**Summary:** A large focus of our work (though not all; see below) is directed towards understanding the genetic and molecular mechanisms that regulate and bring about cell death. Specifically, we are using *Drosophila melanogaster* as a model system to identify genes that function to regulate cell death, and to identify important roles that cell death plays in normal development. Important cellular regulatory pathways are evolutionarily conserved; thus molecules identified as important regulators of cell death in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second set of goals is to take the molecules and pathways uncovered in *Drosophila* and apply this information to the study of cell death in vertebrates, with the ultimate goal of determining the role that aberrations in this process play in human pathologies. In this context we see *Drosophila* as a powerful tool for uncovering conserved components and modes of death regulation.

Apoptosis is a form of regulated cell death in which superfluous or harmful cells are removed from an organism. Apoptotic cell death is required for many aspects of normal development, tissue size homeostasis, and as a defense against potentially harmful cells, such as self reactive cells in the immune system, virally infected cells, cells that have damaged DNA, or cells that are being induced to proliferate inappropriately. Because cell death is widespread during the development and normal function of organisms, deregulation of this pathway has dire consequences. Inappropriate cell death is associated with degenerative neurological diseases such as Alzheimer's disease and Parkinson's disease; inhibition of normally occurring cell death can contribute to the development of auto immunity, persistent viral infections, and can set the stage for cancer by preventing the death of cells that would normally die, allowing them to undergo mutations that could lead to transformation.

Although the signals and stimuli that trigger cell death are diverse, once initiated, apoptosis is thought to proceed via one or several common pathways. The identification in worms, flies and mammals of homologous proteins that function similarly to regulate cell death indicates that, as with other important signal transduction pathways, components and modes of death regulation are likely to be conserved throughout evolution (reviewed in Vernooy et al., 2000). *Drosophila* is an ideal system in which to do screens for genes important in death signaling because it is a complex organism with multiple life stages in which death plays important roles, it has powerful genetics, and we are able to manipulate death signaling in individual tissues using tissue-specific promoters.

**Genetic screens for regulators of cell death in the fly.**

Since normally occurring cell death is essential for *Drosophila* viability, genetic approaches to cell death must take account of this fact as well as the potentially important role of maternal effect gene products. We have circumvented these problems by creating a sensitized system that exploits a tissue dispensable for viability and fertility, the eye. In this system a cell death signaling pathway is made hyperactive (giving rise to flies with small eyes) or partially nonfunctional (giving rise to flies with large, rough eyes). In this sensitized background a decrease in gene dosage of 50% in genes functioning downstream or in parallel to the point at which the signaling pathway has been manipulated might be expected to result in an eye phenotype change, if these components are now rate limiting.
To carry out this approach we use a P element expression vector, pGMR (Glass Multimer Reporter), which drives eye-specific expression of reporter genes (Hay, et al., 1994). To identify genes important in cell death regulation, proteins from Drosophila or other systems that are known to be able to activate or inhibit cell death in some context are expressed in the eye under GMR control, and their capacity to perturb the normal patterns of cell death determined. To the extent that the ability of these proteins to manipulate cell death is conserved, such expression should result in the creation of flies with visible phenotypes (increased cell death=small eye; decreased cell death=large, rough eye). These flies can then be used as screening backgrounds to identify interacting genes.

**Loss of-function modifier screens.** We have carried out several loss-of-function dominant modifier screens for enhancers and suppressors of cell death induced by eye-specific expression of the Drosophila cell death activator Reaper. Flies that express Reaper in the developing eye (GMR-Reaper flies) have small eyes. The premise of a loss-of-function modifier screen is that a twofold decrease in signaling strength (making the fly heterozygous at important death regulatory loci) will result in a change in eye phenotype. During one of these screens we identified the cell death inhibitors DIAP1 and DIAP2 (Hay et al., 1995) and the microRNA mir-14 (Xu et al., 2003). Examples of the kinds of eye phenotypes we observe in these screens are shown in the scanning electron micrographs below.

<table>
<thead>
<tr>
<th>Wildtype</th>
<th>GMR-Reaper</th>
<th>GMR-Reaper +/th5</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Wildtype" /></td>
<td><img src="image2.png" alt="GMR-Reaper" /></td>
<td><img src="image3.png" alt="GMR-Reaper +/th5" /></td>
</tr>
</tbody>
</table>

**Gene activation modifier screens.** Mutational inactivation is an important approach to understanding the role a gene plays in a specific process. The approach is limited, however, by the facts that many genes do not have an easily assayable loss-of-function phenotype, and that any phenotype that is observed reflects only that aspect of a gene’s function that is not compensated for by other genes and pathways. An alternative approach to understanding gene function is to characterize phenotypes associated with tissue-specific misexpression of genes at elevated levels in tissues where they are normally expressed, or in tissues in which they are not normally expressed. Misexpression may create phenotypes where inactivation does not, providing a powerful approach to identifying genes. Misexpression also allows one to determine if the presence of a specific gene product is sufficient to drive a process.
To carry out misexpression screens for genes important in a particular process one needs to be able to drive the expression, individually, of large numbers of genes in a specific tissue. It is not feasible to individually misexpress known genes because this requires that one have the full length gene in hand, that these be introduced into the genome one at a time, and that one pre-select candidate genes that are likely to be important for the process under study. Misexpression of random genes from their normal genomic location provides a much more general approach for identifying genes that can affect a process without preconceptions.

