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To cite this version:

Bernard Mignotte, Jessie Colin, Sylvain Brun, Isabelle Guénal. Apoptosis: the fly point of view. A.I. Scovassi. Apoptosis, Research Signpost, pp.69-186, 2005, 81-308-0021-7. hal-03381529

HAL Id: hal-03381529

https://hal.science/hal-03381529

Submitted on 17 Oct 2021

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Apoptosis: the fly point of view

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Running title: Apoptosis in *Drosophila*

Abstract

It is now established that genes involved in the execution of programmed cell death by apoptosis are relatively well conserved throughout evolution. However, the control of commitment to apoptosis exhibits some differences between organisms. In *C. elegans*, genetic studies have led to the identification of the *ced* genes (cell death) *ced-3* and *ced-4* that are essential to trigger cell death, and *ced-9* that antagonizes the activities of *ced-3* and *ced-4*. CED-3 is a caspase (cysteinyl aspartase) and CED-9 is homologous to proteins of the Bcl-2 family. In mammals, two main pathways of apoptosis have been identified. The intrinsic pathway is regulated by the *bcl-2* family genes (the homologs of CED-9) but is more complex than in *C. elegans* and most data suggest that this family controls caspase activation by regulating mitochondrial membrane permeability and the release in the cytoplasm of proapoptotic factors including cytochrome c, which participate in caspases activation. An extrinsic pathway, which is activated by the binding of ligands on death receptors, activates in some cases caspases in a Bcl-2 independent fashion.

In *Drosophila* the way of action of of Bcl-2 homologs is probably different. No release of mitochondrial proteins during *Drosophila* cell death has been demonstrated. Instead, numerous actors involved in the cell death process migrate from cytoplasm to mitochondria. Furthermore, although numerous homologs of actors of the extrinsic pathway exist in *Drosophila*, the signaling cascade seems quite different.

On the other hand, the genetic approach performed in *Drosophila* has resulted in the discovery of new apoptosis regulators encoded by genes located on the 75C region of the third chromosome. The products of these genes (RPR, HID, GRIM and SICKLE) act through binding to IAPs (Inhibitor of Apoptosis Proteins), disrupting inhibition of caspases managed by IAP. Their action on IAPs is similar to that of mammalian Smac/Diablo and Omi/HtrA2 but, unlike these, RPR, HID, GRIM and SICKLE act upstream or independently of mitochondria. More recently, genetic and genomic tools available in *Drosophila* have also allowed discovering micro RNA (*Bantam* and *mir-14*) that regulates apoptosis.

Finally, homologs of various oncogenes and tumor suppressor genes which control apoptosis have also been identified in *Drosophila*. In this paper, we review the regulation of apoptosis in *Drosophila*, and we discuss the extent to which the data obtained in this organism can be helpful to understand apoptosis in other species.

Introduction

The rapid increase of knowledge about apoptosis mechanisms directly results from the diversity of both approaches and organisms used for this purpose. Genetic studies in *Caenorhabditis elegans* have led to the identification of genes involved in programmed cell death (PCD) [1]. Afterward, it has been found that the mammalian genes of the *bcl-2* family are the homologs of *ced-9* and *egl-1* that regulate PCD in the nematode (Table 1). Furthermore, genes encoding caspases (cysteinyl aspartases) and caspase activators are respectively the homologs of *ced-3* and *ced-4* [2, 3]. In contrast to the situation found in *C. elegans*, in mammals, apoptosis control is mediated by multigenic families containing numerous members such as *bcl-2* and caspase families.

		Nematode	Mammals	Drosophila
	Caspases	CED-3	Casp-9, -3…	DREDD, DRONC, DECAY, drICE…
	Adaptators	CED-4	Apaf-1, FADD…	Dark, DFADD
Bcl-2 family	Antiapoptotic proteins	CED-9	Bcl-2, Bcl-x_L...	Buffy
	Proapoptotic proteins		Bax, Bak, Bok...	Debcl
	BH3 only proteins	EGL-1	Bik, Bad…	?
	Inhibitors of caspases		c-IAP1, c-IAP2…	DIAP1, DIAP2, Deterin
	Disruptors of the anti-caspase activity of IAPs		Smac/Diablo, Omi/HtrA2	RPR, HID, GRIM, SICKLE…

Table I. Conservation during evolution of proteins involved in caspase-dependent cell death.
Three families of proteins involved in apoptosis are found in nematodes, *Drosophila* and mammals: CED-3, -4, -9 are homologous to caspases, caspases activators (like Apoptosis activating factor 1, Apaf-1) and proteins of the Bcl-2 (B Cell Lymphoma 2) family. In *Drosophila* and mammals, caspase inhibitors (IAP, Inhibitor of Apoptosis Proteins) and proteins able to abrogate this inhibition also participate in the control of caspase activity (Smac/Diablo, RPR, HID, GRIM…).

Drosophila is one of the most powerful models to study apoptosis. Many genetic tools are available including collections of *Drosophila* lines containing single gene disruptions, gene targeting or the ability to drive gene or RNAi expression in a temporally and spatially regulated manner [4]. Furthermore, the complete annotated sequence of its genome is now available. The aim of the present paper is to focus on the data obtained with *Drosophila* as a model organism.

