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Review

Molecular mechanisms of cell death and phagocytosis in *Drosophila*

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Abstract

The genetic tools available in *Drosophila* have facilitated our understanding of how apoptosis is regulated and executed in the context of the developing organism. All embryonic apoptosis is initiated by the activity of three genes, *rpr, grim* and *hid*. Each of these genes is independently regulated, allowing developmental apoptosis to be finely controlled. These initiators in turn activate the core apoptotic machinery, including the caspases. *Drosophila* counterparts to other conserved components of the apoptotic machinery have been recently identified, and we discuss how these may be integrated into the process of normal developmentally regulated cell death. We also outline the role that phagocytosis plays in the final stages of apoptosis and consider the molecular mechanisms guiding the elimination of apoptotic corpses. *Cell Death and Differentiation* (2000) **7**, 1027–1034.

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In the beginning there was genetics

Many of the molecules important in the apoptotic process were first identified by genetic means. Studies in the worm, *C. elegans*, showed that three genes, *ced3, ced4* and *ced9*, formed the core of the apoptotic machinery.[1,2] The cloning of the *ced3* gene led to the identification of the caspases as central effectors of the apoptotic process.[3] Similarly, the Ced4 protein was found to be an important modulator of caspase activity,[4–7] and the Apaf1 protein was recognized as a mammalian Ced4 homologue with similar activity.[8] The third gene, *ced9*, was discovered to be a homologue of a gene already implicated as a regulator of apoptosis in humans, bcl-2.[9]

Human genetics has also contributed to the discovery of genes involved in apoptosis. The bcl-2 gene was first discovered at the breakpoint of a translocation found in B cell lymphomas.[10] Further work demonstrated that cells overexpressing bcl-2 became less susceptible to apoptosis.[11] The tumor suppressor p53 was also found to affect sensitivity to a variety of death inducers.[12,13] The absence of p53, as seen in many tumors, made cells more resistant to death.

These discoveries demonstrated the value of genetics as a tool to investigate the regulation and mechanisms of apoptosis. In the early 1990's, Hermann Steller and colleagues tested the usefulness of *Drosophila* as a genetic model system to study this process. Although apoptosis had been observed to occur during *Drosophila* development, no systematic attempt had been made to identify genes that acted as central regulators of the process. Initial studies demonstrated that apoptosis in flies resembled apoptosis in other systems, both ultrastructurally and biochemically.[14] Based on these findings, a genetic screen was undertaken to identify mutations affecting the apoptotic process.

All genetic screens are based on assumptions as to the selected phenotype. In *C. elegans*, mutations affecting apoptosis were identified as adults lacking apoptotic corpses.[1] Some assumptions of the *C. elegans* screen were that cell death defective mutations would be viable, and that single gene mutations would detectably disrupt apoptosis. Related assumptions were made in the initial screen for *Drosophila* cell death defective mutations.[15] As mutant embryos were screened for perturbations in the patterns of apoptosis, it was assumed that these embryos would live until the stage when apoptosis could be easily assayed. Rather than screening single gene mutations for defects in apoptosis, a collection of genomic deletions was assayed. It was considered likely that any apoptosis mutations detected would be the result of a single gene mutation.

Although the screen was a success, identifying a single genomic region that was essential for all apoptosis during embryogenesis, both of the above assumptions turned out to be incorrect. Mutants in the *thread/dIAP1* gene, an important antiapoptotic regulator, die very early in embryogenesis and were missed in the initial screens.[16–18] Additionally, multiple genes are responsible for the cell death defective phenotype seen in deletions overlapping the genomic region identified in the screen.[15]

After the identification of these deletions, attempts were made to generate single gene mutations that recapitulated the cell death defective phenotype. Several years of work led to the realization that the deletion of more than one gene was responsible for the defect. Eventually, three genes were identified that seem to act in a cooperative manner to regulate apoptosis in the embryo. These three genes, *reaper (rpr), head involution defective (hid)* and *grim*, share a short stretch of homology at their N-terminus, but are otherwise dissimilar.[15,19,20] Deletions that remove only one of these genes have very weak effects on embryonic apoptosis, while the removal of two has stronger effects.[15,19,20]

