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REVIEW

Apoptosis in *Drosophila*: which role for mitochondria?

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Abstract It is now well established that the mitochondrion is a central regulator of mammalian cell apoptosis. However, the importance of this organelle in non-mammalian apoptosis has long been regarded as minor, mainly because of the absence of a crucial role for cytochrome c in caspase activation. Recent results indicate that the control of caspase activation and cell death in *Drosophila* occurs at the mitochondrial level. Numerous proteins, including RHG proteins and proteins of the Bcl-2 family that are key regulators of *Drosophila* apoptosis, constitutively or transiently localize in mitochondria. These proteins participate in the cell death process at different levels such as degradation of Diap1, a Drosophila IAP, production of mitochondrial reactive oxygen species or stimulation of the mitochondrial fission machinery. Here, we review these mitochondrial events that might have their counterpart in human.

Keywords Apoptosis · Drosophila · Mitochondria · Bcl-2 family proteins · RHG proteins · IAP · Mitochondrial dynamics · Reactive oxygen species

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Introduction

The genetic basis of metazoans programmed cell death was first discovered in *Caenorhabditis elegans* but it is now largely recognized that the core of the apoptotic machinery, including caspases, adaptor proteins and B-cell lymphoma 2 (Bcl-2) family members, has been conserved during evolution including in insects and mammals. In most organisms, caspases are the main effectors of apoptosis. In mammals, 18 caspases have been identified [1] while seven have been counted in *Drosophila* [2, 3]. Indeed, *Drosophila* apoptosis has an intermediate complexity between nematodes and mammals [4]. The initiator caspase Dronc and effector caspases Drice and Dcp-1 are, in *Drosophila*, the main caspases involved in apoptotic events. Opposing forces modulate the activity of caspases which are constitutively present in most cells in an inactive zymogen form. Indeed, adaptor proteins such as Dark in *Drosophila* and APAF1 in mammals act positively on initiator caspases while a negative control is provided by the inhibitors of apoptosis protein (IAP) family members. Several strategies are used to control the level of active caspases. However the importance of these various checkpoints on the status of caspases varies depending on the organism.

In mammalian cells, apoptosis can be initiated either by an intrinsic pathway involving mitochondria, or by an extrinsic pathway triggered by death receptors. This extrinsic pathway can, at times, also be dependent on mitochondria (for reviews: [5, 6]). Although these pathways can act independently to activate caspases in some cellular systems, in many cell types there is accurate coordination and cross-talk between these two pathways [7]. Thus, in mammalian cells, mitochondria are the main regulators of many cell death processes [8]. Indeed, many

proteins involved in apoptosis regulation in mammals act at the level of, or are released from, mitochondria. Moreover, mitochondria undergo numerous alterations during apoptosis such as ultra-structural changes [9]. Contrary to this central role of mitochondria in apoptosis that has been established for a long time in mammals, the scientific community has only recently attached importance to mitochondria in apoptosis control in *Drosophila*. However, controversies still remain concerning the involvement of certain players in this mitochondrial death pathway. In this review, we discuss the controversial data and highlight new elements demonstrating the crucial place of mitochondria in *Drosophila* apoptosis.

A role for cytochrome c in caspase activation in *Drosophila*: myth or reality?

In mammalian cells, in response to an apoptotic stimulus, cytochrome c is released from the mitochondrial intermembrane space to the cytosol where it binds the WD40 motifs of the APAF1 adaptor. In the presence of ATP, this interaction leads to APAF1 oligomerization and therefore to pro-caspase 9 recruitment and activation in the apoptosome. The cytochrome c release is an important phenomenon in many cell death processes in vertebrates.

In *Drosophila*, the possible release of cytochrome c during apoptosis remains a controversial point. Cytochrome c is encoded by two genes in *Drosophila*: *cyt-c-d* and *cyt-c-p*. Original studies by Varkey et al., indicate that cytochrome c remains localized to the mitochondria during apoptosis. However, during apoptosis cytochrome c displays an otherwise hidden epitope, suggesting that it acquires an altered conformation [10]. These data suggest that cytochrome c could be released from the mitochondrial intermembrane space but remains associated with the mitochondrial outer membrane. In contrast, a more recent study showed a rapid spreading of cytochrome c in the cytosol of dying cells [11]. Subcellular fractionation experiments also provide conflicting results concerning the possible release of cytochrome c [10–15]. Technical differences in immunostaining or subcellular fractionation protocols may explain the discrepancy in these observations. Nevertheless, these data highlight that cytochrome c release cannot be used as a good indicator of apoptosis initiation in *Drosophila* as it can be in mammalian cells.

As its mammalian counterpart APAF1, Dark has WD40 motifs that allow the APAF1/cytochrome c interaction [14, 16, 17]. Moreover, in the presence of cytochrome c, formation of a high molecular weight complex containing the caspases Dronc and Drice can be observed in cell extracts [13]. Thus, an apoptosome could be formed in the vicinity of mitochondria despite the lack of cytochrome c release in the cytosol. However, in vitro studies indicated that the *Drosophila* apoptosome assembly (consisting of eight Dark molecules) does not require the presence of cytochrome c [18–20]. Furthermore, structural data showed that cytochrome c cannot form a stable complex with Dark [21]. These in vitro studies may not reflect the behavior of proteins in vivo and should be interpreted with caution. However, they strongly suggest that cytochrome c would not play a decisive role in the activation of the Dark adaptor.