Caspase-dependent cell death occurs in *Drosophila*

Caspases have been implicated in the execution of apoptosis in *Drosophila melanogaster* by the observation that expression of baculovirus p35 protein, a caspase inhibitor, blocks cell death *in vivo* in *Drosophila* [5]. Furthermore, recently, *Drosophila* caspases have also been involved in autophagic cell death [6, 7], a type of developmental cell death that we will not discuss in this review.

Data from the *Drosophila* genome sequence have permitted to identify seven genes encoding caspases [review: 8]. Among them, 3 procaspases contain a long prodomain and have been classified as apical caspases. STRICA contains a serine and threonine rich prodomain of unknown function. Like the mammalian caspase-8, DREDD harbor a DED domain but is rather involved in the immune response [9, 10]. Like the mammalian caspases -9 and -2, DRONC contains a CARD domain. It is the only fly caspase known to be regulated by the molting hormone ecdysone [11] and RNA interference experiments have suggested that DRONC is important for programmed cell death during embryogenesis [12]. Although in some tissues, effector caspase activation and cell death can occur independently of DRONC [13], it appears as a regulator of cell number in certain developmental cell death pathways and during stress-induced apoptosis [14]. Thus, DRONC could be the functional homologue of mammalian caspase-9 but has also other functions not related to cell death [15].

Four caspases without prodomain and classified as effector caspases have been identified in *Drosophila*: DCP-1, drICE, DAMM and DECAY.

DCP-1 has been shown to be essential to some but not all developmental cell death [16] and to nurse cells apoptosis [17, 18]. Furthermore, studies of DCP-1 expression in the eye tissue show that some apoptotic inducers can activate DCP-1 *in vivo* [19].

DAMM and DECAY show a similar pattern of expression [20, 21]. They are ubiquitously expressed in *Drosophila* embryos during early stages of development, in larval salivary glands and midgut, two tissues which undergo histolysis during larval/pupal metamorphosis, and in adult egg chambers, suggesting that they play a role in developmental programmed cell death.

drICE is expressed at all stages of *Drosophila* development at which PCD can be induced [22] and immunodepletion of drICE from S2 cell lysates have shown that drICE is an essential effector of apoptosis. [23]. Furthermore, drICE cleaves both the *Drosophila* homolog of the caspase-activated DNase (CAD) and its inhibitor (ICAD) permitting the activation of CAD [24]. Thus, involvement of drICE as an effector caspase seems well established.

However, in *Drosophila* as well as in other organisms [25] apoptosis is not the sole type of programmed cell death. Furthermore, caspase activation does not always lead to cell death and, instead, like other components of the apoptotic machinery have also functions [26], and important to other biological process. Indeed, as mentioned above, in addition to its function in cell death, DRONC, the initiator cell death caspase in *Drosophila*, may play a direct or indirect role in cell migration [27], sperm differentiation [28], and compensatory cell proliferation [29]. Elsewhere, DREDD is clearly involved in NFκB-Relish transcription factor activation leading to induction of antibacterial peptide genes.

A pathway of caspases regulation identified in *Drosophila*

In *Drosophila*, the study of genes involved in apoptosis signaling has begun in the 90's with the discovery of the essential role of the 75C chromosomal region in developmental or irradiation-induced programmed cell death [30]. This region contains four genes without known homologs in mammals or nematodes. *rpr* (reaper), *hid* (head involution defective) and *grim* can induce apoptosis alone whereas the more recently identified skl (sickle) seems to act in synergy with *grim* or *rpr* [31-33]. All four proteins display a homology in their N-

terminal domain that is involved in their PCD promoting activity and has been called RHG (Reaper Hid Grim). Cell death induced by these proteins can be blocked by caspase inhibitors like the baculovirus p35 protein or IAPs (Inhibitor of Apoptosis Protein: DIAP1, DIAP2, dBRUCE and Deterin in *Drosophila* [34] or mammalian c-IAP1 and c-IAP2 [35]). DIAP1 inhibits caspases by binding of BIR1 or BIR2 (Baculovirus IAP Repeat) domain to caspases. Since RPR, HID and GRIM are able to alleviate this inhibition by their binding to the BIR domains of DIAP1 [36], it has been proposed that RPR, HID and GRIM act mainly by releasing caspases from the inhibitory action of IAPs and by promoting IAP degradation (Figure 1).

More recent studies have given a precise description of the molecular details involved in DIAP1/caspases interactions. DRONC processing occurs in two steps. The first step appeared to occur continuously even in the absence of an apoptotic signal. The second step was observed only in apoptotic cells indicating that the initial step in DRONC processing occurs continuously in *Drosophila* cells and that DIAP1 is required to prevent excess accumulation of this first form of processed DRONC [37]. BIR2 domain of DIAP1 recognizes a 12-residues sequence in DRONC. Furthermore, the DRONC-binding surface on BIR2 coincides with that required for binding to the N-terminal of RHG proteins [38].