Reaper, *hid* and *grim* are central regulators of apoptosis

What is the evidence that these three genes act as apoptotic regulators? First, as stated above, deletions that remove combinations of these genes have significant effects on the amount of cell death in the embryo.[15,19,20] Second, each of these genes is capable of inducing apoptosis when ectopically expressed in the developing fly and in both *Drosophila* and mammalian cultured cells.[19–26] Third, the expression of these genes corresponds to patterns of apoptosis. *Rpr* and *grim* appear to be exclusively and specifically expressed in cells that are doomed to die,[15,19] while *hid* is more broadly expressed, both in cells that live and in cells that die.[20] In addition *hid* is not expressed in the central nervous system, a site of significant levels of cell death in the embryo.

Initial characterization of the phenotype of embryos homozygous for the smallest deletion that removes all three genes (H99) suggested that these genes are not the actual effectors of apoptosis. Apoptosis could be generated in these embryos by high doses of x-ray irradiation, indicating that the core apoptotic machinery was intact.[15] Caspase activation was shown to be essential for killing by each of the genes.[19–21] The baculovirus p35 protein, which acts as a broad-spectrum caspase inhibitor, inhibits death induced by the ectopic expression of each gene. The mechanisms by which *rpr, grim* and *hid* activate caspases are not fully understood. *In vitro* binding experiments suggest that at least part of their proapoptotic activity is the result of their ability to bind to and inactivate the antiapoptotic IAP proteins.

One attractive model for the regulation of cell death during development is that when the combined activity of Rpr, Hid and Grim in an individual cell exceeds a threshold, caspases are activated and the cell undergoes apoptosis. The activity of each of these genes is regulated by different upstream regulatory signals (Figure 1). Thus the decision of a cell to die is the integration of several regulatory pathways. Most of these regulatory pathways are currently unknown, but several have been identified and include developmental cues, cell to cell signaling mechanisms and internal sensing programs.

The transcription of *rpr* is clearly directed by a number of developmental cues, as it is expressed in many different cells that are fated to die during development. *Rpr* expression is also upregulated when cells die due to defects in the normal developmental program.[27] The elaborate expression pattern of *rpr* suggests that its promoter may be responsive to a large number of developmental pathways. Responses to individual pathways may be directly integrated at the promoter, or may reflect the activity of a limited number of transcription factors, which in turn integrate several pathways.

Current data indicate that at least two pathways impact directly on the *rpr* promoter: the steroid hormone pathway and the p53 pathway. The expression of *rpr* is directly regulated by the steroid hormone ecdysone.[28] This expression occurs in a number of obsolete larval tissues just prior to the onset of cell death. In addition, intracellular signaling in response to chromosomal damage leads to the expression of *rpr* and the subsequent death of the cell.[27,29] Recent work has shown that the *Drosophila* homologue of p53, *dmp53*, is responsible for this signaling and acts through a p53-response element within the upstream regulatory region of *rpr*.[29,30]

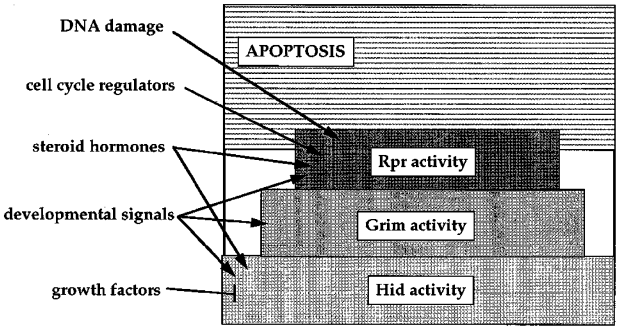

Figure 1 Reaper, Grim and Hid act in a cooperative manner to induce apoptosis. When the combined activity of the three proteins exceeds a threshold, apoptosis is induced. Each of these inducers can be regulated independently. Expression of *rpr* is induced by DNA damage,[29,30] misregulation of the cell cycle,[99] developmental signals[15,27] and steroid hormone levels.[28] *grim* and *hid* expression are also responsive to developmental signals.[19,20] In addition, *hid* activity is negatively regulated by the EGF receptor pathway[31,32]