What about cytochrome c role in caspase activation? Several studies using cultured cells, indicate that cytochrome c is not required for apoptosis in *Drosophila* [11, 15, 22, 23]. However, a moderate caspase activation was observed after addition of cytochrome c to *Drosophila* cell extracts [14] and the addition of Cyt-c-d and Cyt-c-p recombinant proteins led to a strong activation of caspases in mammalian cell extracts. Genetic data indicate a role for *cyt-c-d* in caspase activation in some developmental contexts, while *cyt-c-p* is required for cellular respiration. Indeed, *cyt-c-d* loss of function leads to spermatid individualization defects that involve an activation of caspase independently of apoptosis [24–27]. Moreover the developmental death of interommatidial cells is delayed by *cyt-c-d* loss of function [28]. It is important to note that *cyt-c-d* is not required for cellular respiration, retina cells differentiation or progress of pupal development [28]. Therefore, the cell death defects observed following *cyt-c-d* loss of function may be due to a direct physiological role of cytochrome c in the activation of caspases. In the end, cytochrome c involvement in caspase activation and apoptosis induction in *Drosophila* remains limited and is still a matter of debate.

In mammals, Omi/HtrA2 is sequestered in the mitochondrial intermembrane space. During apoptosis, mitochondrial outer membrane permeabilization allows its release into the cytosol where it can ensure its apoptotic activity by binding and inhibiting the IAP proteins [12, 29, 30] The *Drosophila* homologue of this protein, dOmi/HtrA2, is located in the mitochondrial intermembrane space in living cells. After an apoptotic stimulus, it is released into the cytosol but it remains close to mitochondria. *dOmi/HtrA2* overexpression triggers cell death both in vitro and in vivo [12, 29, 30]. Conversely, the expression of a RNAi directed against *dOmi/HtrA2* delays caspase activation in response to stress in cultured cells [12]. In addition, the *dOmi/HtrA2* loss of function reduces male germ cells death [31]. These data suggest a conservation of dOmi/HtrA2 pro-apoptotic function in *Drosophila*. However, a more recent study, using a *dOmi* loss of function in vivo, does not highlight a disruption of developmental cell death or stress-induced apoptosis in this genetic background [32]. Nevertheless, this observation

does not exclude a function for dOmi in cell death induction in particular contexts such as the death of male germ cells during development.

As a consequence, cytochrome c and dOmi/HtrA2 do not seem to play a crucial role in the cell death control in *Drosophila*. Therefore, the role of mitochondria in apoptosis has long been neglected in this model organism. In *Drosophila*, caspase activity is mainly inhibited by the anti-apoptotic protein Diap1 which is itself negatively regulated by the RHG family proteins.

Crucial role of RHG proteins mitochondrial localization for their pro-apoptotic function

In *Drosophila*, Diap1 is essential for cell survival. Indeed, *diap1* loss of function leads to massive apoptosis in most tissues during development [33–37]. The Diap1 protein, through its BIR (Baculovirus Inverted Repeat) domains, binds and inhibits caspases [38–41]. Furthermore, Diap1 has an E3 ubiquitin ligase activity that allows the degradation of caspases [42, 43]. Diap1 level can be negatively regulated both at transcriptional level by the Hippo pathway and the CREB binding protein (CBP) and at post-transcriptional level by the RNA binding protein Held out of wing (How) (for review: [44]). This anti-apoptotic factor is also post-translationally negatively regulated by the RHG (Rpr, Hid, Grim) family proteins. The RHG proteins show little similarity but they possess a conserved motif called IBM (IAP Binding Motif) in their N-terminal part (Fig. 1). This IBM sequence is necessary for the RHG proteins to fully ensure their pro-apoptotic function [45–50] by allowing them to bind the Diap1 BIR domains [38, 40, 45, 51–55]. Interestingly, similarly to the RHG proteins, mammalian Smac/DIABLO and Omi/HtrA2 contain this IBM and use it to bind and inhibit IAPs. RHG proteins compete with caspases for Diap1 binding, thus allowing active caspases release. In addition, RHG proteins promote Diap1 ubiquitination and its subsequent degradation. As a consequence, the RHG proteins trigger apoptosis on the one hand by releasing caspases from Diap1 by competitive binding and on the other hand by favoring its degradation. Notably, RHG promoters respond to various developmental or environmental signals controlling apoptosis [56–60]. These genes share regulatory regions containing many enhancer or silencer elements that are the target of various transcription factors [61–63]). For example, in response to irradiation, the Dmp53 transcription factor activates the expression of *rpr*, *skl* and *hid* via the irradiation-responsive enhancer region (IRER) located upstream of *rpr* [64].