A similar situation has recently been described for the effector caspase drICE. Inhibition of drICE by DIAP1 occurs only after the cleavage of its N-terminal and involves a conserved surface groove on BIR1. RHG proteins use their N-terminal IAP-binding motifs to bind to the same surface groove, hence relieving DIAP-mediated inhibition of drICE [39].

All these data point out that the main way of controlling proapoptotic caspases activation in *Drosophila* is not the same than in *C. elegans* or mammals. Indeed, caspase activation by activators/adaptators is a crucial control point of caspase-dependent apoptosis signaling in nematodes and mammals (CED-4 in nematodes, FADD or Apaf-1 in mammals). In contrast, in *Drosophila*, the continuous inhibition of DRONC activity seems necessary to maintain cell survival [40, 41].

Another IAP-interacting protein that harbors a conserved N-terminal IAP-binding motif has been identified in *Drosophila*. This protein, called Jafrac2, is a thioredoxin peroxidase that resides in the endoplasmic reticulum but is rapidly released into the cytosol following induction of apoptosis. Similarly to RHG proteins, Jafrac2 displaces DRONC from DIAP1 by competing with DRONC for the binding of DIAP1 suggesting that Jafrac2 triggers cell death by liberating DRONC from DIAP1-mediated inhibition [37].

These results have led to the conclusion that DIAP1 is the principal blockade to abolish in order to activate caspase-mediated cell death in *Drosophila* (Figure 1).

