mitosis (Silke, 2001). The anti-apoptotic BIR domain proteins found in *Drosophila* and vertebrates mostly have C-terminal RING domains that have ubiquitin ligase activities (Yang, 2000). One exception to this is BRUCE, a potent anti-apoptotic protein that contains an Ubiquitin Conjugating Enzyme (UBC) motif instead of RING. These IAPs bind and ubiquitylate major pro-apoptotic proteins to exert their anti-apoptotic function. In addition, they are actively regulated in cells by their inhibitory molecules, referred to as IAP-antagonists. In this review, we will discuss the latest advances in the field, focusing on the roles of IAPs and their antagonists during animal development.

IAP/antagonist interaction

In many cells, IAPs bind and inhibit active caspases to exert their anti-apoptotic function (Devereaux, 1997; Wang 1999; Goyal 2000). Caspases gain full catalytic activity after being proteolytically cleaved, so that the resulting small and large subunits of caspases can assemble to form active catalytic sites. IAPs can inhibit such proteolytically activated caspases (Srinivasula, 2001; Muro, 2002; Shapiro, 2008), and therefore, high levels of IAPs can block apoptosis at the last stage. However, cells with high levels of IAPs can undergo caspase-mediated apoptosis, if IAP antagonizing molecules are around to neutralize IAP function. The so-called IAP-antagonists were first discovered in *Drosophila*, and remains best characterized in this organism. Reflecting their important role in cell killing, these IAP-antagonists were named *grim*, *reaper*, *hid* and *sickle* (Chen, 1996; Christich, 2002; Grether, 1995; Srinivasula, 2002; White, 1994; Wing, 2002).

IAP-antagonists play particularly visible roles in *Drosophila* apoptosis regulation: Virtually all apoptosis is abolished in the absence of these genes, whereas their overexpression is sufficient to kill cells (White, 1994; Chen, 1996; Grether, 1995; White, 1996). Genetic interaction screens have identified DIAP1, DIAP2 and BRUCE as downstream targets (Hay, 1995; Wang, 1999; Goyal, 2000; Lisi, 2000; Vernooy, 2002; Arama, 2003). In living cells of *Drosophila*, DIAP1 normally inhibits both, initiator and effector caspases (Hawkins, 1999; Meier, 2000; Yan, 2004; Tenev, 2005). DIAP1 uses its ubiquitin ligase activity to directly ubiquitylate the initiator caspase DRONC (Lee, 2011; Ryoo, 2004; Wilson, 2002) and also helps to destabilize the upstream adaptor, the apoptosome holoenzyme, which is a protein complex that serves to activate DRONC (Akdemir, 2006; Shapiro, 2008). DIAP1 is a key player as demonstrated by the observation that virtually all somatic cells undergo apoptosis in *diap1* mutant embryos (Goyal, 2000; Lisi, 2000; Wang, 1999). DIAP2 has a more confined role in inhibiting a specific effector caspase (Ribeiro, 2007), and while overexpression of DIAP2 can inhibit IAP-antagonist-induced apoptosis (Hay, 1995), the loss of this gene does not show the dramatic apoptosis phenotype as seen in *diap1* mutants (Huh, 2007; Ribeiro, 2007). BRUCE is also a potent anti-apoptotic gene, and this protein exerts its effect by using its UBC domain to ubiquitylate IAP-antagonists for proteasomal degradation (Arama, 2003; Bartke, 2004; Domingues, 2012; Hao, 2004; Vernooy, 2002). Mammalian IAP antagonists, Smac and Omi/HtrA2, were also identified based on its ability to physically bind to XIAP (Du, 2000; Verhagen, 2000). However, mouse genetics studies indicate that IAP antagonists primarily target c-IAP1 in vivo (Vince, 2007).