Intriguingly, despite the strong conservation of the IBM motif and the role of this domain in the pro-apoptotic function of RHG family members, several studies indicate that Rpr and Grim proteins lacking this domain keep the ability to induce cell death [47, 48, 50, 65–71]. These data suggest that at least another region of Rpr and Grim ensures a pro-apoptotic role. Indeed, a second domain conserved between Rpr, Grim and Sickle was identified [48, 65, 68, 72, 73]. This internal motif corresponding to an α amphipathic helix was named Grim Helix 3 (GH3 domain) [68] or RHG domain 3 (R3 domain) [65] or tryptophan (Trp) block [48] (Fig. 1). This GH3 domain is both required for the pro-apoptotic function of Rpr and Grim and sufficient to induce cell death in vitro [47, 65, 68, 69, 72, 74].

Interestingly, Rpr and Grim localize to mitochondria in a GH3-dependent manner [67–69, 72, 74–76]. Even so, this domain is not a canonical mitochondrial targeting sequence. Freel et al. have shown that the GH3 domain allows Rpr to embed in the outer mitochondrial membrane through interaction of this domain with membrane lipids [74] (Fig. 2). Sandu et al. consider another model in which Hid interaction would be required for Rpr mitochondrial localization [75]. Indeed, as Rpr and Grim, Hid localizes to mitochondria [49, 75]. But unlike the two others, Hid has a mitochondrial targeting sequence in its C-terminal part. Both this sequence and the Cyclin-dependent kinase 7 (Cdk7) protein are required for Hid mitochondrial localization [49, 75, 77]. Hid and Rpr physically interact through the Rpr central helical region which comprises the GH3 domain. Therefore, Rpr would be recruited to the mitochondria thanks to the interaction of its GH3 domain with Hid which possess itself a mitochondrial targeting sequence (Fig. 2).

Fig. 1 Link between RHG structure and their mitochondrial localization. In addition to the IBM N-terminal motif (*in gray*), some members of the RHG family (Rpr, Grim and Sickle) have a helical region called GH3 motif (*in green*). This motif is required for their mitochondrial localization. Hid has a mitochondrial targeting sequence (rich in hydrophobic amino acid) in C-terminal (*in purple*) (Color figure online)

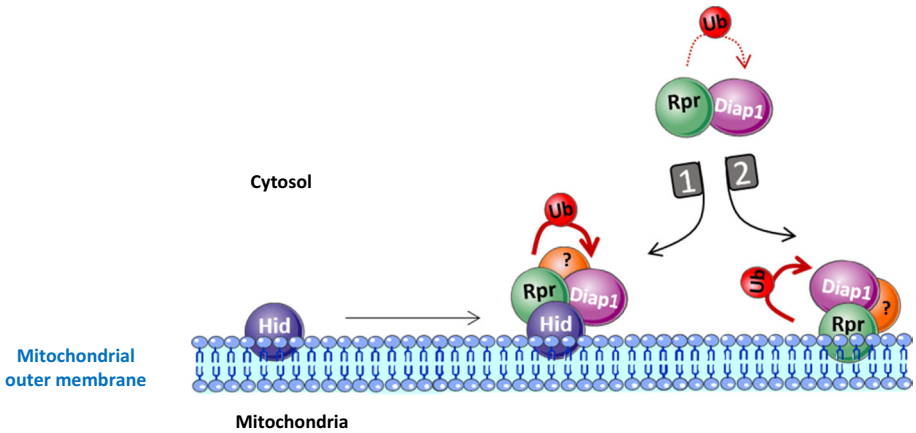

Fig. 2 Model for Rpr mitochondrial localization. Hid possesses in its C-terminal part a sequence which allows its mitochondrial targeting in a Cdk7 dependent manner. There are two models to explain Rpr mitochondrial localization. The first one (*on the left*) shows Rpr mitochondrial recruitment through interaction with Hid which is itself mitochondrial, and potential other factors (*in orange*). The second (*on the right*) shows Rpr direct interaction with the mitochondrial membrane lipids. This interaction allows Rpr integration in the mitochondrial outer membrane. Whatever the recruitment model, Rpr mitochondrial localization increases its ability to stimulate Diap1 ubiquitination (Ub) (Color figure online)

Several studies indicate that the mitochondrial localization of RHG proteins is important for their pro-apoptotic function. $Rpr^{\Delta GH3}$, a form of Rpr devoid of the GH3 domain that is no more mitochondrially localized, has a reduced ability to induce cell death [72]. Interestingly, $Rpr^{\Delta GH3}$ is unable to stimulate the ubiquitination and the degradation of Diap1 although it retains the ability to bind IAPs [72]. However, the addition of Hid mitochondrial targeting sequence to $Rpr^{\Delta GH3}$ is sufficient to restore the Diap1 degradation and an efficient cell death induction [72]. These data indicate that Rpr-induced Diap1 degradation is favored by the mitochondrial localization of Rpr. In addition, Hid, which is involved in Rpr mitochondrial localization, is required for an efficient stimulation of Diap1 ubiquitination by Rpr [75]. Hermann Steller and his collaborators have proposed that the main role of Hid would be to assemble a macromolecular complex in mitochondria in order to recruit Rpr [75]. Within this complex, Rpr would induce Diap1 ubiquitination more efficiently in mitochondria, because of the local concentration of various apoptosis regulators. In particular, it was shown that Dronc and Drice localize to mitochondria [13]. Rpr could therefore be part of a large complex located in mitochondria which regulates the stability of Diap1 and caspase activation. This dependence between Diap1 inhibition and mitochondrial localization is not true in the case of Grim. Indeed, IBM and GH3 domains of Grim activate two distinct cell death pathways, the IBM triggering Diap1 inhibition, while the GH3 acting in a mitochondrial death pathway that remains to be characterized [68]. These two pathways may cooperate in vivo to efficiently induce cell death [68].