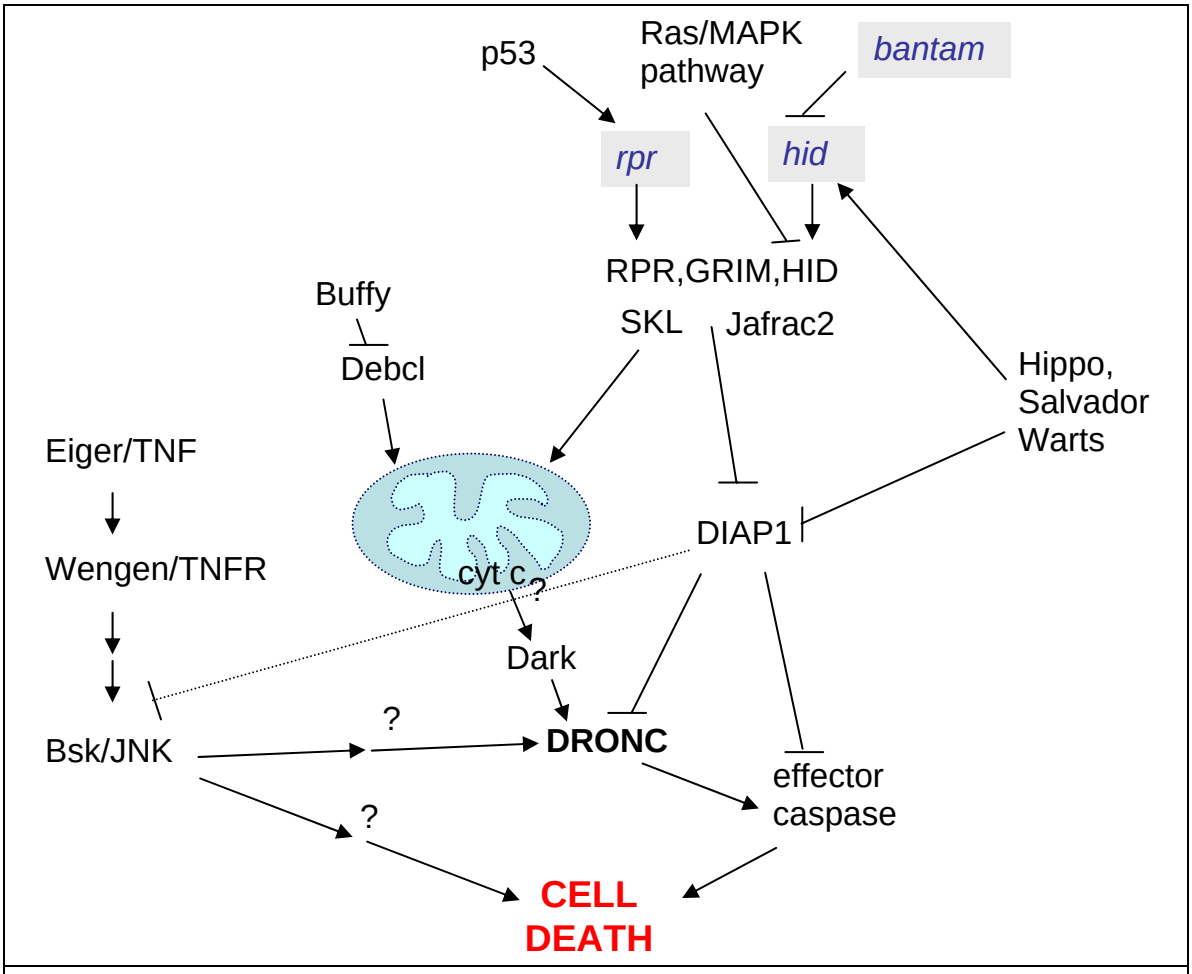

Figure 1. Schematic representation of apoptosis signaling pathways identified in Drosophila.
To survive, *Drosophila* cells must counteract the continuous DARK-mediated processing of DRONC. DIAP1 appears as the major brake able to neutralize this proapoptotic constitutive signal. Thus, it is the key factor to target in order to activate caspase-mediated cell death in *Drosophila*. This survival effect of DIAP1 can be alleviated by several apoptotic signaling. Binding of RHG proteins such as RPR, HID, GRIM or SKL can disrupt the caspase inhibitory action of DIAP1. The endoplasmic reticulum released thioredoxin Jaffrac2 can provide the same effect. Debcl, a proapoptotic member of the Bcl-2 family can stimulate, in a Buffy inhibitable manner, the activation of caspases in a way that could involve mitochondria. RHG proteins could also participate in this mitochondrial pathway. *Bantam* miRNA inhibits apoptosis by downregulating *hid* expression. Tumor suppressor proteins such as p53, Salvador, Warts and Hippo can inhibit cell death by inducing RHG proteins and/or regulating the turn-over of DIAP1. Ras/MAPK pathway controls survival by inhibiting HID. An extrinsic pathway, involving homologs of TNFR, that does not involve a direct activation of caspases, but merely the JNK pathway leads also to cell death in a way that could be inhibited by DIAP1.

IAP proteins are E3 ubiquitin ligases involved in the proteasome pathway

Recently the ubiquitin/proteasome pathway has been implicated in the regulation of apoptosis in mammals and in *Drosophila*. This is one of the main pathways of intracellular proteolysis concerning polyubiquitinated proteins that are degraded by the 26S proteasome.

IAPs carry on their C-terminal end a RING (Really Interesting New Gene) finger domain responsible for a E3 ubiquitin ligase activity. This activity enables IAPs to ubiquitinate caspases bound on their BIR domain [42] and then directs them to the 26S proteasome. Furthermore, as shown in Figure 2, DIAP1 can be auto-ubiquitinated in a UbcD1 E2 conjugase-dependent way under stimulation of apoptosis inducers of the RHG group [43].

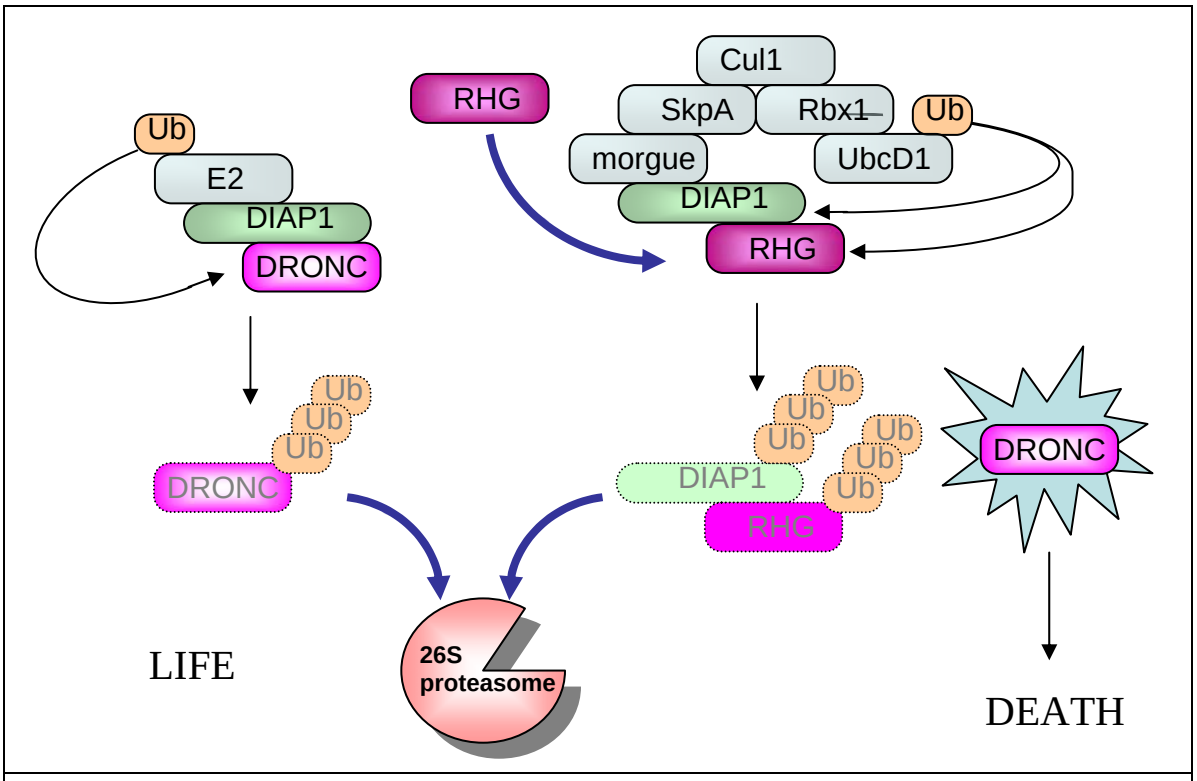

Figure 2. IAP proteins are E3 ubiquitin ligases controlling caspase activation.
In a living cell, DRONC is continuously inhibited and targeted to proteasome by DIAP1 which has a E3 ubiquitin ligase activity, thus mediating DRONC degradation. Upon a death signal, RHG proteins can cooperate with Morgue and a SCF complex to ubiquitinate DIAP1 and the RHG protein. This releases DRONC and promotes caspase activation.

