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INSIGHTS FROM MODEL SYSTEMS
Molecular Prophets of Death in the Fly

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During normal development and aging, tremendous numbers of cells are systematically eliminated by a process referred to as "programmed cell death" (PCD), or apoptosis. This predictable loss of cells serves many diverse functions and has been most extensively studied during morphogenesis, neurogenesis, and maturation of the immune system (reviewed in Jacobson et al. 1997). Usually triggered by systemic hormones, withdrawal of trophic factors, or local cell interactions, apoptotic death is an active choice of fate that requires autonomous genetic functions within the dying cell. Apoptotic cells proceed through a series of changes characterized by membrane blebbing, condensation of nuclear material, cleavage of DNA into nucleosomal size fragments, and fragmentation into "corpses" that are rapidly engulfed by macrophages (Jacobson et al. 1997). The widespread occurrence of this stereotypical form of cell death among vertebrate and invertebrate models, together with more recent genetic evidence, indicates that the central molecular components required for this process are very highly conserved.

A growing body of evidence has begun to clarify the importance of PCD, both during normal development and in human disease. Deletion of interdigital areas and degeneration of neurons are good examples of cell populations eliminated by apoptosis in development (Jacobson et al. 1997). In human disease, misregulated apoptosis constitutes an important mechanism underlying autoimmunities (Rieux et al. 1995), myelomas (Landoweki et al. 1997), and lymphomas (Ashwell et al. 1994). It is also well established that viral infections frequently elicit apoptotic responses in the host cell that are blocked, directly or indirectly, by virally encoded genes (White 1993). Finally, inappropriate cell death may also account for the pathophysiology associated with degenerative diseases of the nervous system (reviewed in Bredesen 1995) and retina (see Travis 1998 [in this issue]).

The earliest genetic evidence that cells actively participate in their own deaths came from work in the nematode *Caenorhabditis elegans,* in which mutations provided the first indisputable evidence that apoptosis was indeed a gene-directed process. Loss-of-function mutations were found that prevented all cell deaths (Ellis and Horvitz 1986). The cloning of genes corresponding to several of these mutations has provided molecular entry points into at least three essential components of a single apoptotic pathway in this model. The nematode *ced-9* gene and its human counterpart, *BCL2*, normally funtion to suppress PCD (Hengartner and Horvitz 1994). Another nematode gene, *ced-3*, is the founding member of a growing family of cysteine proteases (Yuan et al. 1993), or caspases, that play essential roles during apoptotic cell death. Cascades of caspase activity (Cohen 1997) cleave a number of substrates in association with apoptotic death, and, although functional roles for many substrates remain obscure, Enari et al (1998) have recently identified a caspase-activated DNase responsible for the characteristic degradation of chromosomal DNA during apoptosis. A third nematode locus, *ced-4*, links activities of upstream *ced-9/BCL2* family members to downstream caspases (Chinnaiyan et al. 1997; Spector et al. 1997; Wu et al. 1997), and a human *ced-4* counterpart has been identified as well (Zou et al. 1997).

The H99 Genomic Region Is Required for Embryonic Apoptosis in the Fly

A screen for cell death–defective mutations uncovered a 300-kb genomic interval (the H99 region) required for all apoptotic cell deaths during *Drosophila* embryonic development (see sidebar). Although PCD fails to occur in H99 deficiency strains, embryonic development proceeds rather normally. Ultimately, however, these animals accumulate vast excesses of cells, particularly in the CNS (White et al. 1994). Damage-inducible apoptosis is also profoundly defective in H99 homozygotes, suggesting that common elements function in both normal and pathological cell death.