Beyond the mitochondrial localization of Rpr, Hid and Grim, other data support the existence of a mitochondrial death pathway downstream of these pro-apoptotic proteins. Indeed, Rpr, Grim and Hid trigger a permeabilization of mitochondria in *Drosophila* cells, mammalian cells or Xenopus egg extracts [11, 67, 69, 71, 78–80]. In *Drosophila* embryos, a RHG-dependent mitochondrial permeabilization seems to occur during DNA damage-induced apoptosis [11]. Furthermore, *rpr* overexpression leads to mitochondrial defects, such as a loss of the mitochondrial membrane potential. Interestingly, this phenomenon can be counteracted by ectopic over-expression of Bcl-2 [81], a mammalian anti-apoptotic member of the Bcl-2 family. It is interesting to note that the GH3 domain (of RHG proteins) and the BH3 domain (actually a motif present in Bcl-2 family members [82]) share a similar alpha amphipathic helix structure. Furthermore, replacement of the BH3 domain of Bad (a pro-apoptotic BH3 only protein, see below) by the GH3 domain of Grim preserves not only Bad mitochondrial localization but also its ability to induce both cytochrome c release and death of mammalian cells. Taken together, these data might indicate that fly RHG proteins could in some way control a mitochondrial death pathway like mammalian Bcl-2 family members do.

Bcl-2 family proteins function during apoptosis in *Drosophila*

In mammalian cells, permeabilization of the mitochondrial outer membrane is tightly controlled by Bcl-2 family proteins. Members of this family are characterized by the

presence of one to four Bcl-2 homology (BH) domains. Structurally, there are two sub-groups: multi-domain proteins (three to four BH domains) and BH3-only proteins which have only the BH3 domain [83]. BH3-only proteins are pro-apoptotic while the subset of multi-domain proteins includes both anti-apoptotic factors (such as Bcl-2) and pro-apoptotic ones (like Bax or Bak). Members of the Bcl-2 family are key regulators of cell death in mammals. Given the minor role of cytochrome c in *Drosophila* apoptosis, we can wonder about the role of Bcl-2 family proteins in cell death regulation in this model organism.

Two Bcl-2 family members have been identified in *Drosophila*: Buffy and Debcl [84–88]. These two proteins have three BH domains (BH1 to 3) and a hydrophobic domain at the C-terminal allowing their anchoring in membranes. Debcl displays mitochondrial localization and Buffy is found both in mitochondria and the endoplasmic reticulum [86–89]. In the literature, there are divergent data on the existence of a BH4 domain in Debcl, so it is called "weak" BH4 domain [90]. *debcl* and *buffy* have a similar dynamic expression profile throughout all developmental stages [84, 86, 87] but little is known about the regulation of their transcription. One study showed that the transcription factor nuclear factor Y-box B (NF-YB) binds *debcl* promoter region to regulate its expression, at least in cultured cells [91]. Beyond this transcriptional regulation, Debcl protein level is regulated by the ubiquitine/proteasome pathway. Indeed, the β-TrCP homologue Slimb interacts with Debcl and targets it to the proteasome [92]. Moreover, we recently showed that Rbf1 (the *Drosophila* homologue of the pRb tumor suppressor protein) cooperates with the transcription factor dE2F2 to repress *buffy* transcription during wing imaginal disc development [93]. Interestingly, *buffy* and *debcl* expression is correlated with the cell death profile in the developing embryo [85, 87], suggesting a role for these genes in *Drosophila* apoptosis.

Ectopic expression of *debcl* induces cell death in various cultured cell models both from *Drosophila* or other species and in different tissues in vivo during *Drosophila* development [13, 15, 84–89, 92, 94–99] (Tables 1 and 2). Conversely, overexpression of *buffy* counteracts the cell death induced by ectopic expression of *rpr*, *hid* or *grim* in the eye [87]. These data suggest that Debcl is pro-apoptotic whereas Buffy ensures anti-apoptotic functions. Actually, Debcl and Buffy have both pro- and anti-survival properties. For example, it has been shown that *debcl* expression protects neurons from polyglutamine proteins toxicity, whereas *buffy* expression promotes this neuronal degeneration [100]. In addition, *buffy* expression induces apoptosis in *Drosophila* BG2 cells [89] while *debcl* protects these cells from serum deprivation-induced cell death [84]. This dual role of Bcl-2 family proteins is not restricted to *Drosophila* [101]. Indeed, in the nematode, CED-9 carries pro- or anti-apoptotic functions. Likewise, Bax and Bak which are pro-apoptotic proteins in mammals, may, in some cases, promote survival. Whatever the species, the ability of Bcl-2 family proteins to promote or rather inhibit apoptosis probably depends on the cell context.