*Drosophila* is an ideal system in which to carry out such random misexpression screens because transposable elements (P elements) can be mobilized throughout the genome at a high frequency, in a controlled fashion, and because mutagenic P elements have a preference for insertion near the 5' ends of a genes. To carry out this approach we designed a P element vector, known as GMREP, that contains an eye-specific promoter near one P element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay et al., 1997). Flies carrying these insertions can then be tested in various ways to identify those that are misexpressing cell death regulators.

![Diagram of GMREP vector](image)

### GMREP drives eye-specific overexpression of nearby genes

<table>
<thead>
<tr>
<th>suppressor name</th>
<th>genomic location</th>
<th>suppresses reaper</th>
<th>suppresses grim</th>
<th>suppresses hid</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.2</td>
<td>X</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PEG</td>
<td>2R</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KV1</td>
<td>2R</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.1</td>
<td>3L</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HS1</td>
<td>3L</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E3</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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<td>RS1</td>
<td>3R</td>
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<td>-</td>
<td>+</td>
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<td>dBruce</td>
<td>3L</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GS5</td>
<td>3R</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GS1</td>
<td>3R</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DIAP1</td>
<td>3L</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

We have carried out several large screens for cell death inhibitors and have identified 11 interesting loci. One of these, the Bruce locus (others named it this in mammalian systems, not us), encodes a very large protein with E2 ubiquitin conjugation activity. *Drosophila* Bruce acts as a very potent inhibitor of RPR- and GRIM-dependent cell death (Vernooy et al., 2002). Interestingly, and consistent with a possible role of Bruce as an
oncogene, mammalian Bruce is upregulated in multiple human cancers. A second group of four cell death inhibitors encode small, noncoding microRNAs (Xu et al., 2003). Several other modifiers remain to be characterized.

**Genetic screens for regulators of cell death using yeast.**

Site-specific proteolysis plays a number of critical roles in regulating cellular processes. For each of these cleavage events we would like to know what the protease is that is doing the cleavage, what the targets of the protease are, how the activity of the protease is regulated, and where and when the protease is active? A useful tool for addressing these questions would be a reporter for protease activity whose presence or absence can be visualized and quantitated. We have devised such a protease reporter that uses living cells. The basis for our approach is the creation of chimeric proteins that consist of two protein domains separated by a protease target site. One of the two domains, domain A, when released from association with domain B, is able to act as a signal transducer. Domain B functions to inhibit the signal transducing function of domain A when they are linked. Thus, in the absence of a protease that cleaves between domains A and B, no signal is transduced, while in the presence of the appropriate protease, the target site is cleaved, releasing domain A to transduce a signal which is subsequently detected.

![Diagram of protease activity reporter](image)

There are a number of possible ways of constructing such a system and we have pursued several of these. One approach that works well involves creating a fusion
protein that consists of a transcription factor (domain A) tethered to the intracellular domain of a type 1 plasma membrane protein (domain B). In this fusion, the C-terminus of the transmembrane protein is separated from the transcription factor by a linker domain that contains protease target sites. In the absence of a protease that can cleave the target site, the transcription factor remains at the membrane where it is nonfunctional. When the target site is cleaved by an introduced or endogenous protease, the transcription factor is released from the membrane and is free to go to the nucleus and activate transcription of a reporter.

A central step in carrying out apoptosis is the activation of members of a family of cysteine-dependent, aspartate specific proteases, known as caspases. In many cell types, caspases sufficient to carry out cell death are expressed ubiquitously, indicating that their activation and activity must be tightly controlled in normal cells. Because caspases are central to cell death regulation, they are important potential therapeutic targets. In order to take advantage of this potential, it is important to identify and characterize the organismal complement of caspases, and to understand how their activity is regulated by other cellular factors. With these goals in mind, we have developed yeast that act as reporters for caspase activity. Yeast provides an ideal background in which to screen for caspases and their regulators because they can be transformed with high efficiency, allowing for the screening of large numbers of proteins for activity in any particular assay; 2) Strains with a number of nutritional markers are available, allowing for the introduction and maintenance of a number of different plasmids at defined copy number, 3) Powerful selections can be carried out, and 4) It is likely that the intracellular environment of yeast constitutes a null, or close to null, state with respect to the activity of higher eukaryotic cell death regulators, including caspases.