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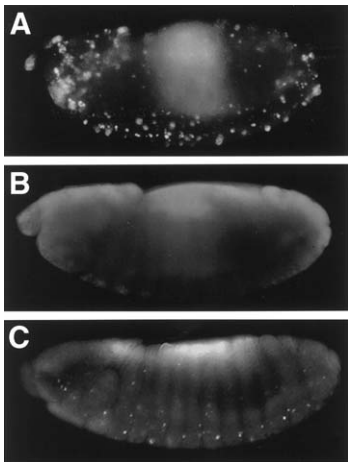
Courting death: Using synthetic deletions to define death genes in the fly

The *Drosophila* embryo has proved to be an ideal system for studying the genetic and molecular basis of development. Mutant strains are readily obtained, and divergence from normal developmental processes can be easily recognized with an extensive collection of cell type–specific markers, antibodies, and reporter genes. Some of the most powerful features of this model derive from the existence of well-characterized stocks, bearing gross chromosomal alterations such as deletions, duplications, or translocations. These altered chromosomes typically carry visible "marker" genes (affecting eye morphology or body color, for example), making it straightforward to identify flies with the desired alteration. The set of available "deficiencies" covers about half of the *Drosophila* genome, and, traditionally, these strains were tools to expedite complementation analysis and map new mutations. More recently, these strains have facilitated a merging of genetic and physical maps of the fly genome; for many of the strains, deletion breakpoints have been reconciled with a physical map derived originally from polytene chromosome banding patterns. Still more analytic power comes from cross breeding these altered strains to create, for example, "synthetic deletions," animals with two distinct, but overlapping, chromosomal deletions. This approach allows the rapid definition of a minimal genomic interval that is implicated in a given phenotype. We have exploited these same tools to tease apart the separate roles of several related and tightly linked genes in a gene complex needed for PCD.



We initially used acridine orange staining to detect abnormal patterns of PCD in a set of chromosomal deletion strains. Panel *A* shows the pattern of cell death in a wild-type embryo. Panel *B* shows the complete loss of PCD in an embryo identified from our screen; the deficiency in this homozygous mutant includes a region, H99, which is required for all embryonic PCD (White et al. 1994). When we created trans-heterozygous embryos, carrying deletions that break within H99, we noted a partial loss of PCD. Intermediate cell death–defective phenotypes, observed in a series of synthetic deletions, suggested an additive effect of multiple cell-death genes that all mapped to the H99, a supposition that was confirmed with the cloning of three related, and partially redundant, genes, *reaper*, *grim*, and *hid*. When *grim* alone is restored to an embryo that carries the homozygous H99 deletion (as in panel *C*), the defect in PCD is intermediate between the wild-type and H99 phenotypes.

Activators of Cell Death in the H99 Chromosomal Region

Three cell-death genes have been found in the H99 chromosomal interval, all of which are transcribed in the same orientation, have a common motif at their N-terminus, and appear to encode partially redundant functions.

reaper

reaper, the first cell-death gene identified in the H99 interval, encodes a 65–amino acid cytoplasmic protein bearing no obvious similarities to known proteins. This gene product is selectively expressed in cells that will later die (White et al. 1994), and its expression is sufficient to trigger apoptosis in both transgenic (Hay et al. 1995; White et al. 1996) and cultured cells (Chen et al. 1996*a*; Pronk et al. 1996). Alignments between *reaper* and the death domain of tumor necrosis factor receptor 1 (TNFR1) family members have led to suggestions that these proteins might share an ancestral function (Golstein et al. 1995). However, empirical tests have, so far, failed to obtain evidence for a function shared by *reaper* and the TNFR1 death domain (Chen et al. 1996*a*; Vucic et al. 1997*b*).

As in mammalian tissues, ionizing radiation triggers ectopic apoptosis in *Drosophila*. In mammals, p53 is a central mediator of ionizing radiation–induced apoptosis (reviewed Canman et al. 1994), and, although no *Drosophila* p53 homologue has yet been identified, the similar reaction to irradiation suggests the presence of an analogous pathway. It is also of interest to note that *reaper* plays a role in apoptosis induced by ionizing radiation and in other contexts of aberrant development. Irradiation of fly embryos prior to the stage at which PCDs first appear results in massive apoptosis (Abrams et al. 1993), preceded by a striking induction of *reaper* (Nordstrom et al. 1996). Similar inductive responses are also seen in congenital mutants in which aberrant development leads to massive apoptosis among cells that would otherwise survive (Nordstrom et al. 1996). These observations, together with studies on the behavior of reporter transgenes, establish that *reaper* mRNA behaves as a transcriptional "prophet of death" when cells are provoked to die by "unnatural causes." Recently, *reaper* has also been shown to induce apoptosis in *Xenopus* (Evans et al. 1997), and this finding fuels speculation concerning the existence of vertebrate *reaper* homologues.