Moreover, a genetic screen of modifiers of a chimeric RPR/GRIM protein-induced cell death has enabled the discovery of Morgue (Modifier of Rpr Grim, Ubiquitously Expressed), an F-box protein with a non-functional E2 conjugase domain. F-box proteins are adapter proteins able to recruit a specific substrate protein in a multimeric E3 called SCF (SkpA-Cul1-F-box protein) complex. In the presence of the death inducer RPR, Morgue stimulates DIAP1 ubiquitination. Indeed, DIAP1 has a short half-life and *morgue* mutants present high levels of DIAP1. Thus, Morgue is also implicated in regulating the turn-over of DIAP1. These studies have also pointed out the roles of Uba1, an E1 ubiquitin activating enzyme, SkpA, a component of the SCF complex and Fat Facets, a deubiquitinating enzyme, in Morgue-induced targeting of DIAP1 to the proteasome [44, 45]. Thus, ubiquitin/proteasome-mediated destruction of DIAP1 is a key event during the initiation of caspase-mediated apoptosis in *Drosophila* [review: 46].

In addition, flies also possess an apoptosis signaling pathway related to the Jun kinase pathway of mammals. Wengen is a *Drosophila* type III membrane protein with conserved cysteine-rich residues in the extracellular domain, a hallmark of the TNFR superfamily (Figure 1). Wengen can bind to the *Drosophila* homolog of TRAF2 (TNF Receptor-Associated Factor 2) [47] and act as a receptor of Eiger [48], a type II transmembrane protein with a C-terminal TNF homology domain which induces cell death through a process which completely depends on its ability to activate the JNK pathway but may not require new transcripts controlled by Jun [49]. Although this cell death process is blocked by DIAP1, it does not require the activity of the caspase-8 homolog DREDD [50]. Indeed, DIAP1 can also modulate cell death by inhibiting JNK activation through the ubiquitin/proteasome pathway and the binding of RHG proteins to DIAP1 can also activate this pathway [51].

Mitochondria and apoptosis in *Drosophila*

In *C. elegans*, mitochondria seem to play a minor role in apoptosis. In contrast, in mammals, proapoptotic members of the Bcl-2 family like Bax trigger the release of various key proapoptotic factors from the inter-membrane space of mitochondria (review: [52] and Vandenabeele, this book). These factors include cytochrome c, Apoptosis Inducing Factor (AIF), Endonuclease G, Smac/DIABLO (Second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI), the serine protease Omi/HtrA2 and several caspases. Once released in the cytoplasm, cytochrome c binds to the WD40 domain of Apaf-1 (that is missing in *C. elegans*) and leads to the formation of a cytochrome c/Apaf-1/caspase 9 complex called "apoptosome" in which caspase-9 is auto-activated and then initiates a caspases cascade. Antiapoptotic members like Bcl-2 inhibit this release and apoptosis induced by Bax or many other death stimuli [53].

The role of mitochondria in *Drosophila* programmed cell death remains elusive [review: 54]. Most data show that cytochrome c is not released from mitochondria during apoptosis *in vivo*. However, some studies suggest that cytochrome c might regulate apoptosis without being released in cytoplasm [55-57]. Interestingly, the *Drosophila* proapoptotic Bcl-2 homolog Debcl (Death Executioner Bcl-2 Homologue), which localizes to mitochondria, triggers cytochrome c release and apoptosis when expressed in mammalian cells, while in *Drosophila* cells, it triggers the translocation of DRONC to mitochondria and caspase activation without cytochrome c release, suggesting that

cytochrome c might participate in apoptosis without being released from mitochondria. However, a very recent study shows in a cell-free system that recombinant *Drosophila* cytochrome c DC3 or DC4 (the products of the two cytochrome c genes found in *Drosophila)* failed to activate caspases in *Drosophila* cell lysates, but induced caspase activation in human cell extracts [58]. Furthermore, silencing expression of either or both DC3 and DC4 had no effect on apoptosis or activation of DRONC and drICE in *Drosophila* cells suggesting that caspase activation occurs independently of cytochrome c in these cells [58].

