

## **Caspases Downstream of DIAP1 in *Drosophila* S2 Cells: In What Order are They Activated and What Role Does the Proteasome Play in Their Degradation?**

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### **Specific Aims**

**SA1.** Determine which of the seven identified *Drosophila* caspase genes are expressed in *Drosophila melanogaster* S2 cells.

**SA2.** Identify which of the expressed caspases are downstream of DIAP1 in the apoptosis pathway of S2 cells.

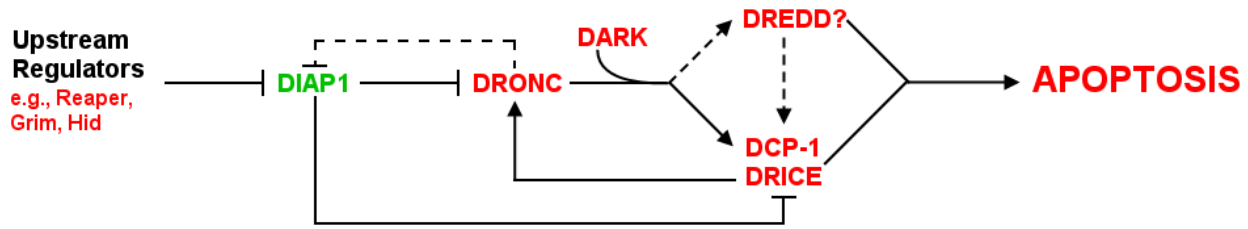
**SA3.** Explore the order in which expressed initiator and effector caspases are activated and study the function of proteasomal degradation in regulating the initiator caspase DRONC.

### **Background and Significance**

The apoptotic pathway is critically dependent on the class of cysteine proteases known as caspases, of which seven have been identified in the *Drosophila* genome (Kumar and Doumanis, 2000; see Table 1). Caspases are synthesized as zymogens and activated by proteolysis upon pro-apoptotic signaling. Apical or “initiator” caspases primarily activate the downstream “effector” caspases whose protease activity is directed toward destruction of cellular machinery. Each caspase has a specific amino acid sequence as its preferred substrate; although caspases may cleave related sequences at lower rates, at minimum a distinction can be drawn between initiator and effector caspases based on substrate preference (Thornberry et al., 1997). For example, the initiator caspase DRONC is the only *Drosophila* caspase reported to cleave after the amino acid sequences TQTE or IETD (Hawkins et al., 2000), whereas effector caspases generally cleave DEVD (Kumar and Doumanis, 2000).

The key regulatory protein DIAP1 (*Drosophila* inhibitor of apoptosis), which can function as an E3 ubiquitin ligase, blocks the activation of the caspase cascade in non-apoptotic

cells by binding to and ubiquitinating the initiator caspase DRONC (Palaga and Osborne, 2002). DIAP1 has also been reported to bind and inhibit DRICE without ubiquitinating it (Yan et al., 2004). In apoptotic cells, DIAP1 is itself inhibited by upstream regulatory proteins thought to compete with DRONC for its DIAP1 binding site, thereby preventing DRONC ubiquitination (Chai et al., 2003). See Figure 1 for an outline of the pathway.



**Figure 1. The *Drosophila* apoptosis pathway.** Red proteins are pro-apoptotic and green are anti-apoptotic. Dashed lines represent less well-established interactions. Adapted from Richardson and Kumar 2002.

After removal of DIAP1 inhibition, DRONC recruits the activator protein DANK to form a structure called an apoptosome, which is specialized for further caspase activation (Martin, 2002). Alternatively, DANK may be required for early processing of DRONC into its active form (Muro et al., 2002). Activated DRONC is capable of activating DRICE, which in turn cleaves DRONC and renders it invulnerable to inhibition by DIAP1 (Yan et al., 2004). The remaining caspases are less well studied; DCP-1 and DREDD are reported to be expressed in S2 cells, but DECAP, DAMM, and STRICA are relatively unknown quantities (Kumar and Doumanis, 2000). The proposed series of experiments seeks to establish which of the seven *Drosophila* caspases are expressed and function downstream of DIAP1 in S2 cells. Further, we intend to explore the sequence in which the expressed caspases are activated and to investigate whether the initiator caspase DRONC is active after ubiquitination but before proteasomal degradation. Knowledge of these key apoptosis parameters as manifested in S2 cells will improve our understanding of the apoptotic pathway in *Drosophila*.