IAP-antagonists share a conserved N-terminal 4 – 8 residues that directly bind to a groove within the IAP BIR domain, allowing caspases to be liberated from IAPs (Wu, 2000; Wu, 2001). Furthermore, they promote the auto-ubiquitination and degradation of IAPs (Li, 2011; Ryoo, 2002; Yoo, 2002). Notable in this interaction is the fact that the first methionine of the N-terminal IAP binding motif must be lost, and the new N-terminus must start with an alanine residue, in order to fit into an IAP BIR groove (Wu, 2000).

Mitochondrial Association of IAP Antagonists

How can cells make peptides that do not start with an N-terminal methionine residue? In mammals, the IAP antagonist Smac encodes an N-terminal mitochondrial localization motif followed by an IAP-binding motif that is similar to the N-terminal residues of GRIM, REAPER, HID and SICKLE (Du, 2000; Verhagen, 2000) (Figure 1). Omi/HtrA2 is a mitochondrial protease that has a similar IAP-binding motif (Hegde, 2002; Martins, 2002; van Loo, 2002) – although in *Drosophila*, Omi/HtrA2 does not appear to regulate IAPs and downstream caspases (Yacobi-Sharon, 2013). The N-terminal mitochondrial localization sequences of Smac and Omi/HtrA2 are cleaved off while being trafficked into the mitochondrial intermembrane space, generating new N-terminal motifs that start with alanine residues and with the ability to bind to BIR domain grooves. Because mammalian IAP-antagonists are sequestered into the mitochondrial intermembrane space, they do not inhibit IAPs that reside in the cytoplasm during non-apoptotic conditions (Du, 2000; Verhagen, 2000; Hegde, 2002; Martins, 2002; van Loo, 2002). Only when Bcl-2 family proteins help release the mitochondrial proteins into the cytoplasm during apoptosis do these proteins get into contact with IAPs in the cytoplasm, neutralizing their target IAPs.

In case of the *Drosophila* IAP-antagonists, there are no N-terminal mitochondrial localization sequences, and it remains unclear how these *Drosophila* proteins lose their N-terminal methionine residues. *Drosophila* IAP-antagonists do not enter the mitochondrial intermembrane space, but localize to the mitochondrial outer membrane. Hid contains a C-terminal tail anchor sequence that inserts into the mitochondrial outer membrane, with the IAP-binding motif facing the cytoplasm (Abdelwahid, 2007; Haining, 1999). GRIM and REAPER each contain an amphipathic helix that are required for their mitochondrial outer membrane localization (Claveria, 2002; Olson, 2003a; Sandu, 2010). These proteins form multimers with each other, and such association is important for their mitochondrial localization (Sandu, 2010). Mutating their mitochondrial localization sequences disrupt their pro-apoptotic function (Abdelwahid, 2007; Claveria, 2002; Olson, 2003a). More recently, it was found that *cdk7* mutants block the mitochondrial localization of Hid, and such conditions abolished Hid's cell killing activity (Morishita, 2013). Why should these proteins localize to the mitochondrial outer membrane to trigger apoptosis? Certain studies have implicated pro-apoptotic roles of IAP-antagonists that are independent of DIAP1 (Abdelwahid, 2007; Thomenius, 2011; Thress, 1999). On the other hand, DIAP1 overexpression almost completely blocks IAP-antagonist-induced apoptosis in vivo (Hay, 1995), suggesting that DIAP1 independent effects of IAP-antagonists are likely to be subtle, at best. A different explanation was proposed recently, suggesting that the IAP-binding activity of the antagonists is linked to the mitochondrial localization. Specifically, subcellular fractionation studies indicate that only the mitochondria-associated pool of Hid,

but not the cytoplasmic pool, has the ability to bind to recombinant DIAP1 proteins (Morishita, 2013).