However, mammalian Bcl-2 inhibits both developmental and radiation-induced cell death *in vivo* [59]. Furthermore, *rpr*-induced apoptosis in *Drosophila* is inhibited by Bcl-2 and, as observed in mammals, this inhibition is associated with the preservation of mitochondrial homeostasis [60]. Interestingly, mitochondria seem to be targeted by RHG domain proteins. Although the three genes *rpr*, *hid* and *grim* are unknown in mammals, they are functional and their action is associated with their mitochondrial localization *in cellulo* and/or cytochrome c release in a vertebrate acellular essays [61-63].

Furthermore, the study of RHG proteins has revealed a functional role for these proteins at the mitochondrial level in *Drosophila*. In addition to their N-terminal RHG domain, RPR, GRIM and SKL contain an internal domain able to target them to the mitochondria. First identified in the third helix of GRIM, this domain has been called GH3 (Grim Helix 3) [64]. It is predicted to be structured in amphipathic alpha-helixes with one hydrophobic and one hydrophilic face and has been found also in RPR and SKL.

During apoptosis induction, Grim relocalizes to mitochondria in a GH3-dependent but N-terminal- and caspase activity-independent manner. *In vivo*, the RHG and the GH3 domains cooperate for apoptosis induction. Indeed, RHG and GH3 domains of GRIM can activate independently apoptosis and synergize to induce programmed cell death efficiently [64]. The role of the GH3 domain has also been studied in mammalian cells. In this system, the GH3 domain is essential for Grim proapoptotic activity, whereas the RHG domain is dispensable. The GH3 domain is required and sufficient for Grim targeting to mitochondria and for cytochrome c release in a Bax, Bak, caspase- and IAP-insensitive manner [65].

The C-terminal mitochondrial targeting sequence of Bcl-xL can alleviate GH3 deficiency [66]. Furthermore, the C-terminal domain of HID, although not containing a GH3, is somehow similar to the C-terminal mitochondrial targeting sequence of Bcl-xL and can substitute to the GH3 of RPR. Studies of RPR also revealed that its GH3 domain is involved in its mitochondrial localization, the induction of IAP degradation and cell killing [66]. These data suggest that IAP destabilization by RPR and probably by HID requires their localization to mitochondria.

All these data strongly suggest that mitochondria play an important role in apoptosis signaling in *Drosophila* even if further investigations are needed to understand their exact contribution.

Drosophila death adaptor proteins: Dark and dFADD, the homologs of Apaf-1/Ced-4 and FADD

An homolog of the Apaf-1/Ced-4 protein, called DARK, HAC-1 or Dapaf-1, has been identified [67-69]. Study of Dark mutants and gene silencing by RNAi showed that this gene is required for normal developmental apoptosis [67, 68]. Furthermore, Dark

cooperates with RHG proteins to induce cell death in the eye [68, 69]. Numerous data suggest that Dark is responsible for the basal level of DRONC activation [41]. However, the molecular details of caspase activation by Dark remain elusive [70]. Some data suggest that two isoforms of Dark can be produced by alternative splicing [67]. A short isoform, structurally related to CED-4 could induce caspase activation and cell death autonomously as CED-4 does in *C. elegans* [71]. However, the most abundant isoform contains an additional C-terminal region comprising multiple WD repeats (WD-40 domain) that could mediate protein-protein interactions. This long isoform is in this way similar to mammalian Apaf-1. Indeed, Dark and cytochrome c seem to cooperate with DRONC and drICE in an apoptosome-like complex localized at mitochondria and responsible for the processing of both caspases DRONC and drICE in *Drosophila* cells [57, 67-69, 72].

A *Drosophila* homolog of FADD, called dFADD, that contains a death domain homologous to the mammalian FADD death domain has also been identified [73]. dFADD acts downstream of IMD in the pathway that controls the expression of the antibacterial peptide genes [74, 75]. dFADD contains a death domain homologous to the FADD death domain and binds to DREDD thus enhancing the cell death activity and proteolytic processing of DREDD suggesting the existence of a FADD-like apoptosis pathway in *Drosophila* [73]. However, dFADD is probably not involved in Eiger (the *Drosophila* homolog of TNF)-induced apoptosis. Rather, as mentioned above Eiger seems to induce cell death indirectly by triggering JNK signaling, which, in turn, causes activation of DRONC [50] (Figure 1).

Two *Drosophila* homologs of the Bcl-2/Ced-9 family

Surprisingly, no fly homolog of the *bcl-2*/*ced-9* gene family has been found in genetic screens. Only the analysis of the genome permitted to identify two *Drosophila* members of the *bcl-2* family [76]. *Debcl* (also called *dborg-1*, *dbok* or *drob-1*) is the first studied member and it has been shown to be proapoptotic [77-81]. More recently, the second one, called Buffy (or dBorg-*2*), has been shown to be merely antiapoptotic [82].

