

# DIAP1-Mediated Apoptosis in *Drosophila* S2 Cells Relies on the Caspases DRONC, DRICE, and DCP-1

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## Abstract

The caspase cascade in *Drosophila* is controlled primarily by DIAP1 (*Drosophila* inhibitor of apoptosis), which exerts its regulatory effect by targeting the initiator caspase DRONC for proteasomal degradation. Pro-apoptotic signaling stimulates auto-ubiquitination by DIAP1 and frees DRONC to activate the caspase cascade. We used RT-PCR for expression profiling of *Drosophila* S2 cells and determined that all *Drosophila* caspases save DECAY are expressed. Furthermore, we employed the technique of RNA interference (RNAi), complemented by biochemical caspase activity assays, to determine that suppression of DRONC, DRICE, and DCP-1 by RNAi largely rescues cells from apoptosis induced through the DIAP1 pathway.

## Introduction

The apoptotic pathway in *Drosophila* 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 are activated by proteolysis upon pro-apoptotic signaling. Apical or “initiator” caspases primarily activate the downstream “effector” caspases whose protease activity is directed toward the deconstruction of cellular machinery.

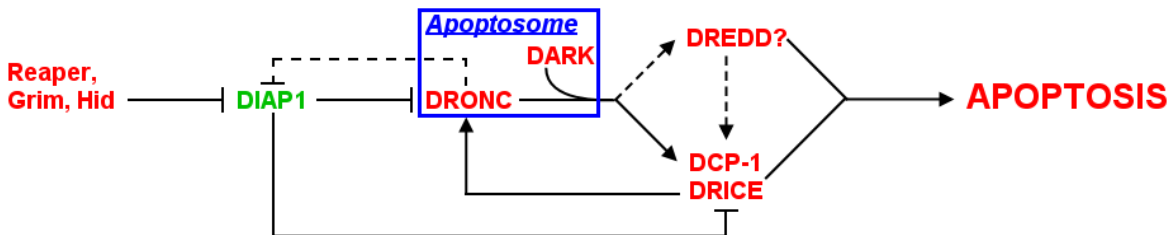
**Table 1: The seven known *Drosophila* caspases and their functions.**

<i>Caspase</i>	<i>Function</i>	<i>Prodomain</i>	<i>Caspase</i>	<i>Function</i>	<i>Prodomain</i>
DRONC	Initiator	Long	DECAY	Unknown	Short
DREDD	Initiator?	Long	DAMM	Unknown	Short
DRICE	Effector	Short	STRICA	Unknown	Long
DCP-1	Effector	Short			

Adapted from Richardson and Kumar, 2002; Kumar and Doumanis, 2000.

This classification scheme is correlated with the size of structural elements called prodomains which lie N-terminal to the caspase active sites and frequently contain protein interaction motifs. Initiator prodomains are typically long and effector prodomains generally short (Kumar and Doumanis, 2000).

Although the caspase activation pathway has yet to be fully characterized, the roles of certain critical regulatory molecules are well-established. DIAP1 is understood to be the single most important regulatory element in the caspase cascade (Figure 1). Its primary mode of regulation is the ubiquitination of the initiator caspase DRONC, which is thus targeted for proteasomal degradation (Palaga and Osborne, 2002). DIAP1 has also been reported to bind to and inhibit the effector caspase DRICE without ubiquitinating it (Yan et al., 2004).



**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. See Discussion for a revised model based on our data.

In apoptotic cells, DIAP1 is itself inhibited by upstream regulatory proteins known as Reaper, Hid, and Grim (RHG); when these proteins are active, DIAP1 ubiquitinates itself and is degraded by the proteasome. A partial negative feedback loop exists in this interaction – DIAP1 is capable of ubiquitinating Reaper as well (Olson et al., 2003). After removal of DIAP1 inhibition, DRONC recruits the activator protein DARK to form a structure known as the apoptosome, which is specialized for further caspase activation (Martin,

2002). Alternatively, DARK may be required for early processing of DRONC into its active form (Muro et al., 2002). Once activated, DRONC is capable of activating DRICE, which in turn cleaves DRONC and renders it invulnerable to DIAP1 inhibition (Yan et al., 2004). Finally, DCP-1's position as a general-purpose downstream protease is relatively uncontroversial, although it has not been reported to participate in any feedback mechanisms (Richardson and Kumar, 2002).

The roles of the remaining caspases in the activation sequence are less well-studied. DREDD is reported to be an initiator caspase, although no clear position in the pathway has been assigned to it. One review suggests that it may also play a role far upstream in the pro-apoptotic signaling of the innate immunity pathway (Richardson and Kumar, 2002). It has even been suggested that DREDD concentration is a rate-limiting factor in apoptosis induced via RHG activation (Kumar and Doumanis, 2000). DECAY has been reported to be an effector caspase similar to DCP-1 (Danial and Korsmeyer, 2004), but this assignment is tenuous; the roles of DECAY, DAMM, and STRICA in the caspase cascade at this point remain ambiguous.