The role of mammalian IAP-antagonists

Genetic analysis of mammalian IAP-antagonists have shown varying outcomes. Perhaps Smac has drawn the most attention, but a knockout study brings into question its biological significance: The Smac-deficient mice grow normally, and the knockout cell lines respond normally to the apoptotic stimuli that were tested (Okada, 2002). Omi/HtrA2 mutations are associated with Parkinson's disease (Strauss, 2005), which is more consistent with its role in mitochondrial homeostasis, but not with IAP-antagonist function. However, there is another mammalian IAP-antagonist, ARTS. This is a splice isoform of Septin4, originally identified in a retroviral insertion screen (Larisch, 2000). Subsequent studies have revealed that this protein binds to XIAP, but not through an N-terminal sequence as found in other IAP-antagonists. Instead, the nine C-terminal end residues of ARTS serve as the XIAP1 binding motif (Reingewertz, 2011). Upon binding, ARTS promotes the ubiquitin-mediated degradation of XIAP, similar to the *Drosophila* IAP antagonists (Gottfried, 2004). ARTS knockout mice have elevated levels of XIAP, resulting in enhanced cell death resistance. Perhaps as a result, these animals have increased numbers of hematopoietic stem cells and hair follicle stem cells. On the negative side, these mice are more prone to develop tumors (Garcia-Fernandez, 2010). On the positive side, they display marked improvement in wound healing and regeneration (Fuchs, 2013).

Transcriptional regulation of *Drosophila* IAP-antagonists

Unlike mammalian IAP-antagonists, which are initially segregated into the mitochondrial intermembrane space, transcriptional induction of IAP-antagonists in *Drosophila* is sufficient to trigger apoptosis. In fact, the transcription of *grim*, *reaper* and *sickle* foreshadows apoptosis induction in this organism (White, 2004; Chen, 2006). As a result, there is much interest in understanding the transcriptional regulation mechanisms of *Drosophila* IAP-antagonists.

Stress-activated pathways mediated by the tumor suppressor gene p53 respond to a number of distinct stress conditions, including DNA damage (Brodsky, 2000; Ollmann, 2000) and viral infection (Liu, 2011, 2013). p53 directly binds to a regulatory sequence that lies between *reaper* and *sickle* (Brodsky, 2000) (Figure 2). This site is part of a broader irradiation-responsive element that controls the induction of multiple IAP-antagonists, including *hid* that lies more than 250kbp away (Zhang, 2008). Interestingly, this locus is active only during early embryogenesis, and subsequently becomes silenced through epigenetic regulation, thereby making cells insensitive to irradiation-induced apoptosis (Zhang, 2008).

Jun N-terminal Kinase (JNK) signaling is also tightly associated with IAP-antagonist induction in *Drosophila* (Figure 2). The heterodimeric transcription factors Jun and Fos form the AP-1 complex to mediate the transcriptional response to this pathway. In fact, IAP-antagonist expression triggers a transcriptional feed back loop that induces other IAP-

antagonists to augment their pro-apoptotic effects, and this is mediated by JNK signaling and p53 (Kuranaga, 2002; Shlevkov, 2012). Analysis of the *reaper* upstream sequence in fact shows functionally significant binding sites. However, JNK is also involved in cell migration and other physiological events without the induction of apoptosis. Why certain cells evade JNK-induced apoptosis has been a mystery up until recently. It has now been found that JNK-mediated apoptosis signal is integrated at the *reaper* regulatory sequences, together with other anti-apoptotic signals. DPP signaling, mediated by the transcription factor Schnurri, represses JNK-mediated *reaper* expression through a binding site located in between those of AP-1 (Beira, 2014) (Figure 2). During embryonic development, dorsal closure is regulated by JNK signaling, but these cells do not undergo apoptosis, due to Dpp-Schnurri mediated repression of IAP-antagonist gene expression.

The steroid hormone ecdysone, which triggers the onset of metamorphosis, is also well known to induce massive cell death during metamorphosis. The effect is, in part, due to the transcriptional induction of *grim*, *reaper* and *hid* by the Ecdysone Receptor (EcR), which binds to a *reaper*-upstream enhancer sequence (Jiang, 2000). In addition to the control of IAP-antagonist, a recent study shows that Ecdysone signaling is required for the proper induction of downstream caspases. That study noted that the resulting increase in caspase expression renders cells sensitive to *reaper* and *hid*-induced apoptosis during the late 3rd instar stage of *Drosophila* development, as ecdysone signaling becomes active at this stage (Kang, 2014). In addition, loss of EGF Receptor/MAP Kinase survival signaling pathway induces *hid* transcription (Kurada, 1998).