## Research Design and Methods

### *Cell Culture and Survival Assays*

*Drosophila* S2 cells will be cultured in liquid Schneider's media supplemented with inactivated fetal calf serum and penicillin-streptomycin to aid in the prevention of bacterial contamination. Cultures will be maintained at 37°C and split once every 3-4 days. All transfections will be performed in six-well plates incubated under the same conditions. Transfected cultures will be assayed for cell survival by staining with Trypan Blue, which is excluded from viable cells but taken up by those that are nonviable. Although this assay makes no distinction between apoptotic and necrotic cells, a properly maintained culture should have a minimal amount of necrosis. Viable cells are counted by visual inspection of a hemacytometer grid with a light microscope.

### *Identification of Expressed Genes*

DNA and mRNA will be extracted from a population of non-apoptotic S2 cells. The mRNA will be subjected to two-step reverse transcriptase-PCR (RT-PCR), allowing the isolation of a pool of newly synthesized DNA which can be used in multiple PCR reactions aimed at amplifying different target genes. PCR primers will be designed based on those in the Heidelberg RNAi database (Boutros, 2005) for the genes of interest. Results will be visualized on an agarose gel in which each lane corresponds to a different gene target; the intensity of a band of appropriate size in any lane correlates to the degree to which that gene is expressed.

Name	FlyBase ID	Name	FlyBase ID
DRONC	FBgn0026404	DAMM	FBgn0033659
DREDD	FBgn0020381	STRICA	FBgn0033051
DCP-1	FBgn0010501	DARK	FBgn0024252
DRICE	FBgn0019972	DIAP1	FBgn0003691
DECAY	FBgn0028381		

**Table 1. Caspases and related regulatory proteins identified in the *Drosophila* genome.** Caspases are shown in black; related regulatory proteins in blue. Each of the seven caspases will be subjected to gene expression analysis.

The extracted DNA will serve as one of the several controls necessitated by this experimental design; PCR reactions with the extracted DNA as the template will be performed using the same primer sets as above, thus giving each RT-PCR reaction a corresponding DNA-based control. Failure to produce a band, or the production of a band wildly different in size from that observed for the corresponding RT-PCR, indicates the primers' failure to properly amplify the gene of interest. Amplification by RT-PCR of a gene known to be expressed using known-good primers will serve as a second positive control to confirm the success of the RT step. Negative controls not expected to produce a gel band will consist of performing PCR without any template, and performing PCR on unprocessed mRNA without an intervening RT step. Observation of a band in these lanes would imply contamination of the PCR reagents or mRNA.

#### *Identification of Downstream Caspases*

Caspases acting downstream of DIAP1 will be identified by double-knockdown experiments using RNA interference (RNAi; Hannon, 2002). Since knocking down DIAP1 alone results in apoptosis, simultaneously knocking down a required caspase should lead to cell survival, allowing the identification of the caspases required for apoptosis in S2 cells. RNAi probes directed toward each expressed caspase gene will be designed based on probes in the Heidelberg RNAi database (Boutros, 2005) and purchased as dsDNA, on which *in vitro* transcription will be performed to produce dsRNA suitable for use in transfection.

The transfection reagent FuGENE will be used to promote dsRNA uptake by healthy cells seeded in a six-well plate to a standard concentration per well. Double transfections may be performed asynchronously to account for differences in protein half-lives; in most cases transfection with dsRNA specific to the target caspase will be performed before the DIAP1 transfection to ensure maximal depletion of the caspase. Transfection with dsRNA targeting GFP

– a protein known not to be expressed in S2 cells – will serve as a negative control against the possibility that apoptosis might be induced by the transfection procedure itself.