The regulation of *hid* appears to be somewhat more complex than that of *rpr. hid* expression is observed in a variety of cells that do not undergo apoptosis, although ectopic over-expression of *hid* is a potent inducer of death.[20] The activity of *hid* is modulated by growth factor signaling, acting through the Ras/Raf/MAPK pathway.[31,32] MAPK activity inhibits *hid* both transcriptionally and post-translationally, probably through direct phosphorylation. In the developing eye and in the embryonic midline glia the epidermal growth factor receptor (EGFR) activates this antiapoptotic activity of Ras.[31–37] Thus, cell survival is favored when cells are exposed to high levels of EGFR ligands, and cells undergo *hid*-induced apoptosis in the absence of this activity. Little is known about the regulation of *grim* expression, although it is also transcriptionally regulated during development, and *rpr* and *grim* expression appear quite similar.[19]

Rpr, Grim and Hid kill by inactivating IAPs

While it is clear that programmed cell death in the *Drosophila* embryo is initiated by the death regulators *rpr, grim* and *hid*, and that death itself is mediated through the proteolytic action of caspases, the mechanisms connecting the initiation of apoptosis to caspase activation are less clear. One class of anti-apoptotic molecules that has been shown to play a role in this process is the IAPs, or Inhibitors of Apoptosis Proteins. Originally identified in baculoviruses, the IAPs have been found in virtually all multicellular organisms that have been examined to date.[38,39] IAPs are characterized by a well-conserved BIR domain (Baculovirus IAP Repeat). Many also have a well-conserved RING domain located near the C-terminus of the protein. Both of these structural domains

consist of arrangements of cysteines and histidines that coordinate zinc ions.

In *Drosophila*, three IAPs, *dIAP1, dIAP2* and *deterin*, have been characterized.[40,41] Ectopic expression of either *dIAP1* or *dIAP2* suppresses killing by ectopically expressed *rpr, grim* or *hid* and also partially suppresses normal developmental cell death.[41] When expressed in cultured cells *deterin* suppresses killing by over-expressed *rpr* and by cytotoxic insult.[40]

DIAP1 directly binds to and inhibits a number of *Drosophila* caspases.[42–44] There are five characterized caspases in *Drosophila*. Two of these, Dredd and Dronc, appear to be apical caspases with long prodomains, while Dcp-1, drICE and Decay appear to be effector caspases. The inactive proforms of both Dronc and Dcp-1 are efficiently bound by DIAP1, while binding to drICE appears to occur only following cleavage of this caspase into active subunits. Thus, it seems that at least part of the function of DIAP1 is to complex with and inactivate previously activated caspases (i.e. drICE) or to maintain caspase proforms in their inactive states.

This caspase inhibitory activity is consistent with the observation that homozygous loss of function alleles of the *th/dIAP1* gene are lethal very early in embryogenesis due to massive apoptosis.[16–18] While the protective mechanism of DIAP1 can perhaps be entirely explained by its ability to interact with and inhibit caspases, this has not yet been shown for either DIAP2 or Deterin. It may be that these IAPs, as well as DIAP1, function through an as yet unidentified mechanism. In addition, *Drosophila* IAPs may have functions unrelated to apoptosis, as they do in other systems.[45,46]

When expressed in cultured cells, both DIAP1 and DIAP2 bind to the apoptotic inducers Rpr, Grim and Hid through their BIR domains.[47,48] This has led to the model that Rpr, Grim and Hid kill by binding to and suppressing the protective function of IAPs. This model is strongly supported by the demonstration that DIAP1 directly binds to and inhibits the activity of the caspase DCP-1 and that the binding of Hid abrogates this caspase inhibition.[18] Further support for such a mechanism comes from studies of mutant alleles of the *th/dIAP1* gene which suppress the rough eye phenotype induced by ectopic expression of *rpr* and *hid*. These mutations result in DIAP1 molecules that can no longer be bound by either Rpr or Hid. Thus Rpr and Hid can no longer disrupt DIAP1-mediated inhibition of caspase activity.[16]