Post-transcriptional regulation of IAP-antagonists

When IAP-antagonists were first discovered, it had been noted that the transcripts of *hid* are distributed more broadly than the actual pattern of apoptosis, suggestive of post-transcriptional regulatory mechanisms (Grether, 1995). Ever since, distinct types of regulatory mechanisms have been elucidated.

As in many other cellular mechanisms, kinases play roles in IAP-antagonist regulation. A well-characterized example is MAP Kinase, which phosphorylates Hid to inhibit its cell killing activity (Bergmann, 1998; Bergmann A, 2002). This EGF Receptor/MAP Kinase survival signaling pathway also regulates *hid* transcription (Kurada, 1998). More recently, CDK7 was identified as a gene required for IAP-antagonist-induced cell death. However, it remains unclear whether CDK7 directly phosphorylates IAP-antagonists (Morishita, 2013).

Many IAPs and their antagonists are regulated through ubiquitylation. As introduced earlier, both mammalian and insect IAPs undergo auto-ubiquitylation when bound by IAP-antagonists (Yang, 2000; Yoo, 2002; Ryoo, 2002). IAP-antagonists are ubiquitylated by IAPs (Olson, 2003b). Lysine-deficient REAPER is more stable, but it was recently demonstrated that BRUCE can add ubiquitins on REAPER even at non-lysine residues and target it for degradation (Domingues, 2012). In the developing eye discs of *Drosophila*, sensitivity to apoptosis changes as cells transition from an unspecified state to differentiated photoreceptors. Unspecified cells are more vulnerable to apoptosis, as DIAP1 is kept low

through ubiquitylation by the Cullin-3 complex in these cells. Differentiated photoreceptors accumulate DIAP1 to gain apoptotic resistance (Fan, 2014).

The role of micro RNAs (miRs) in various cellular processes has been intensely studied in the past decade. Not surprisingly, IAP-antagonists are also targets of miRs in *Drosophila*. One of the first to be discovered was the miR, bantam, which targets *hid* for translational suppression (Brennecke, 2003). *bantam* mutants are homozygous viable but are smaller in size due to reduced tissue growth. Consistently, overexpression leads to overgrowth of tissue due to an increase in cell numbers. GFP-reporter studies show that *bantam* binds to at least five sites in the *hid* 3'UTR region and target it for degradation by RNAi. Similar reporter studies show miR-2 to target sites in *reaper*, *grim* and *sickle* transcripts (Stark, 2003) and miR-14 to target *reaper* (Xu, 2003). More recent studies have found miR-6 and 11 as additional regulators of all four IAP-antagonists (Ge, 2012; Truscott, 2011). Though single mutants are viable, miR-6/miR-11 double mutants are embryonic lethal and show defects in the CNS, suggesting that they may have overlapping roles. Broadly, spatial expression patterns of miRNAs and their targets contribute to regulation of cell death, but an additional layer of complexity is added by competition amongst miRNA for the same targets.

The role of IAP-antagonists in nervous system development

Arguably, a majority of apoptosis observed during development occurs in the nervous system. Reinforcing this view, recent studies on the *Drosophila* mutants for *grim*, *reaper* and *hid*, revealed their intricate roles in neuronal development. In the ventral nerve cord of the central nervous system, most neuroblasts (neural stem cells) in the abdominal segments undergo apoptosis during late embryogenesis, and a few additional dying cells are observed in the late third instar larval stage. *grim* and *reaper* double mutants show a dramatic impairment of neuroblast cell death in this tissue, leading to a significantly enlarged ventral nerve cord (Peterson, 2002; Tan, 2011). This indicates that, in wild type animals, developmental cues confer death as a fate to the abdominal segment neuroblasts. Apoptosis is induced specifically in the abdominal segment neuroblast due to the regulation of *grim*, *reaper* and *hid* by the Hox gene expressed in that region, AbdA (Bello, 2003). A temporal series of transcription factors that are expressed in these cells make up a combinatorial code to determine the timing of apoptosis (Maurange, 2008). In addition, there appears to be a cell death signal originating from the progeny: Those progeny express the Notch ligand, delta, and the resulting Notch signaling contributes to AbdA induction to express IAP-antagonists (Arya, 2015).