The Debcl protein is closer to Bok than to any other mammalian member of the Bcl-2 family. Like Bok, it contains BH1, BH2 and BH3 domains and a putative domain of membrane anchoring. Using RNAi methods, this protein has been shown to be necessary to developmental cell death [79]. Like most members of the family, Debcl is located at intracellular membranes and mostly at the outer mitochondrial membrane both in mammalian and *Drosophila* cells [80, 81]. In mammalian cells [81], but not in *Drosophila* [57], Debcl triggers cytochrome c release. Experiments with mammalian cells has also allowed to show that Debcl-induced cell death is inhibited by Bcl-2 and Bcl-x_L [79] and that Debcl specifically binds to the antiapoptotic members of the Bcl-2 family. However we failed to detect any protection offered by Bcl-2 against Debcl *in vivo* in *Drosophila* (Colin *et al.*, unpublished results). Genetic interactions between *debcl* and other apoptosis regulators suggest that *debcl* and *dark* act in the same apoptotic pathway whereas, RHG proteins would act independently of *debcl* [79]. However, whether or not apoptosis induced by *debcl* is caspase-dependent merits further investigations. It is possible that Debcl, like Bax in mammals, can induce both caspase-dependent and -independent cell death. In this respect, it is noteworthy that mammalian Bax induces caspase-independent apoptosis in *Drosophila* [60].

On the contrary, although Buffy does not contain evident BH4 domain, and is structurally related to Debcl [83], ablation of Buffy by RNA interference leads to ectopic apoptosis, whereas overexpression of Buffy results in the inhibition of developmental and gamma irradiation-induced apoptosis thus exhibiting antiapoptotic properties [82]. Furthermore, Buffy interacts genetically and physically with Debcl to suppress Debcl-induced cell death. Studies of genetic interactions suggest that Buffy acts downstream of RPR, GRIM and HID to block caspase-dependent cell death. In addition, similarly to Bcl-2, Buffy inhibits cell cycle progression [82].

Genetic control of cell death by micro RNAs

Recently, a new way of apoptosis regulation involving the so called micro RNAs (miRNAs) has been pointed out using *Drosophila.* miRNAs are small RNAs encoded by the genome. Transcripted but not translated, they adopt a hairpin structure which undergoes a maturation process that consists in a cleavage by a type III RNAse called Dicer (also involved in RNAi maturation). Maturation produces RNAs of about twenty nucleotides which, once charged in a complex similar to the RISC (RNA-Induced Silencing Complex, involved in RNAi), are able to inhibit translation of target mRNAs. In animals, this process seems to occur through a mechanism that preserves the stability of the mRNA target [84-86]. Recognition is made by partial complementarities with a sequence of the target mRNA 3'UTR which allows one miRNA to inhibit several genes. Hundreds of miRNA genes have been found in diverse animals, and many of these are phylogenetically conserved [85].

Two loci encoding miRNAs involved in apoptosis regulation have been identified in *Drosophila*: *bantam/clochette* and *mir-14* (reviewed in [87]).

bantam has been found in a screen of genes involved in growth regulation [88, 89]. Deletion of this locus gives a dwarf phenotype (*bantam* was the name of a dwarf chicken) due to both a cell proliferation and an apoptosis regulation role of *bantam*. *bantam* stimulates proliferation by driving both G1/S and G2/M transitions and inhibiting apoptosis induced by this overproliferation or by overexpression of E2F/DP [90]. Indeed, five target sequences of *bantam* are in the 3'UTR of the *hid* mRNA and the miRNA of *bantam* can abolish *hid* translation without affecting its mRNA level [90]. The target(s) responsible for the proliferative effect of *bantam*, that should be an inhibitor(s) of proliferation, remains to be identified. Interestingly, *bantam* miRNA expression is controlled by developmental signals.

mir-14 has been identified as a modifier of *rpr*-induced apoptosis in *Drosophila* eye [91]. Indeed, mutated *mir-14* increases *rpr*-induced apoptosis. What is (are) the target(s) of *mir-14*? In agreement with an antiapoptotic effect of *mir-14*, its overexpression inhibits *rpr-*, *hid-*, *grim-* and *dronc*-induced apoptosis. However, target sequences of *mir-14* have not been found in the mRNA of these genes. On the other hand, *mir-14* does not affect apoptosis induced by Strica or Tramtrack (an inhibitor of Ras). Thus, one possibility is that *mir-14* inhibits the *drice* gene that is an effector common to *rpr*, *hid*, *grim* and *dronc.* In agreement with this possibility, *mir-14* mutants exhibit a high level of drICE. However, a direct action of *mir-14* on *drice* remains to be demonstrated.

Apoptosis control by oncoproteins and tumor suppressor proteins in *Drosophila*

Numerous genes involved in tumorigenesis act on cell proliferation and/or apoptosis. Some of them, as *myc* and *e2f* activate both process. As a consequence, proliferating cell are more susceptible to death signals [92], and, in many cases, inactivation of antiapoptotic tumor suppressors like p53 cooperate with oncogene activation in tumorigenic transformation. In *Drosophila*, genes that control both proliferation and apoptosis have also been found. Some of them are homologs of known mammalian oncogenes (*e2f* [93], *myb* [94], *Src* [95] etc…) or tumor suppressor genes (for example l(2)Tid [96]). Noteworthy, *hid* is downregulated by a Ras-dependent survival signaling [97, 98]. Others cancer related genes have been identified through genetic screens performed in the fly. We will focus on two examples of tumor suppressor genes for which links with the apoptotic machinery have been established: DmP53 and Salvador/Hippo/Warts.