Cells will be assayed with Trypan Blue to determine the extent of apoptosis in a given sample. Since the efficacy of RNAi against DIAP1 is known, rescue from apoptosis in a double-knockdown sample implies that the targeted caspase is a required component of the apoptosis pathway in S2 cells. Extensive apoptosis suggests that either the targeted caspase is not obligatory for apoptosis, or the caspase gene is not susceptible to RNAi. RT-PCR performed on extracts of such cells would reveal the extent to which mRNA for the targeted caspase is present and thus distinguish between the two possibilities.

#### *Caspase Ordering and DRONC Regulation*

The order of caspase activation in apoptosis is difficult to determine genetically because the caspases all contribute to the same end effect – cell death. However, caspase substrate specificity can be exploited to assign relative upstream and downstream positions. Commercially available activity assays (e.g., BIOMOL Quantipak, AK-004) couple a cleavable peptide substrate to a *p*-nitroanilide moiety that is colored only when free in solution. Because solution color is directly proportional to the number of free *p*NA molecules, the optical density of a cell-free lysate treated with caspase substrate provides a proxy measure of caspase activity. Used on cell lysates prepared from double-knockdown RNAi experiments, caspase activity assays can dissect the pathway of caspase activation; for example, if DIAP1 and Caspase A are knocked down and no apoptosis is observed, but Caspase B is active in the cell lysate, one may conclude that Caspase B is upstream of Caspase A. The power of this method is limited by imperfect caspase specificity but should be sufficient to classify caspases as initiators or effectors.

Additionally, an RNAi knockout of a selected proteasome subunit (e.g., Wojcik, 2002)

will be assayed using a substrate specific to the initiator caspase DRONC, which is degraded by the proteasome in non-apoptotic cells. If DRONC is active in proteasome knockouts, the hypothesis that it is ordinarily inhibited by ubiquitin-mediated proteasomal degradation is supported. A double knockdown of both DRONC and the proteasome subunit will address the (somewhat unlikely) possibility that proteasomal dysfunction itself induces apoptosis via a caspase-independent mechanism.

A number of controls are required under this experimental paradigm. Testing the optical density generated by each substrate in a lysate made from non-apoptotic cells accounts for background protease activity. A peptide-anilide construct that is an unsuitable caspase substrate will serve as the primary negative control. If purified enzymes are available, incubation of each expressed caspase with its preferred substrate will function as a positive control.

## References

- Boutros M. (2005). GenomeRNAi Drosophila Resources. <http://rnaï.dkfz.de/>
- Chai J, Yan N, Huh JR, Wu JW, Li W, Hay BA, and Shi Y. (2003). Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent DRONC ubiquitination. *Nat. Struct. Biol.* **10(9)**: 892-8.
- Hannon GJ. (2002). RNA interference. *Nature* **48**: 244-51.
- Hawkins CJ, Yoo SJ, Peterson EP, Wang SL, Vernooy SY, Hay BA. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem.* **275(35)**: 27084-93.
- Kumar S and Doumanis J. (2000). The fly caspases. *Cell Death Differ.* **7**: 1039-44.
- Martin SJ. (2002). Destabilizing influences in apoptosis: Sowing the seeds of IAP destruction. *Cell* **109**: 793-6.
- Muro I, Hay B, and Clem RJ. (2002). The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J. Biol. Chem.* **277(51)**: 49644-50.
- Palaga T and Osborne B. (2002). The 3 D's of apoptosis: Death, degradation, and DIAPs. *Nat. Cell Biol.* **4**: E149-51.
- Richardson H and Kumar S. (2002). Death to flies: *Drosophila* as a model system to study programmed cell death. *J. Immunol. Methods* **265**: 21-38.
- Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, and Nicholson DW. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **272(29)**: 17907-11.

Wojcik C and DeMartino GN. Analysis of *Drosophila* 26S proteasome using RNA interference.

*J. Biol. Chem.* **277(8)**: 6188-97.

Yan N, Wu JW, Chai J, Li W, and Shi Y. (2004). Molecular mechanisms of DrICE inhibition by

DIAP1 and removal of inhibition by Reaper, Hid, and Grim. *Nat. Mol. Struct. Biol.*

**11(5)**: 420-8.