Analogous to the example of cell death induction in the ventral nerve cord, a recent study has found that a combinatorial transcription code induces *grim* and *reaper* to confer death as a fate to certain differentiating cells. In the region of the optic lobe Outer Proliferation Center (tOPC), neuroblasts temporally express a series of transcription factors to confer different fates to the differentiating neurons. In one of the early lineages, a specific transcription factor helps the cells interpret Notch signal as an apoptotic signal. In a later lineage, a different transcription factor helps the cells to perceive Notch signaling in an opposite way – as a survival signal (Bertet, 2014). Such a strategy allows a diverse array of neuronal subtypes to be established in this tissue.

IAPs and their antagonists in sculpting morphogenesis

In *Drosophila*, mutant alleles of DIAP1 are referred to as *thread*, as one of the hypomorphic alleles, *thread^l* causes the fly antenna tip to appear as thin as a thread. Normally, that region of the antenna, which is called the arista, has many branches, which disappear in *thread^l* mutants due to excessive death of cells in the larval antennal discs. Conversely, loss of the IAP-antagonist *hid*, causes excessive branches to appear in the arista (Cullen, 2004). These observations indicate that apoptosis is regulated in antennal discs to regulate the morphogenesis of the arista during development.

One of the important functions of apoptosis is to sculpt body structures. An example is the role of the *Drosophila* Hox gene Deformed (Dfd) in inducing *reaper* to kill cells along the segment boundary (Lohmann, 2002). A more recent study has found that *Drosophila* joint boundaries also undergo apoptosis. How do the tissue know where to make the joint boundary? The data seem to indicate that the Dpp morphogenic gradient is a key determinant. Dpp is secreted from the organizers to form a gradient, but when there is a sharp discontinuity in the gradient, it is known to activate JNK signaling and the induction of IAP-antagonists. This is what happens at the joints, leading to the death of those boundary cells (Manjon, 2007). Notch pathway also contributes to tarsal joint development. This pathway induces the transcription factor Dys, which in turn induce *reaper* and *hid* to help sculpt joints (Cordoba, 2014).

Recent work on *Drosophila* IAP-antagonists has revealed a surprising role of apoptosis in unexpected morphogenic processes, such as tissue rotation. It had been noted that *hid* mutants can survive to adulthood, and the surviving males have their genitalia rotated in abnormal angles. A live imaging study of the developing male genitalia in the pupal stage helped elucidate this rotation process in detail. Two distinct domains, each rotating 180 °C, have the incremental effect of rotating 360 °C. These two domains are initially part of the same epithelial layer, and the investigators found that *hid*-induced cell death allows the two domains to separate and rotate away from each other. A failure to separate the two domains leads to a rotational defect (Suzanne, 2010).

Non-apoptotic roles of IAPs in morphogenesis, cell migration and proliferation

Caspases were primarily studied as proteins dedicated to apoptosis induction, but now, there are increasing numbers of studies implicating caspases in diverse non-apoptotic roles. Since caspases in *Drosophila* are tightly regulated by DIAP1, IAP-antagonists and DIAP1 are also involved in those non-apoptotic processes. The examples include their roles in regulating non-autonomous cell proliferation, cell migration and dendritic morphology regulation.