grim

As the physical map of the *reaper* region was compiled, evidence suggesting that the H99 interval might harbor more than a single cell-death gene began to

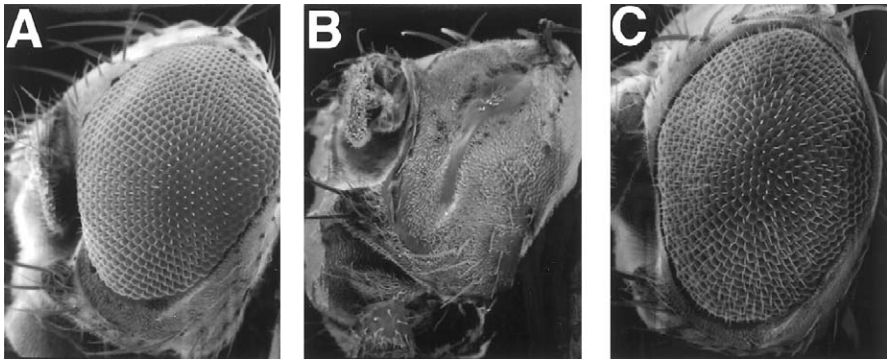


Figure 1 Manipulation of tissue by engaging elements of the apoptotic machine. Selective expression of an apoptotic activator, *grim*, in the developing fly retina provokes massive cell death and deletion of this tissue. Compare wild-type eye (*A*) and ablated eye of the pGMR*grim* strain (*B*). These deaths are completely prevented (*C*) by coexpression of p35, a viral antiapoptotic protein that inhibits caspase action. The result (*C*) demonstrates that preservation of apoptotic tissues may be achieved through targets of caspase function. (These panels are reprinted here from the article by Chen et al. [1996*b*, p. 1773], with the permission of *Genes & Development* and the Cold Spring Harbor Laboratory Press.)

emerge (see sidebar). The most definitive observation in this regard was that fly strains bearing deletions internal to the H99 breakpoints displayed markedly less severe phenotypes when placed in *trans* to the H99 deletion. This complexity could be explained by presuming that at least one, or possibly two, additional cell-death functions map to this region. Direct tests for such a function led to the identification of *grim* (Chen et al. 1996*b*), a cell-death gene that exhibits many features in common with *reaper*.

The deduced open reading frame for *grim* is a protein of 138 amino acids with no predicted transmembrane domains and no extensive homologies to any sequence in the current GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The amino terminal end of *grim* shares notable similarity to *reaper*, but *reaper* (Chen et al. 1996*b*) and *grim* (P. Chen and J. M. Abrams, unpublished data) mutants deleted for these residues are still able to provoke apoptosis in cell-culture assays (albeit with less potency). The shared N-terminal motif is therefore not absolutely essential for killing but may confer functions associated with protein stability and/or regulation.

Several lines of evidence established that *grim* encodes an activator of PCD during development. First, germline transformation of genomic DNA spanning the *grim* locus resulted in partial restoration of cell death to H99 mutant embryos. Also, induction of *grim* in either cultured cells or ectopic tissues triggered extensive apoptosis, and these deaths were prevented by p35, a viral anti-apoptosis protein that targets essential components of the death machinery (fig. 1). Finally, expression of *grim* selectively anticipates PCD during embryonic development (Chen et al. 1996*b*) and in metamorphosis (Robinow et al. 1997). These observations suggest that, like *reaper, grim* is both a prophet of death and an activator of apoptosis.