As the only conserved regions shared by Rpr, Grim and Hid are contained within the first 14 amino acids of each molecule, it would be reasonable to expect that binding to DIAP1 and the initiation of cell death would be mediated by these domains. This appears to be the case for Hid, as removal of the first 14 amino acids abolishes both the ability of Hid to interact with DIAP1 and to kill cultured cells.[47] Oddly, deletion of the first 15 amino acids from either Rpr or Grim does not prevent either of these molecules from initiating cell death,[49–51] and both N-terminally truncated Grim and Rpr are still able to co-precipitate with DIAP1. (Bangs and White, unpublished observations; and[47]) Coupled with the fact that the N-terminal portion of Grim, like that of Hid, is sufficient to both bind DIAP1 and induce killing, these data indicate that there are at least two domains in Grim that are capable of interacting with DIAP1 and inducing cell death.[47] Further differences in the ways in which Rpr, Grim and Hid interact with DIAP1 are seen with certain gain of function mutations in the *th/dIAP1* gene. One class of mutations suppresses killing by ectopically expressed *rpr* and *grim*, but has no effect on killing by *hid*. The other class of *th/dIAP1* mutations instead suppresses killing by ectopically expressed *hid*, but has no appreciable impact on *rpr* or *grim* killing.[17] One might expect that these mutations would also exhibit differential binding capabilities with respect to Rpr and Grim on the one hand and Hid on the other.

It is important to note that the recently identified SMAC/DIABLO protein in mammalian systems may be a functional homologue of Rpr, Hid and Grim.[52,53] This protein is released from the mitochondria on receipt of a death-inducing signal, and potentiates caspase activation. SMAC/DIABLO has been shown to bind to IAPs, and thus may augment caspase activity by inactivating the caspase-inhibitory function of mammalian IAPs. SMAC/DIABLO does not have any sequence homology to Rpr, Hid and Grim, but it seems to promote apoptosis through the same mechanism.

Caspases are required for Rpr, Grim and Hid killing

Currently, five caspases have been characterized in *Drosophila*, and three others have been predicted based on genomic sequence information. Of these, the caspases Dredd/Dcp-2[54,55] and Dronc[56] have the long N-terminal prodomains consistent with initiator caspases, while Dcp-1[57], drICE[58] and Decay[59] have the shorter prodomains associated with the executioner caspases. In addition, one of the three predicted, but uncharacterized, caspases has a long 'initiator' type prodomain, while the other two appear to be of the short prodomain 'executioner' class.[60]

The caspase Dronc mediates cell death initiated by *rpr, grim* and *hid*, as a reduction in the dosage of Dronc, or expression of a catalytically inactive Dronc, greatly suppresses the eye ablation phenotype due to ectopic *rpr, grim* or *hid* expression.[44,61] Further, ectopically expressed Dronc itself can kill in yeast, cultured cells and cells in the developing *Drosophila* eye.[44,56,61] Dronc effectively cleaves drICE[44,61], as predicted for an apical caspase. Dronc is expressed highly in the embryo, and in tissues that will degenerate in response to the steroid hormone ecdysone. Thus it is expressed at the right time to be involved in apoptosis initiated by *rpr, grim* or *hid*.

Dredd appears to be an apical caspase by virtue of its relatively long prodomain and its association with the ced4/Apaf-1 homologue Ark.[55,62] Reductions in the dosage of *dredd* have a suppressive effect on killing by ectopically expressed *rpr* and *grim*, indicating that Dredd, like Dronc, mediates killing by these death activators. Intriguingly, Dredd may not play a significant role when cell death is initiated by *hid*, as reduction of *dredd* does not appear to appreciably affect killing by ectopically expressed *hid*.

Among the *Drosophila* caspases that have been characterized thus far, only *dredd* expression is linked specifically to cell death, and transcripts accumulate in cells that are specified to die. This accumulation in doomed cells is apparently coupled to the activity of the cell death inducers *rpr, grim* and perhaps *hid*, as embryos homozygous for the H99 deletion, thus lacking *rpr, grim* and *hid*, do not show wild-type accumulation of *dredd* mRNA. The mechanism by which cells accumulate *dredd* mRNA in response to *rpr, grim* and *hid* is presently unknown.