The loss of DIAP1, or activation of IAP-antagonists, not only induces caspase activation, but also activates a pathway that triggers mitogen expression and the proliferation of neighboring cells. This phenotype is dramatically augmented to cause tissue overgrowth if effector caspases are blocked with p35. It turns out p35 does not inhibit the initiator caspase DRONC, and latter has an apoptosis-independent function in activating the JNK pathway to

promote the expression of mitogenic genes such as *wingless* and *dpp* (Ryoo, 2004; Huh, 2004; Perez-Garijo, 2004. Kondo, 2006; Fan 2008).

DIAP1's target DRONC is involved in a number of other non-apoptotic roles. One of the non-apoptotic cellular processes that resemble apoptosis is neuronal dendrite pruning. In order to make, or break, proper synaptic connections, certain dendrites have to undergo dramatic morphological changes. Certain neurons have adopted the caspases regulatory network to eliminate, not the entire cell, but specific dendrites. For example, the *Drosophila* initiator caspase DRONC can promote dendrite pruning during development, and this process is inhibited by DIAP1 (Kuo, 2006). Other regulators of dendrite pruning have been discovered, and in one example, it was discovered that the AAA ATPase, VCP, regulates dendrite pruning by binding to DIAP1 and facilitating its degradation (Rumpf, 2011).

Sensory organ development of *Drosophila* also involves caspases in a non-apoptotic mechanism. During the formation of the precursor cells, caspases cleave Shaggy, a negative regulator of Wnt signaling. The cleavage by caspase converts the substrate to an active kinase, promoting the formation of sensory organ precursors (Kanuka, 2005). Not surprisingly, such caspase activity is under the control of DIAP1. DIAP1 is regulated in these cells, not by IAP-antagonists, but through phosphorylation by IKK epsilon. Loss of this kinase results in the stabilization of DIAP1, which in turn, blocks caspase-mediated shaggy cleavage and activation (Kuranaga, 2006). The IKK epsilon/DIAP1/caspase cascade is also involved in F-actin turnover at the cellular margin and contributes to the morphogenic changes of cultured cells (Oshima, 2006).

Such effects of DIAP1 on the cytoskeleton are not limited to morphological changes, but can also affect cell migration. In the *Drosophila* ovary, border cells migrate during ovary development to a specific position, and this process requires the GTPase Rac that helps to rearrange the cytoskeleton during this process. Evidence indicates that DIAP1 and DRONC regulate Rac, and without DIAP1, border cells fail to migrate properly (Geisbrecht, 2004). In an analogous mechanism, XIAP1 and c-IAP-1/2 regulate mammalian cell motility by ubiquitinating C-RAF (Dogan, 2008). The non-apoptotic roles of DIAP1 bring up an important question. How can some cells regulate DIAP1 without triggering apoptosis? Live imaging of DIAP1 in the sensory organ precursor cells of *Drosophila* indicates that the turnover rate of DIAP1 varies between cell types, and such temporal regulation of DIAP1 may determine whether the downstream caspases are utilized for apoptotic or non-apoptotic roles (Koto, 2009).

Spermatid differentiation is a process that involves dramatic morphological changes, including the removal of the bulk of cytoplasm along the elongating spermatids through a process termed "spermatid individualization". Caspases have been adopted in these cells to mediate the massive cytoplasmic removal in this differentiation process (Arama, 2003). In *Drosophila*, caspases form a gradient in their activity to regulate spermatid differentiation, so that the regions of the spermatids that are the last to individualize have the lowest caspase activity. This gradient is formed by a counter gradient of the IAP protein, BRUCE (Kaplan, 2011). Consistently, *Drosophila bruce* mutants show male sterility (Arama, 2003). An analogous regulation of caspases occurs during mammalian spermatid differentiation

through XIAP1 and its antagonist, ARTS. In fact, mice lacking the ARTS/Septin 4 locus show defects in sperm cell maturation (Kissel, 2005).