Head Involution Defective (hid)

The *hid* gene was originally described as a locus required for proper head and genitalia formation (Abbott and Lengyel 1991). Embryos carrying single-gene *hid* mutations do not exhibit gross cell-death defects that phenocopy the H99 deletion, but they do manifest partial failures in apoptosis (Grether et al. 1995). The incidence of PCD is noticeably reduced in these mutants, and supernumerary cells are observed in the larval optic nerve. It is also clear that expression of *hid* is sufficient to trigger extensive apoptosis in both transgenic animals (Grether et al. 1995) and cell culture (J. Varkey and J. M. Abrams, unpublished data). Also, like the previous models described for *reaper* and *grim*, these deaths are also prevented by coexpression of p35. The predicted *hid* gene product is a novel 410–amino acid protein rich in serine and proline residues. Although *hid* mRNA occurs in many regions that are coincident with patterns of PCD, it does not behave like a prophet of death, because expression of this gene occurs in many cells that do not ultimately die.

How do *reaper, grim,* and *hid* function to elicit apoptosis? Investigating this question is certain to be a high priority for the future, yet already several observations permit us to make some predictions in this regard. First, conditional expression studies show that each gene is sufficient to elicit extensive cell death when the other two are absent. Therefore, each can function in parallel to trigger apoptosis and does not require the functions

of the other two. However, combinatorial requirements and selective constraints are likely to specify patterns of PCD in development (Zhou et al. 1997). For instance, during early embryogenesis, prior to the onset of normal PCD, expression of *grim* is sufficient to elicit apoptosis, whereas *reaper* is not (Chen et al. 1996*b*). Candidates for regulators of signaling include members of the inhibitors of apoptosis (IAP) gene family. These proteins occur in baculovirus, mammals, and *Drosophila* and can modulate apoptosis in a number of contexts, including *reaper*-, *grim*-, and *hid*-induced cell killing (Hay et al. 1995; Vucic et al. 1997*a*). As in mammalian systems, multiple activators in the fly can function independently through parallel circuits that ultimately converge on a common set of effectors. Moreover, these circuits can function coordinately and may be subject to distinct sets of inhibitors.

Inhibitors of Cell Death

IAPs

The IAP proteins were first identified as virally encoded modulators of apoptosis (Clem and Miller 1994) and later as endogenous IAPs in mammals (Liston et al. 1996). More direct evidence that *Drosophila* shares similarities to mammalian apoptosis came with the identification and cloning of two fly IAP genes (Hay et al. 1995). IAPs typically encode a RING finger motif at their carboxy terminus and two or more baculovirus IAP repeats at the amino terminus. The former may constitute a negative regulatory domain, whereas the latter motif is essential for the antiapoptotic activity associated with these proteins. At least one mammalian IAP, NAIP, has thus far been implicated in the pathophysiology of a human neurodegenerative disorder (Liston et al. 1996).

In a screen for modifiers of *reaper*-mediated apoptosis in the eye, Hay and colleagues identified mutations in the *Drosophila* inhibitor of apoptosis 1 (*diap1*). Heterozygous mutations at this locus enhanced the *reaper* death phenotype, suggesting that IAPs inhibit activator-induced apoptosis in a dose-dependant manner. Sequence analyses prompted by the discovery of *diap1* led to the identification of a related gene, *diap2*. Unlike *diap1*, heterozygous deletions at *diap2* do not enhance *reaper*-mediated retinal cell death. Overexpression of *diap1* or *diap2* not only suppressed normal PCD in the eye but also prevented excessive apoptosis due to overexpression of *reaper, hid* (Liston et al. 1996) or *grim* (P. Chen and J. M. Abrams, unpublished data).

Expression of *diap1* is widespread throughout the embryo and the developing eye disc, but embryos homozygous for loss-of-function mutations in either *diap1* or *diap2* show no obvious cell-death phenotypes, possibly owing to extensive maternal contributions of these proteins and/or functional redundancies. However, clonal analyses in postembryonic development and in the germ line suggest that *diap1* could be required for cell survival.