There is some evidence that specific executioner caspases act downstream of individual death inducers, as ectopic expression of *rpr* and *grim* seems to promote the activation of Dcp-1.[63] In addition some caspases may play unique roles in specific developmental processes. In the absence of *dcp-1*, oogenesis is abnormal.[64] However, no mutant alleles have been characterized for many of these caspases. In general, the characterization of *Drosophila* executioner caspases lags significantly behind the mammalian studies.

The mitochondrial pathway

Until recently, no *Drosophila* homologues for either ced4/Apaf-1 or ced9/bcl-2 had been found. The discovery of *Ark*, a ced4/Apaf1 homologue[62,65,66] and *debcl* and *Buffy*, ced9/bcl-2 family members[67–70], has solidified the concept that apoptotic mechanisms are well conserved across species. In both the worm and in mammalian systems, ced4/Apaf1 activation promotes caspase activation.[71] Pro- and anti-apoptotic members of the ced9/bcl2 family modulate this activation. In mammalian systems Apaf1 activity is upregulated by binding to cytochrome *c*, which is released from the mitochondria upon induction of death. This 'mitochondrial pathway' seems to enhance killing by a number of inducers, and may act as an amplifier of an apoptotic signal.

The *Drosophila* ced4/Apaf1 homologue *dark/Dapaf-1/HAC-1* is listed in sequence databases as *Ark*.[62,65,66] Like its counterparts in worms and mammalian cells, Ark has an apparent nucleotide binding site in the N-terminal Ced-4 domain, and like Apaf-1, but distinct from Ced4, the C-terminal domain contains two series of WD repeats. This WD domain seems to act as a negative regulator of Ark function. Cytochrome *c* binding releases this inhibition. An alternatively spliced isoform of Ark lacking this inhibitory WD domain has also been reported, although it is unclear whether it is expressed at significant levels.[65]

Ectopic expression of *Ark* in *Drosophila* S2 cells induces a moderate amount of cell death at best, but expression of a truncated version lacking WD repeats results in a substantial increase in cell killing.[62,65] Genetic evidence indicates that *Ark* is an important component of death pathways activated by *rpr, grim* and *hid*.[62,65,66] The normally robust eye ablation phenotypes observed for ectopically expressed *rpr, grim* and *hid* are substantially suppressed by *Ark* mutations.[62,65] Decreased *Ark* also suppresses death in eyes that ectopically express *dcp-1*.[66] Interestingly, a strong synergistic effect between ectopically expressed *rpr* and *dcp-1* is not suppressed to any significant degree in *Ark* heterozygotes. Taken together, these latter two observations suggest that Dcp-1 activation by Ark and Rpr may occur through independent pathways.

Embryos mutant for *Ark* exhibit reduced cell death, particularly in the CNS and epidermal regions, and the larval nervous systems are substantially larger than wild-type and have excess cells.[62,65,66] Adult flies have a number of relatively mild defects including abnormal wings, a few extra bristles, occasional melanotic tumors and extra photoreceptors and/or pigment cells.[62,65] In addition, approximately 47% of the males homozygous for a null mutation are sterile.[62] These relatively mild and weakly penetrant phenotypes and the observations of at least some developmental cell death in these mutants, indicate that *Ark* is not absolutely necessary for normal cell death to occur, but may instead contribute greatly to the efficiency of the process.