debcl or *buffy* homozygous mutants are viable, fertile and develop normally without apparent defect of developmental program cell death [102]. Therefore, Debcl and Buffy do not seem essential for most developmental cell death processes. However, their function was revealed to be required specifically in certain tissues. Debcl and Buffy are required to induce the death of germ cells during oogenesis and spermatogenesis [31, 103, 104]. Furthermore, *buffy* is required for glial cells apoptosis in the microchaete lineage [105]. Therefore, Debcl and Buffy role in the control of developmental cell death is limited to certain tissues and developmental stages. Additionally, these proteins have a role in the DNA damage response. Indeed, Debcl and Buffy modulate the embryonic apoptotic response to irradiation. *debcl* mutant embryos have fewer apoptotic cells in response to irradiation compared to control embryos, whereas *buffy* mutant embryos exhibit a slight increase of the number of apoptotic cells [102]. Moreover, *debcl* loss of function animals were slightly compromised for viability after genotoxic stress [106]. Otherwise, Debcl plays a crucial role in Rbf1-induced apoptosis whereas Buffy protects against this cell death [93, 94]. In addition, Buffy and Debcl regulate autophagy in response to amino acids deprivation [107] and *buffy* is required for the nutrient stress response at the larval stage [108]. Therefore, although the role of *Drosophila* Bcl-2 family members in the control of developmental cell death appears minor, these proteins seem to play a major role in the apoptosis induced by stress or by a tumor suppressor protein homolog such as Rbf1.

Debcl physically interacts with Buffy [87]. Thus, it is likely that these two proteins act in a manner analogous to that of their mammalian counterparts by binding and neutralizing each other. Genetic data are consistent with this idea. The overexpression of *buffy* inhibits Debcl-induced cell death both in the wing and in the eye [87]. Likewise, *buffy* overexpression attenuates the dendritic phenotype induced by *debcl* overexpression [109]. Moreover, in response to polyglutamine proteins Buffy inactivates Debcl to induce neuronal degeneration [100]. Finally, the use of *buffy* and *debcl* double mutant embryo showed an antagonistic action of these two genes in irradiation-induced cell death [102]. However, Buffy and Debcl do not always ensure opposite roles. Indeed, since both *buffy* and *debcl* mutant show a striking defect in cell death in the ovary, they can, in this case, cooperate to induce cell death [31, 103].

Table 1 Summary of Debcl pro and anti-survival functions

debcl pro-cell death functions			
debcl triggers cell death when ectopically expressed in cultured cells	In *Drosophila* cells	Zimmerman et al. 2002	[12]
		Colussi et al. 2000	[71]
		Igaki et al. 2000	[72]
		Dorstyn et al. 2002	[10]
		Doumanis et al. 2007	[75]
	In chinese hamster ovary cells	Brachmann et al. 2000	[70]
	In mammalian cells	Colussi et al. 2000	[71]
		Zhang et al. 2000	[74]
	In insect cells (other than *Drosophila*)	Zhang et al. 2000	[74]
debcl triggers cell death when ectopically expressed in various tissues during *Drosophila* development	In embryos	Brachmann et al. 2000	[70]
	during eye development	Brachmann et al. 2000	[70]
		Igaki et al. 2000	[72]
		Kanda et al. 2011	[84]
		Park et al. 2010	[83]
		Copeland et al. 2007	[86]
		Quinn et al. 2003	[73]
	During wing development	Quinn et al. 2003	[73]
		Grusche et al. 2011	[85]
		Colin et al. 2014	[78]
		Colin et al. 2015	[81]
		Clavier et al. 2015	[80]
	In larval brain	Brachmann et al. 2000	[70]
	In salivary glands	Brachmann et al. 2000	[70]
debcl is required for developmental cell death	At embryonic stages	Brachmann et al. 2000	[70]
		Galindo et al. 2009	[93]
		Senoo-Matsuda et al. 2005	[87]
		Colussi et al. 2000	[71]
	During oogenesis	Tanner et al. 2011	[90]
	During spermatogenesis	Yacobi-Sharon et al. 2013	[28]
debcl is required for DNA damage induced cell death	*debcl* expression enhances irradiation induced cell death during eye development	Brachmann et al. 2000	[70]
	debcl is required for irradiation induced cell death at embryonic stage	Sevrioukov et al. 2007	[89]
debcl is required for tumor suppressor induced cell death	*debcl* is required for Rbf1-induced cell death	Clavier et al. 2015	[80]
debcl pro-survival functions			
debcl counteracts CED-3 induced cell death in *Drosophila* cells		Brachmann et al. 2000	[70]
debcl promotes survival following serum deprivation		Brachmann et al. 2000	[70]
debcl knockdown enhances respiratory inhibitor-induced cell death		Senoo-Matsuda et al. 2005	[87]
ectopic expression of *debcl* suppresses the neurodegeneration caused by expanded polyglutamine		Senoo-Matsuda et al. 2005	[87]