The precise function of the *Drosophila* IAP proteins during normal development remains obscure because, although they exhibit potent activities in sensitized transgenic backgrounds, their loss-of-function effects on normal apoptosis have yet to be determined. Two important clues shed light on IAP functions. First, the IAP proteins directly bind to *reaper* and inhibit apoptosis provoked by this activator (Vucic et al. 1997*a*). Second, in the mammalian system, the X-linked IAP inhibits apoptosis by a direct interaction with caspase-3 and caspase-7 (Deveraux et al. 1997). Taken together, these results suggest that the IAPs may be involved in bridging the activators of apoptosis with the actual cell-death machine.

p35

Baculovirus p35 is another protein that engages the death pathways triggered by *reaper, grim,* or *hid*. Miller and his colleagues established that this protein functions to suppress apoptosis that would otherwise occur among infected host cells (Clem and Miller 1994). p35 bears no obvious similarity to known proteins, yet it is capable of suppressing cell death in mammalian (Rabizadeh et al. 1993) and invertebrate contexts, including apoptosis by *reaper, grim,* and *hid* (Grether et al. 1995; Chen et al. 1996*b*; Nordstrom et al. 1996; White et al. 1996). This baculovirus protein is an irreversible inhibitor of (and pseudosubstrate for) caspases, which are thought to be universal and requisite effectors of apoptotic circuits throughout the animal kingdom (reviewed in Cohen 1997).

The Cell-Death Machine: Caspases

Cell killing induced by *reaper, grim,* or *hid* triggers induction of proteolytic activity characteristic of members of the caspase gene family. This activity is strikingly similar to mammalian counterparts because extracts from *Drosophila* cultures exhibit signature cleavage activity against the human death substrate, poly-ADP-ribose-polymerase, a protein that is also found in flies (Uchida et al. 1993).

At least three members of the caspase family have been identified thus far in *Drosophila*. Two of these, *Drosophila* caspase 1 (DCP1; Song et al. 1997) and *Drosophila* interleukin-1B-converting enzyme (drICE) (Fraser and Evan 1997), are most closely related to CPP-32 and Mch2, whereas the third, DREDD (P. Chen, A. Rodriguez, and J. M. Abrams, unpublished data) is most closely related to ced-3 and ich-1/nedd2. DREDD is

unique among the *Drosophila* caspases because of features found in its long prodomain and a novel residue found at its active site. Activation of these caspases occurs during *reaper*-, *grim*-, and *hid*-induced cell killing (P. Chen and J. M. Abrams, unpublished data), consistent with the notion that these three genes act as parallel switches that stimulate apoptosis via a common circuit of downstream effectors. A precise determination of the role of these caspases during PCD will require detailed genetic and biochemical analyses. Precedents from genetic studies on *dcp1* (Song et al. 1997), however, suggest that sophisticated analyses for characterizing multiply mutated individuals will be required because of potential confounding effects associated with functional redundancies and/or maternal contributions.

Concluding Remarks

During the past several years, apoptosis research has witnessed spectacular growth. A general lesson to be drawn from our knowledge thus far is that the parallels between apoptosis in *Drosophila* and mammals are striking. In both systems, the death of cells is regulated by hormones, occurs in reproducible patterns in development, and is associated with a characteristic cytomorphology. Similarly, in both systems, ectopic apoptosis can be observed in association with defective development and induced by ionizing radiation. These parallels persist at the molecular level as well. In both systems, cell death is associated with caspase activity, DNA degradation, and modulation by IAPs. Persuasive evidence for evolutionary conservation of these components argues that understanding apoptosis in *Drosophila* will have profound relevance to human pathologies in both gene discovery and disease treatment. For instance, *Drosophila* death activators, *reaper, grim,* and *hid,* might furnish excellent tools for the discovery of therapeutic drugs intended to induce apoptosis in diseased human tissues. In addition, the fly model will continue to afford us with a valuable system in which to identify precise determinants that initiate and amplify cascades of caspase activity. Whereas studies in mammalian cells have uncovered a complex meshwork of distinct proteolytic cascades involving >10 caspases, a reduced complexity of caspase networks in *Drosophila* may facilitate the complex task of understanding caspase circuits at both genetic and biochemical levels. It is similarly likely that valuable information will also be obtained from studies of IAP gene function. For instance, taking observations from insect and mammalian models together, an attractive model might propose that IAPs normally hold caspase activation under represssion and that apoptosis activators provoke cell death by inactivating this inhibition. The sophisticated level of genetic tools available in *Drosophila* will permit direct tests of this and other possible models.

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