Considering the conservation of the apoptotic machinery from worms to humans, it comes as no surprise that *Drosophila* would have Bcl-2 family members as part of its cell death repertoire. Several laboratories have identified and characterized the proapoptotic Debcl/dBorg-1/dRob-1.[67–69] This protein contains the BH1, BH2 and BH3 domains as well as a C-terminal transmembrane domain that localizes it to intracytoplasmic membranes, primarily the mitochondria. Ectopic expression of *Debcl* increases cell death in a variety of tissues and in the eye produces a rough to severely ablated phenotype in a dose-dependent manner. This phenotype is dramatically suppressed by the caspase inhibitor p35 demonstrating that Debcl functions in a caspase-dependent manner in the fly.[67,69]

In cultured cells, Debcl has a potent killing activity that is conserved across several species. Interestingly, several cell types, including *Drosophila* S2 cells and COS cells are relatively refractory to killing by Debcl, and in these cells Debcl may in fact be protective when expressed at levels that do not induce death.[67] When Debcl does induce killing in S2 cells, caspase activation is also observed.[68] In contrast to what is seen in *Drosophila* eyes, p35 does not appear to prevent killing by Debcl in S2 cells although caspase activation is abolished. This suggests that in some situations, Debcl may induce cell death by both caspase-dependent and caspase-independent mechanisms.

As is the case for other members of the Bcl2 family, the mechanism of Debcl function is unclear. One possibility is that Debcl binds to and neutralizes the activity of anti-apoptotic Bcl2 homologues. This hypothesis, hampered somewhat by the fact that no pro-survival Bcl-2 homologues have yet been reported in *Drosophila*, is supported by the observation that Debcl co-precipitates with a number of the anti-apoptotic Bcl2 family members from other species.[69] Genetic studies show that Debcl interacts with the *Drosophila* Apaf1/ced4 homologue Ark and that reducing the dosage of Ark significantly suppresses the rough eye phenotype caused by ectopic expression of *Debcl*. Debcl also interacts with the apoptotic inhibitor *th/dIAP1* as shown by the fact that when the gene dosage of *th/dIAP1* is halved, the rough eye phenotype produced by *Debcl* is strongly enhanced. This does not appear to be true for interactions between *Debcl* and *dIAP2*. Reducing the dosage of the cell death-inducing genes *rpr, grim* and

hid does not appear to have any effect on killing by *Debcl*, suggesting that the activity of these genes is not rate limiting in Debcl-induced death.[69]

A second *Drosophila* bcl-2 family member, Buffy, has also been reported, although it has not been thoroughly characterized.[67,69,70] Other family members may also be found in the newly completed genome sequence.

How do the mitochondrial pathway and the Rpr, Grim, Hid/DIAP pathway interact?

The mechanisms by which Rpr, Grim and Hid kill are not fully understood, but a model can be made based on the existing data (Figure 2). Assuming that experiments done *in vitro* accurately reflect the situation *in vivo*, it is likely that *rpr, hid* and/or *grim* expression or Hid activation, above a threshold level, initiates a cascade of caspase activity by binding to the protective molecule DIAP1 and disrupting its caspase-inhibitory activity. Given the fact that Dronc is among those caspases that are regulated by DIAP1, it is expected that Dronc activation would occur fairly early in the process, presumably by self-cleavage, and that an active Dronc would subsequently process other caspases as well.[44] Circumstantial evidence suggests that Dredd, whose expression is up-regulated following the expression of *rpr, grim* or *hid*, would be an early target of the active Dronc.[55]

It is unclear whether the Bcl2-related Debcl or the Apaf-1 homologue Ark are absolutely necessary for the preceding scenario to occur or whether they are utilized instead to improve the efficiency or timing of the process. The connection between the cell death regulators Rpr, Grim and Hid on the one hand and Debcl and/or Ark on the other is also murky. One possibility is that the pathways are initiated independently by the death stimuli, and only interact at the level of caspase activation. Another possibility is that the *Drosophila* homologue of *Xenopus* Scythe (CG7546 in the GadFly genome annotation database) connects Rpr, and possibly Grim and Hid to caspase activation by Debcl and Ark. *Xenopus* Scythe has been shown to bind to Rpr.[72] As a consequence of the interaction of Rpr with *Xenopus* Scythe, an unidentified proapoptotic factor is released from Scythe.[73] This unidentified factor possesses a cytochrome *c* releasing activity when combined with mitochondria, and could therefore be responsible for cytochrome *c* dependent activation of an Apaf-1-like molecule in *Xenopus*. A possible scenario is that *Drosophila* Scythe sequesters a proapoptotic factor that is released when one of the cell death activators interacts with Scythe.