The roles of IAPs in the innate immune response

Early studies of mammalian IAPs, XIAP and c-IAP-1/2 focused on their ability to regulate effector caspases and apoptosis, but in vivo studies of these IAPs increasingly point to their important roles in TNF α signaling and the innate immune response. Upon infection by virus or pathogenic bacteria, various cells in our body produce TNF α to initiate immune response signaling. There are at least three distinct pathways that can be activated downstream of TNF α receptors: The extrinsic cell death pathway mediated by caspase-8 and -3; NF- κ B signaling that leads to cytokine production; and RIP (receptor interacting protein)-1 and -3/MLKL mediated “necroptotic cell death (Silke, 2011). As introduced earlier, c-IAP1 and 2 are mammalian IAP proteins initially identified based on their physical association with TNF α receptor 2 (Rothe, 1995; Uren, 1996). c-IAP1/2 specifically bind to RIP1 while in a complex with TNF α receptor. Upon TNF α stimulation, c-IAP1 and 2 ubiquitylate RIP1 to activate NF- κ B and MAP kinase signaling (Bertrand, 2008). Loss of c-IAP1/2, or deubiquitination of RIP1 triggers the formation of a different TNF receptor signaling complex that, instead of promoting NF- κ B signaling, activates the caspase-8 mediated apoptosis or RIP3/MLKL-dependent necroptosis (Tenev, 2011; Vince, 2007). c-IAP1 and 2 also ubiquitylate a different, yet related protein, RIP2, and such ubiquitination promotes NF- κ B signaling and cytokine production (Bertrand, 2009). In *Drosophila*, DIAP1 and 2 are the closest homologs of c-IAP1/2, and while DIAP1 primarily regulates apoptosis, DIAP2 promotes the activation of the *Drosophila* NF- κ B homolog, Relish, as part of an innate immune response to gram positive bacteria infection (Huh, 2007).

XIAP was originally characterized as an anti-apoptotic protein that primarily inhibits at least two effector caspases, caspase-3 and -7 (Deveraux, 1997). There was a slight disappointment to the field when it was first reported that XIAP1^{-/-} mice do not show obvious developmental abnormalities (Olayioye, 2005). Subsequent studies revealed subtle cell death phenotypes: XIAP1 deficient sympathetic neurons are more vulnerable to apoptosis after cytochrome c injection (Potts, 2003), and the mutant fibroblasts are sensitized to TNF α -induced apoptosis (Schile, 2008).

Interestingly, more recent studies also implicate XIAP in TNF α signaling and immune response. Mutations in human XIAP (also referred to as BIRC4) have been found to underlie immunodeficiency with aberrant activation of macrophages and dendritic cells, and the accumulation of activated T lymphocytes after viral infection (Rigaud, 2006; Damgaard, 2013; Marsh, 2010; Pachlopnik Schmid, 2011). Similarly, XIAP knockout mice show reduced ability to clear infectious pathogens (Bauler, 2008; Prakash, 2010). These immunodeficiency phenotypes are difficult to explain through XIAP's ability to inhibit effector caspases. Recent studies have found that XIAP1 has an inhibitory effect on TNF α signaling, a process that is also regulated by c-IAP1/2. However, XIAP1 and c-IAP1/2 have different mechanisms of action: Whereas c-IAP1/2 ubiquitylate RIP1 while bound with TNF receptor to activate NF- κ B signaling, XIAP1 appears to ubiquitylate RIP1 at a later stage of signaling, within a distinct complex. The loss of such XIAP activity results in abnormally

high inflammasome activity, caspase-1 activation, and IL-1 β secretion from dendritic cells (Yabal, 2014). Human patients with XIAP mutation suffer from hyperinflammation, and XIAP's effect on inflammasome provides a molecular explanation.