A single homolog of the p53 protein, called Dmp53, has been identified in 2000 by three groups [99-101]. The DNA-binding and transactivation domains of this protein are similar to that of its mammalian counterpart. However, it contains a specific N-terminal domain and is notably different in the MDM2 binding motif (a negative regulator of p53 that has not been found in *Drosophila*). As mammalian p53, Dmp53 is not required for developmental apoptosis but is involved in radiation [102, 103] and reactive oxygen species [104] -induced apoptosis. However, may be in relation to a role of Dmp53 in DNA repair, in some circumstances Dmp53 can also protect from DNA damage-induced cell death [105].

The *rpr* and *skl* genes are targets of Dmp53 [99, 102] but apoptosis induced by Dmp53 does not require RPR [106] and is not inhibited by p35 [99, 100], suggesting that, like its *C. elegans* homolog [107] and like the mammalian p53 in some cases [108], Dmp53 could be able to induce an effector caspase-independent cell death pathway. Furthermore, Dmp53, unlike its mammalian counterpart but like *C. elegans* p53 is not involved in the cell cycle arrest observed after irradiation [109]. These observations suggest that cell cycle inhibition by the proteins of the p53 family has appeared after the arthropod/vertebrate dichotomy. The study of Dmp53-induced apoptosis could then provide a model p53-induced apoptosis disconnected from the cell cycle.

Screens for *Drosophila* loss of function mutations that result in tissue overgrowth has allowed to discover new tumor suppressor genes in *Drosophila*. Three interesting genes that promote both cell cycle exit and cell death have been identified, *salvador* (*Sav*, also called *Shar-pei*), *Warts* (also called *LATS*) and *Hippo* (also called *dMST*) [110, 111]. Interestingly, the human orthologs of these genes have been found mutated in some cancer cell lines. These three genes negatively regulate expression of Cyclin E, downregulate the *Drosophila* inhibitor of apoptosis protein DIAP1, and induce *hid* expression. Sav binds to the Warts serine/threonine kinase of the myotonic dystrophy protein kinase family [111]. Sav also binds to a regulatory domain of Hippo that is essential for its function. Since DIAP1 is phosphorylated in a Hippo-dependent manner in *Drosophila* S2 cells and Hippo can phosphorylate DIAP1 *in vitro*, Hippo could promote apoptosis by reducing cellular amounts of DIAP1. In addition, Sav is an unstable protein that is stabilized by Hippo [112]. These results indicate that Hippo, Warts and Sav function together to restrict tissue growth *in vivo* both by favoring cell cycle exit and by inducing cell death. This control of PCD is

done through a reduction of cellular amounts of DIAP1 and induction of *hid* expression [113-116].

Concluding remarks

Mechanisms involved in the control and execution of apoptosis in *Drosophila* exhibit numerous common traits with what exists in mammals. However, conservation during evolution of most of the genes involved in cell death is not associated with the maintenance of the mechanisms involved.

This is true for both the intrinsic and the extrinsic pathway. In mammals, the release from mitochondria of proapoptotic factors including cytochrome c, AIF and Endo G (see the chapter "Proteins released from mitochondria during apoptosis" by P. Vandenabeele) is a decisive event of the intrinsic pathway of cell death, controlled by the Bcl-2 protein family. In contrast, although homologs of Bcl-2 proteins are found in *Drosophila*, no such protein release occurs during *Drosophila* cell death. Rather, numerous actors involved in the cell death process migrate from cytoplasm to mitochondria. Since Debcl can trigger cytochrome c release from mammalian mitochondria, it could be that the ability of some mitochondrial proteins to cooperate with Debcl to permeabilize outer mitochondrial membrane has been acquired during subsequent stage of evolution.

Concerning the extrinsic pathway, although *Drosophila* contains homologs of TNFα, FADD and caspase-8, an evolutionary conserved extrinsic pathway of apoptosis does not seem to function. On the one hand, the homolog of TNF activates the JNK pathway, which in turn activates caspases. On the other hand, the homolog of FADD can function as an activator of the caspase-8 homolog (DREED) but it does not seem to be involved in apoptosis regulation. Therefore, direct activation of effector caspases by the FADD/Caspase-8 pathway is also an evolutionary recent event.

In spite of these dissimilarities, *Drosophila* has been shown to be a powerful model-system to identify cell death regulators. Indeed, identification of proapoptotic regulators acting as disruptors of IAP inhibition of caspases has been possible first in *Drosophila* and functional analogs of these proteins (Smac/Diablo and Omi/HtrA2), although displaying different cellular localization (and relecolization) have subsequently been found in mammals. The power of the genetic approach with this organism let us glimpse that it will provide us much more other advances to our knowledge of apoptosis regulation among the life kingdom.

Acknowledgements

We thank S. Gaumer for his critical reading of the manuscript. Our work was supported in part by grants from the Association pour la Recherche contre le Cancer (#3367) and from the Ligue Contre le Cancer (Comité des Yvelines).

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