Mammalian pro-apoptotic Bcl2-family members such as Bid and Bim are sequestered from mitochondria until activated by cleavage or signaling.[74] Debcl (and Buffy) might also be sequestered in living cells, as they are broadly expressed. It is possible that *Drosophila* Scythe might play a role in this sequestration, or that caspase activation downstream of IAP inactivation might result in the release of the proapoptotic Bcl-2 proteins. These proteins could then mediate either a release of cytochrome *c* from the mitochondria or participate in the altered display of cytochrome *c* observed with mitochondria in the early stages of programmed cell death in *Drosophila*.[75] Once cytochrome *c* was available, cytochrome *c*-dependent interactions between Ark and either Dredd or Dronc could result in the activation of these caspases and an amplification of the caspase cascade.

While the preceding scenario is obviously speculative, it attempts to tie many of the known components of the *Drosophila* apoptotic machinery with the apoptotic pathways of other systems. Clearly, more work needs to be done to reach a decent understanding of the molecular mechanisms that govern and execute this important process. The genetic tools available in *Drosophila* will be invaluable for the identification of both pro- and anti-apoptotic molecules and for the delineation of the apoptotic pathways in the living organism.

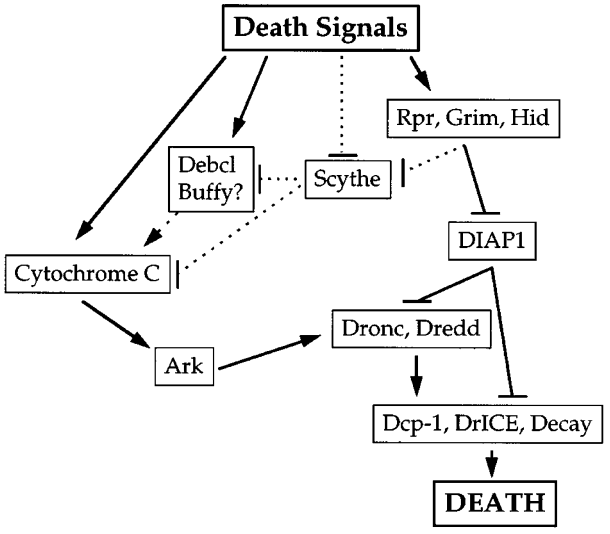

Figure 2 A model for the interactions between the inducers Rpr, Hid and Grim, and the apoptotic effector machinery. The interactions between Rpr, Grim and Hid and DIAP1 and the caspases are supported by experimental data (represented by solid lines, see text). Dotted lines represent purely speculative interactions between these inducers and the mitochondrial/Ark pathway, including the bcl-2 family homologues and the scythe homologue. The pro- or anti-apoptotic activity of Buffy has not yet been reported

Phagocytosis is a crucial final step in apoptosis

Cells that are programmed to die must be cleared rapidly. This is an essential element of the apoptotic program, as persistent corpses may release cytotoxic substances and damage neighboring developing tissues.[76,77] In all multicellular organisms, apoptotic cells are removed through phagocytosis. Phagocytosis is the process by which cells engulf and digest large particles that they recognize as 'non-self' or effete self. In vertebrates, phagocytosis can be carried out either by 'professional' or by 'non-professional' phagocytes, i.e. by cells whose major function is engulfment or by cells with other functions that are capable of engulfment.[78] In *C. elegans*, apoptotic corpses are phagocytosed by neighboring cells.[79] In the *Drosophila* embryo there is some evidence of phagocy-

tosis by glial cells.[80] However, the most efficient embryonic phagocytes are professional 'macrophages', the phagocytic hemocytes or blood cells.

Drosophila embryonic macrophage precursors originate from the procephalic mesoderm approximately 2 h after gastrulation.[81] These 40 precursors give rise to approximately 700 hemocytes. This complement of hemocytes remains constant throughout embryogenesis, independent of the amount of apoptosis in the embryo. Emerging hemocytes are scattered round or irregularly shaped cells that migrate freely in the hemocoel (plasma) and spread throughout the embryo.