Our caspase reporter yeast express a fusion protein substrate for caspase cleavage in which the type 1 transmembrane protein CD4 has linked to its cytoplasmic tail the transcription factor LexA-B42 (LB). Separating these two domains is a short linker (DG6)
consisting of six different caspase cleavage sites that bracket the specificities of known caspases and the serine protease granzyme B, which cleaves caspases at sites of similar sequence. The emerging N-terminus following cleavage at any of the caspase target sites is a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast. When this molecule, referred to as CLBDG6 is expressed in a yeast strain that carries a plasmid in which expression of the lacZ gene is under the control of a LexA-B42-dependent promoter (designated as the LexA/B-gal reporter strain), expression of lacZ, requires caspase cleavage at one or more of the introduced target sites, which releases LexA-B42 from membrane association, allowing it to activate lacZ transcription. We have used these strains, as well as the fact that overexpression of caspases in yeast kills these cells, to identify and characterize regulators of caspase activity in flies and worms (Hawkins et al., 1999; Wang et al., 1999; Hawkins et al., 2000).

Cell-killing proteins (caspases) and their inhibitors (the IAPs). The core of the cell death machine.

The core of the cell death machine are members of a family of proteases known as caspases. Caspases become activated in response to many different death signals. They then cleave a number of different cellular substrates, leading ultimately to cell death. Most if not all cells constitutively express caspase zymogens (inactive precursors) sufficient to bring about apoptosis. Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Deregulation of caspase activation, and thus cell death, is associated with many human diseases. For example, inappropriate inhibition of caspase activity is associated with cancer and autoimmunity, while ectopic caspase activation is associated with many neurodegenerative diseases. The only known cellular caspase inhibitors are members of the Inhibitor of Apoptosis (IAP) family of proteins, which therefore constitute a last line of defense against caspase activation. Because of their critical role, determining how IAPs function to inhibit cell death, and how these activities are regulated, is important for understanding how cell death is regulated in health and disease. We have described three different mechanisms by which death activators disrupt IAP function (see figure above). However, a number of questions remain unanswered. 1) What are the mechanisms by which apoptosis inducers shorten IAP half life, and what are the contexts in which these mechanisms are important? 2) Are there unknown IAP binding partners that promote or inhibit IAP anti-apoptotic function through modulation of IAP-caspase interactions, effects on IAP half life, or through other mechanisms? 3) What is the source of the apical cell death signal that DIAP1 must fight against to maintain cell survival? and 4) Are there other important pathways that serve to modulate critical steps
in this death circuit, such as apical caspase activation, IAP efficacy as a caspase inhibitor, or IAP turnover rate, that have not been identified?

**MicroRNA regulators of cell death**

In the early 1990s Ambros and colleagues, working in *C. elegans*, identified an odd developmental regulatory gene, *lin-4*, that encoded an approximately 70 nucleotide hairpin-forming transcript which was ultimately processed into a 22 nucleotide product (Lee et al., Cell 75: 843-854). Observations by this group and others over the years argued for a model in which the RNA products of the *lin-4* and *let-7* genes (*let-7* is a second *C. elegans* gene encoding a short hairpin-forming transcript) bound to target transcripts through imperfect basepairing. This then led, through still mysterious mechanisms, to translational repression of the target gene (reviewed in Ambros, Cell 107: 823-826). See figure below.

More recently it has become clear that *C. elegans* *lin-4* and *let-7* are not alone in their oddity. A number of groups, using computational and cloning approaches designed to identify small RNAs, have identified a large number of small noncoding RNAs (roughly 21-23 nucleotides). Many of these are also predicted to arise from a slightly larger stem loop precursor, much like *lin-4* and *let-7*. It is currently estimated that the genomes of complex organisms such as worms, flies and humans contain on the order of at least several hundred miRNAs (see the microRNA registry for details; http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml). At this point very little is
known about the regulation and functions of these newly identified RNAs, known generically as miRNAs.

We identified four microRNAs that function as cell death suppressors in an eye-based dominant modifier gene activation screen (see Xu et al., 2003 for a description of one of these). We are working to 1) identify new miRNA cell death regulators, 2) understand how these microRNAs function, 3) to identify the contexts in which they are important, and 4) to determine if they play similar roles in mammals.

**Non-cell death roles for cell death regulators in spermatogenesis - the control of male fertility.**

Spermatozoa throughout the animal kingdom are generated and mature within a germline syncytium. Differentiation of haploid syncytial spermatids into single motile sperm requires the encapsulation of each spermatid within an independent plasma membrane and the elimination of most sperm cytoplasm, a process known as individualization. Little is known about how individualization is carried out. However, the importance of one aspect of this process for human fertility, the elimination of excess cytoplasm, is suggested by the fact that many conditions or treatments resulting in infertility disrupt this process.

We recently reported that multiple caspase family proteases and their activators were required for spermatid individualization in *Drosophila* (Huh et al., 2004). These observations were striking because caspases are the core of the evolutionarily conserved, apoptotic cell death machine. Once activated they typically cleave a number of cellular substrates that ultimately lead to cell death and corpse phagocytosis. Our observations raise a number of questions. In particular, 1) What are caspases doing to facilitate spermatid differentiation? 2) How is it that spermatids avoid death in the presence of active caspases that would induce apoptosis in other cells? 3) What are the pathways that mediate caspase activation in spermatids? 4) There are strong hints that caspases and their activators are also likely to be present in mammalian spermatids. Is their activity required for proper sperm function, and does deregulation of this activity underlie cases of male infertility?
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