Invertebrate IAPs and their antagonists also respond to viral infection as part of an innate immune response. Mosquitos and *Drosophila* induce IAP-antagonists when infected with DNA or RNA viruses, which helps to kill cells infected with virus and block their propagation (Liu, 2013). Certain virus have evolved to inhibit such innate immune response by evolving IAPs in their genome (Clem, 2005), and the best characterized examples of viral IAPs include *Orgyia pseudotsugata* Op-IAP, which primarily inhibit initiator caspase activity (Birnbaum, 1994; LaCount, 2000). It appears that these viral IAPs have a more stable anti-apoptotic activity than their cellular homologs: Whereas XIAP, c-IAP1/2 and DIAP1 have short half-lives and undergo auto-ubiquitination and degradation upon binding to IAP-antagonists (Yang, 2000; Ryoo, 2002), or after cleavage by caspases near the N-terminus (Yokokura, 2004; Ditzel, 2003), Op-IAPs lack the N-terminal degrons found in cellular IAPs and exhibit more stability (Cerio, 2010; Vandergaast, 2015). Restoring apoptosis by introducing *Drosophila reaper* into Sindbis virus impaired their ability to infect mosquitoes, and a gradual negative selection against *reaper* expression in the recovered virus (O'Neill, 2015). Together, these observations indicate that IAPs and IAP-antagonists regulate the degree of viral propagation in insect hosts.

Concluding Remarks

It has been more than two decades since IAPs and their antagonists were first discovered, but dramatic new discoveries continue to be made in this field. A number of them are particularly notable: For example, although it had been thought that IAP antagonists play no obvious roles in mammalian development, knockout of ARTS revealed defects in stem cell death. Exciting biological roles of IAPs and IAP-antagonists in innate immune response, in contributing to neuronal diversity and numbers, and playing unexpected roles in morphogenesis, have been discovered only recently. There are still many unanswered questions. We still do not fully understand why *Drosophila* IAP-antagonists must localize to the mitochondrial outer membrane, and how it is trafficked to that site. The intricate regulatory mechanisms that converge on transcription, translation, and post-translational levels, are only beginning to be understood. We hope to see major advances in these areas in coming years.

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Inhibitors of Apoptosis (IAPs)Viral Fly Mammalian **IAP antagonists** | = IAP Binding Motif (IBM)Fly Mammalian **Figure 1. Domain maps of IAPs and their antagonists from various model systems**

All known IAPs contain at least one Baculovirus IAP-Repeat (BIR) domain. In addition, most have RING domains. BRUCE is the largest IAP (as indicated by the break in sequence in figure) and an exception in that it does not contain a RING domain but instead has an Ubiquitin Conjugation domain (UBC). Most IAP-antagonists contain a short 5–10 amino acid IAP-Binding Motif (IBM) at their N-terminii, usually immediately after the Methionine, which is cleaved to expose the IBM. The mammalian IAP antagonists, Smac, ARTS and Omi/HtrA2, localize to the mitochondria for their function and hence contain a Mitochondria Localization Sequences (MLS) amongst other domains. ARTS belongs is a non-canonical IAP-antagonist that does not have an N-terminal IBM, and instead uses the C-terminal sequences to bind IAPs. Domain maps to scale, source: www.uniprot.org.

Figure 2. A schematic showing the *Drosophila* H99 locus and its regulation

The 3L chromosome arm contains all four of the IAP-antagonists, *hid*, *grim*, *reaper* (*rpr*) and *sickle* (*skl*), clustered in the H99 locus. A ~12kbp region upstream of *rpr* is the hub of most transcriptional regulation and controls transcription of *rpr* and *hid*, although the *hid* locus is more than 250kbp away. It contains a ~11kbp Irradiation-Responsive Enhancer Region (IRER) containing a 20bp p53 binding site critical for radiation induced apoptosis. The IRER region is heavily epigenetically regulated during development by histone deacetylases (HDAC) and methylases. The *rpr* upstream promoter region contains sites for binding several transcription factors, notably Jun/Fos and Schnurri (Shn), which mediate context dependent JNK-driven apoptosis.

Figure 3. A schematic diagram of IAP-antagonists and their targets

Upper panel shows the relationships between *Drosophila* genes, whereas the lower panel shows mammalian IAPs and their antagonists.