Most hemocytes become phagocytic macrophages as they encounter apoptotic cells.[81] Recent work has shown that there are in fact two major classes of hemocytes in the embryo: the macrophage lineage (also called plasmatocytes), and the crystal cell lineage.[82] Crystal cells participate in the sequestration of large particles by melanization.[83] It is interesting to note that the differentiation of these lineages requires the activity of specific transcription factors, similar to those in mammalian systems. The GATA homologue *serpent* is required for all hemocyte differentiation[84], while the AML1 homologue *Lozenge* is required for the crystal cell lineage.[82] The *gcm* transcription factor plays a role in macrophage differentiation.[85]

The macrophage lineage can be distinguished by the expression of Peroxidasin, an extracellular matrix protein produced by these cells.[86] By the end of embryogenesis approximately 90–95% of the Peroxidasin-positive hemocytes are phagocytic.[81] This number can rise to 100% in the presence of excess apoptosis, suggesting that the presence of apoptotic corpses might be important for the terminal differentiation of macrophages and/or to trigger their phagocytic activity. Although macrophage-induced apoptosis is evident in the developing mouse eye,[87] there is no evidence that *Drosophila* macrophages play an active role in apoptosis. Indeed apoptosis seems to proceed normally in mutants that lack all head mesoderm and are devoid of hemocytes.[81]

A critical step in phagocytosis is the recognition of the target to be engulfed. This requires receptors that recognize specific biochemical patterns at the surface of the target.[88] Croquemort (Crq), a *Drosophila* member of the CD36 family of receptors, is one such receptor.[89] Crq is expressed exclusively on macrophages during embryogenesis. It is most prominently expressed at the membrane surface of subcellular vesicles that contain apoptotic corpses. Moreover, *crq* expression is sufficient to trigger the recognition and engulfment of apoptotic cells in a heterologous system. Importantly, in the absence of *crq*, macrophages do not efficiently remove apoptotic corpses.[90] However, *crq*-deficient macrophages are able to carry out all other known macrophage functions, including the engulfment of bacteria. These results argue that *crq* specifically plays a role in phagocytosis of apoptotic corpses in the embryo.

The homology between Crq and mammalian CD36 suggests conservation in the mechanisms underlying phagocytosis of apoptotic corpses throughout evolution.[90–92] CD36 was shown to act in concert with the vitronectin receptor, an integrin, in the recognition of apoptotic corpses via a bridge of thrombospondin, an extracellular matrix protein.[92] Whether Crq requires other partners to assemble in a phagocytic complex remains to be explored. Perhaps most interesting will be the elucidation of the molecular nature of the pattern recognized by this phagocytic complex. In addition, the role of *crq* in the phagocytosis of apoptotic corpses at later stages of development is unknown. However, Crq is expressed on plasmatocytes, the phagocytic hemocytes of larval and pupal stages.[82,90]

Other receptors that may play a role in the recognition of apoptotic cells in the embryo are the *Drosophila* phosphatidylserine (PS) receptor,[93] and the *Drosophila* scavenger receptor, DSR-CI.[94] PS exposure is a common feature of apoptotic death,[95] and a recently identified mammalian PS receptor appears to be both necessary and sufficient to promote the engulfment of apoptotic corpses in some cell types.[93] The *Drosophila* homologue is highly conserved, but its function has not yet been characterized. Although the physiological significance of scavenger receptor activity remains unknown in the fly, a number of scavenger receptors have been proposed to participate in phagocytosis of apoptotic cells in mammals.[96–98]

Genetic strategies can clearly be used to evaluate the requirement of specific molecules in apoptosis as well as phagocytosis of apoptotic corpses in *Drosophila*. The elaborate regulation of apoptosis during development and the role of apoptosis in a variety of developmental processes are just beginning to be examined. In addition it is apparent that considerable work is still to be done to understand how the various components of the death machinery interact to kill cells. Finally, the genetic dissection of phagocytosis in the fly will provide important information on the recognition and clearance of apoptotic cells, as well as pathogens. It is clear that the apoptotic process is highly conserved, and insights gained in such model systems will have implications in other organisms.

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