Until recently, the molecular basis of Debcl pro-apoptotic activity was poorly described and data in the literature were often contradictory. Indeed, depending on the study, the cell death induced by *debcl* ectopic expression is totally, partially or not at all inhibited by a caspase inhibitor [15, 84–86, 88]. These data suggest that Debcl induces cell death by pathways dependent and independent of caspases. Similarly, Dark involvement in Debcl-induced cell death is a debated topic: if two studies agree that Dark is required for Debcl-induced apoptosis in vivo [85, 106], a third study performed in *Drosophila* cultured cells rejects its involvement [15]. Similarly, depending on the publications, the BH3 domain of Debcl can be described as crucial or not important for its ability to trigger apoptosis

Table 2 Summary of Buffy pro and anti-survival functions

Buffy pro-cell death functions			
Buffy is required for cell death	Induced by a respiratory inhibitor in *Drosophila* cells	Senoo-Matsuda et al. 2005	[87]
	Of germ cells during spermatogenesis	Yacobi-Sharon et al. 2013	[28]
	Of germ cells during oogenesis	Tanner et al. 2011	[90]
	Of glial cells in microchaete lineage	Wu et al. 2010	[92]
	Induced by *grim* expression during eye development		
Ectopic expression of *buffy* enhances the neurodegeneration caused by expanded polyglutamine		Senoo-Matsuda et al. 2005	[87]
Buffy ectopic expression triggers apoptosis in *Drosophila* cells		Doumanis et al. 2007	[75]
Buffy pro-survival functions			
Buffy protects against cell death	Induced by irradiation both in embryos and wing imaginal discs	Quinn et al. 2003	[73]
	Induced by *diap1* loss of function during embryonic development		
	Induced by *rpr* or *hid* expression		
	Induced by *grim* expression	Quinn et al. 2003	[73]
		Sevrioukov et al. 2007	[89]
	Induced by *rbf1* expression	Clavier et al. 2014	[79]
		Clavier et al. 2015	[80]
Buffy loss of function enhances irradiation-induced apoptosis in embryos		Sevrioukov et al. 2007	[89]
Buffy is required for embryonic cell survival		Quinn et al. 2003	[73]

[85, 88, 89]. Although this has not been systematically tested, it seems that the RHG proteins are not required for Debcl pro-apoptotic properties and vice versa Debcl would not be involved in the pro-apoptotic activity of the RHG proteins. We recently shed light on some aspects of Debcl mechanism of action. We performed a genetic screen in order to identify gene products that modify Debcl-induced cell death. Among the suppressors identified, the Glycerophosphate oxidase-1 participates in Debcl-induced apoptosis by increasing mitochondrial reactive oxygen species (ROS) accumulation [95]. Therefore, our data highlight that mitochondrial oxidative stress takes an important place in Debcl-induced cell death. More precisely, we showed that this oxidative stress was linked to impaired mitochondrial dynamics [94]. Indeed there are close links between Bcl-2 family members and mitochondrial dynamics as discussed in the following paragraph.

In summary, some controversies remain about the precise function of Bcl-2 family proteins in *Drosophila* cell death. Further in vivo studies are required to fully characterize the role of these proteins in *Drosophila* apoptosis. It is possible that, in *Drosophila*, Debcl, Buffy and the RHG proteins could jointly control a mitochondrial death pathway and ensure together a comparable function to the one played by Bcl-2 family members in mammals. In line with this hypothesis, Grim was shown to physically interact with Buffy and Debcl [105] even if the consequences of this interaction remain to be determined.

Mitochondrial dynamics and apoptosis in *Drosophila*

A mitochondrial fragmentation is observed during apoptosis

Mitochondria network undergoes fragmentation early in the course of cell death in mammals [110]. This fragmentation can also be observed in *Drosophila* cells during apoptosis [11, 79, 94, 106]. This disruption of mitochondrial dynamics occurs in vivo during developmental cell death [79, 106] and in cultured cells after exposure to different apoptotic stimuli like etoposide, cycloheximide or *rpr* and *hid* ectopic expression [11, 76, 79]. Thus, mitochondrial dynamics seems to be altered during apoptosis, both in *Drosophila* and in mammals.

The mitochondrial fragmentation plays a role in the apoptosis induction

The molecules involved in mitochondrial fusion and fission have been identified and are conserved during evolution. Several GTPases of the dynamin family: OPA1 and the mitofusins (Marf and Fuzzy onions in *Drosophila* and Mfn1 and Mfn2 in mammals) are involved in mitochondrial fusion whereas Drp1 is the main effector of mitochondrial fission.

There are several arguments in favor of an active role for mitochondrial fragmentation in the commitment to apoptosis. Firstly, this fragmentation occurs upstream of caspase activation, either in mammalian or *Drosophila* cells [79]. Moreover, whatever the species, inhibition of caspases does not block mitochondrial fragmentation [79, 110]. Conversely, and interestingly, the inhibition of the pro-fission gene *drp1* leads to an inhibition of caspase activation and cell death, again, both in *Drosophila* and mammalian cells [11, 79, 94, 110–114]. This suggests that mitochondrial fission is required for apoptosis. However, these experiments do not exclude the existence of a potential function of Drp1 in the cell death process that would be independent from its role in mitochondrial dynamics. Similarly, both depletion and overexpression of mitofusins modulate the response of cells to apoptosis [76, 111, 115, 116]. It has been demonstrated that Rpr induces mitochondrial fragmentation by binding and inhibiting the pro-fusion protein Marf [76]. *marf o*verexpression induces mitochondrial elongation and counteracts apoptosis induced by either irradiation or *rpr* expression [76]. This suggests that mitochondrial fragmentation would be required for Rpr apoptotic activity.

Relationship between the Bcl-2 family members and mitochondrial dynamics during apoptosis in Drosophila

In mammals, Bcl-2 family proteins modulate the mitochondrial dynamics and this aspect of their activity is important for the regulation of apoptosis [83, 117, 118]. In *Drosophila*, far less is known about Bcl-2 family members activities. Thus, the possible link of these proteins with mitochondrial dynamics remains elusive. A study conducted by John Abrams team indicates that *debcl* loss of function does not induce defects in the organization of mitochondria in living cells [106]. Similarly, mitochondrial fragmentation, which occurs during the developmental cell death of salivary glands, is not changed after *debcl* loss of function [106]. However, *buffy* expression suppresses the mitochondrial changes associated with the apoptosis induced by *pink1* loss of function [119], a gene encoding a mitochondrial kinase whose loss of function in humans is associated with mitochondrial dynamics alteration in Parkinson's disease. Buffy and Debcl are required for the death of nurse cells during oogenesis. In this context, *debcl* or *buffy* loss of function leads to mitochondrial network elongation [103, 104], underlying their ability to affect mitochondrial fusion or fission processes. All these data suggest a link between Bcl-2 family members, mitochondrial dynamics and cell death in *Drosophila*. We recently showed that Debcl and Drp1 can physically interact and that Buffy inhibits this interaction [94]. Notably, Debcl favors Drp1 mitochondrial localization during apoptosis. Moreover, Debcl-induced apoptosis requires Drp1 and involves a mitochondrial fragmentation. This mitochondrial dynamics alteration induced by Debcl through Drp1 triggers an accumulation of ROS which in turn activates the c-Jun N-terminal kinase (JNK) signaling pathway and triggers apoptosis [94]. This evolutionarily conserved kinase cascade has crucial roles in the regulation of cell death in response to many stimuli [120]. Therefore, these results shed light on a link between Bcl-2 family members and mitochondrial dynamics in vivo during cell death in *Drosophila*.

Other ROS related events and mitochondrial factors involved in *Drosophila* apoptosis

Various stimuli can induce an accumulation of mitochondrial ROS leading to apoptosis. However, depending on the inducer, the resulting activated cell death pathway may vary. For example, a null mutation in the *selD* gene causes an impairment of selenoprotein biosynthesis, a ROS burst and an apoptosis driven by the caspase-dependent Drosophila p53 (Dmp53)/Rpr pathway [121]. Dmp53 is able to promote cell apoptosis and also cell division in apoptosis-induced proliferation [122]. Interestingly, there is a cross talk between these JNK and Dmp53 pathways. The normally rapid JNK-dependent apoptotic response to genotoxic stress is significantly delayed in Dmp53 mutants. This suggests that Dmp53 potentiates the JNK-dependent response; however, the mechanism whereby Dmp53 stimulates JNK activity remains undefined [123].

Beyond the RHG proteins and the Bcl-2 family members, other mitochondrial proteins appear to play a role in *Drosophila* apoptosis. For instance, the mitochondrial i-AAA protease mutation causes abnormal mitochondria, increased level of reactive oxygen species (ROS) and leads to a caspase-mediated apoptotic cascade in the photoreceptor cells [124].In particular, loss of function of apoptosis inducing factor (*AIF*) reduces the embryonic cell death leading to the presence of supernumerary cells [125]. Furthermore, dMiro, a GTPase of the mitochondrial outer membrane, is required for caspase activation and cell death induced by the expression of an RNAi against *diap1* in *Drosophila* cultured cells [126]. Moreover, *drosophila* translocator protein (dTSPO), another mitochondrial outer membrane protein is required for apoptosis induced by irradiation or oxidative stress in the larval brain [127]. Thus, a large number of mitochondrial proteins are involved in *Drosophila* apoptosis which reinforces the importance of mitochondria in this death process.

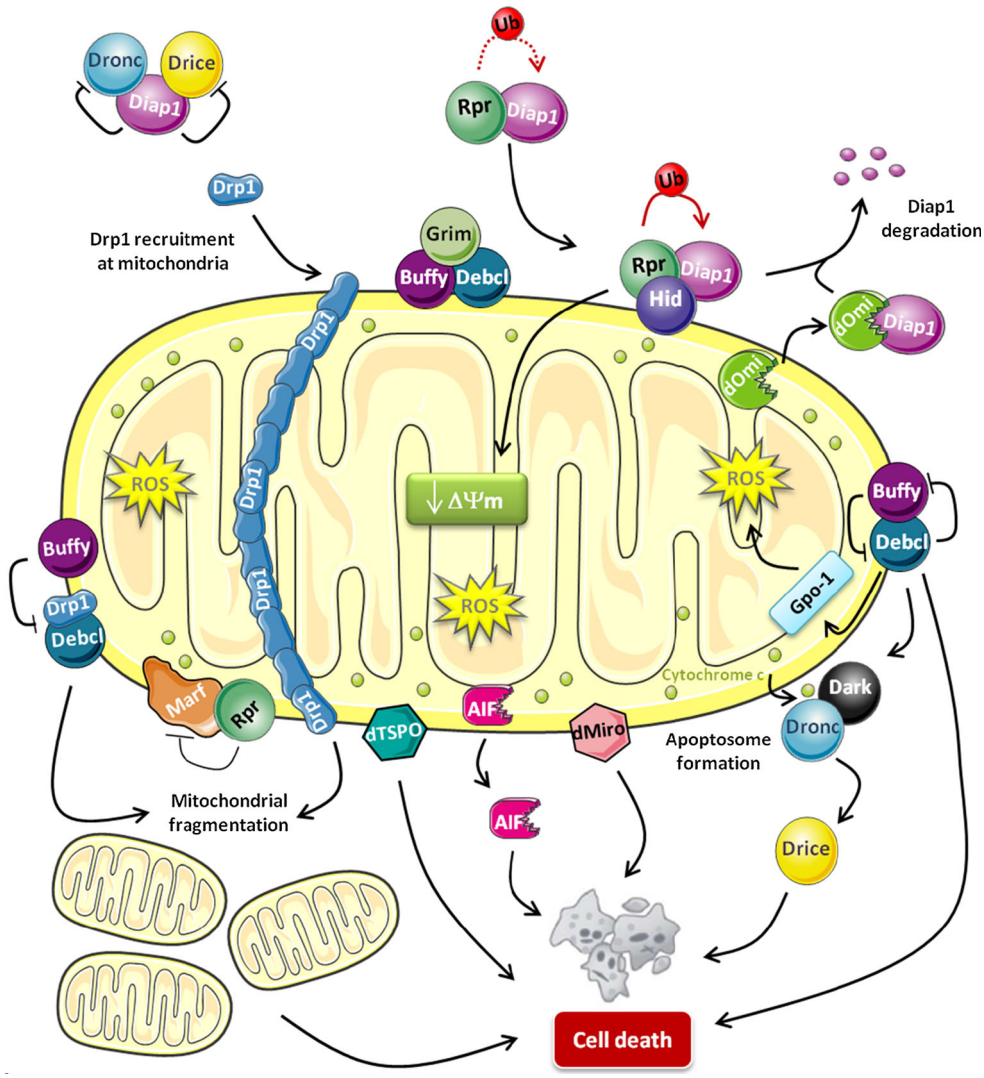

Fig. 3 Mitochondrial cell death pathway in *Drosophila.* In response to an apoptotic stimulus, dOmi/HtrA2 is released from mitochondria. Once in the cytosol, it degrades Diap1 by cleavage. AIF is also released from mitochondria and is involved in cell death induction. Mitochondrial dTSPO and dMiro proteins are localized at the mitochondrial outer membrane and are also involved in the cell death program. Cytochrome c is exposed to the mitochondrial surface in response to apoptotic stimuli. This exposure would promote the formation of an apoptosome near the mitochondria. Debcl and Buffy play a role in stress-induced cell death. Gpo-1 participates in Debcl-induced cell death by increasing ROS accumulation. Debcl and Buffy interact with Grim but the biological significance of this interaction is unknown. Rpr is recruited to mitochondria in response to apoptotic stimuli which increases its ability to induce Diap1 degradation. Rpr can also alter the mitochondrial membrane potential. In addition, Rpr promotes mitochondrial fragmentation by inhibiting pro-fusion protein Marf. Debcl also triggers a mitochondrial fragmentation by interaction with the pro-fission protein Drp1. This mitochondrial fragmentation is required for efficient cell death

Conclusion

Contrary to what foreshadowed by the first studies on *Drosophila* apoptosis, mitochondria have a prominent place in the control of cell death in this model organism as is the case in mammals (Fig. 3). However, unlike what is observed in mammalian cells, in *Drosophila,* few proteins seem to be released from mitochondria during apoptosis, and the existence of a permeabilization of the mitochondrial outer membrane remains today a subject of debate. Nevertheless, several mitochondrial proteins are involved in the death program and some *Drosophila* proteins are recruited to mitochondria in apoptotic conditions. In view of these data, it was proposed that the apoptotic cascade is reversed between *Drosophila* and mammals: in mammals the actors of apoptosis are released from the mitochondria to the cytosol while in *Drosophila* the regulators of apoptosis undergo an inverse relocation: they are concentrated in or near the mitochondria during apoptosis [128]. Moreover, like during mammalian cells apoptosis, a

mitochondrial fragmentation is observed during apoptosis in *Drosophila* and the involvement of the fission/fusion machinery in the mitochondrial death pathway appears to be conserved between these species. One possible hypothesis is that the mitochondria, particularly fission sites, serve as docking sites to recruit and concentrate the proteins involved in cell death [129]. Mitochondria thus appear today in *Drosophila* as major regulators of the cell death process. *Drosophila* proves to be a powerful model system for understanding human diseases involving mitochondrial death pathway, or associated with altered mitochondrial function [129–131]. The apparent discrepancy of the mitochondrial cell death pathways between *Drosophila* and mammals could be due at least in part to the incomplete state of our knowledge. Therefore it appears essential to continue research on the contribution of mitochondria in cell death in *Drosophila*.

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