A critical element of the caspase activation pathway is the substrate specificity of each caspase. The canonical caspase substrate is the peptide sequence DxxD, where x represents any amino acid; however, individual caspases have more specific substrate requirements. Effector caspases like DCP-1, DRICE, and DECAY typically recognize the sequence DEVD (Kumar and Doumanis, 2000). DRONC, the only known *Drosophila* caspase capable of cleaving a peptide ending in either glutamate or aspartate, can cleave TQTE, TETD, and IETD as well as DEVD (Hawkins et al., 2000). Because DRONC must proteolytically activate itself as well as other caspases, it must be capable of recognizing a site toward which proteases (including the other caspases) have no activity. Were this not the case, DRONC would be susceptible to activation even in the absence of pro-apoptotic signaling.

The technique of RNA interference (RNAi) has been fruitfully applied to the study of cellular processes in *Drosophila* (Clemens et al., 2000). Because the regulatory molecule DIAP1 is an ideal RNAi target, we used the technique to examine patterns of caspase activation in *Drosophila* S2 cells. We first determined which of the seven *Drosophila* caspases are expressed in S2 cells and found that DECAY is the only caspase absent. We next used RNAi targeted toward each caspase in conjunction with DIAP1 in an attempt to produce partial

rescue from DIAP1-induced apoptosis. Consistent with expectations, we found that DRONC, DRICE, and DCP-1 confer strong resistance to apoptosis triggered through the DIAP1 pathway. Finally, we employed biochemical assays of caspase activity to explore the order and magnitude of caspase activation in apoptotic cells.

## Materials and Methods

### Cell Culture and Survival Assays

*Drosophila* S2 cells were cultured in liquid Schneider's media supplemented with inactivated fetal calf serum and penicillin-streptomycin to aid in the prevention of bacterial contamination. Cultures were maintained at 25°C and split once every 3-4 days. Cultures were 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 does not distinguish between apoptotic and necrotic cell death, well-maintained cultures should have a minimal amount of necrosis. Viable cells were counted by visual inspection of a hemacytometer grid with a light microscope.

### Expression Profiling by RT-PCR

Total RNA was extracted from a population of non-apoptotic S2 cells (7.16 RNeasy protocol). The resulting RNA was briefly treated with DNase to remove contaminating DNA before subjected to two-step reverse transcriptase-PCR (RT-PCR; 7.16 RT-PCR protocol) to produce a cDNA pool. PCR was performed in 25 $\mu$ L volume per reaction with the primers shown in Table 2. Stock primers for the ribosomal RNA RpS17 were used as a control to confirm PCR success and to semi-quantitatively compare RNA volume between extracts. The PCR procedure was adapted from Hild et al. (2003) and consisted of 30 seconds denaturation at 94°C, 30 seconds annealing at either 55°C (A) or 65°C (B), and 90 seconds extension at 72°C. After an initial 5-minute denaturation step at 94°C, 10 cycles of procedure A and 25 cycles of procedure B were run, followed by a final 5-minute extension at 72°C.

## Preparation of Double-Stranded RNA

*In vitro* transcription (IVT) was used to prepare double-stranded RNA (dsRNA) for RNAi transfections. Commercially available DNA clones were purchased from OpenBiosystems where available; DNA for the expressed caspase for which no clone was available (STRICA) was obtained by performing PCR on total RNA extractions using primers with T7 extensions. Due to persistent low yields, slight modifications to the standard protocol (7.16 IVT protocol) were made: we used 3 $\mu$ g of DNA template, precipitated the resulting RNA with 30 $\mu$ g LiCl solution, and washed each pellet twice with 70% ethanol in DEPC-treated water. Concentration was determined by measuring the light absorbance at 260nm, and each product was diluted to a final concentration of 1 $\mu$ g/ $\mu$ L for later use.

**Table 2: Primers and reference numbers for expression profiling of each caspase.**

<i>Caspase</i>	<i>Forward Primer</i>	<i>Reverse Primer</i>	<i>CG number</i>
DRONC	GACAATGGTGACCTCCTCGT	GCCATCACTTGGCAAACTT	CG8091
DREDD	ACTACTTGCCGCATATCG	TGCTAACATTCCGGTGAAAC	CG5486
DCP1	GCGGTGGCCATACTCTC	GAACAGCTTCGGCTTGC	CG5370
DRICE	GTACAACATGCGCCACAAG	CAGGAACCGTTCGAGTAGG	CG7788
DECAY	CTCGGGAGGATCACAGTC	AATGTGGAGTAGAAGACGAG	CG14902
DAMM	GCTGGATTTGTCCTCTTTATC	TTTTTGGCTTGCCAGATAAG	CG18188
STRICA	AGAGTGGACCTCAAGGGAA	CGCAAGACCTTCACATCC	CG7863

CG numbers reference the caspases in the FlyBase and OpenBiosystems databases. All primers except DRONC were taken from Hild et al. (2003); the DRONC primers shown were tested and confirmed good by previous 7.16 students. STRICA, for which an OpenBiosystems clone was not available, was amplified using primers with the T7 attachment GAATTAATACGACTCACTATAGGGAGA at the 5' end. All primers are written 5' to 3'.

## RNA Interference Transfections

RNAi transfections were performed using serum starvation (7.16 serum starvation protocol) to encourage cells to take up exogenous dsRNA. Healthy cells cultured as described previously were centrifuged at 1000rpm for 3 minutes to remove them from serum-supplemented media and resuspended to a concentration of 1 million cells/mL in serum free media (SFM). Aliquots of 1mL were placed into each well of a 6-well plate and 5-20 $\mu$ g of dsRNA was added dropwise. Cells were incubated in SFM for approximately 30 minutes before 2mL of serum-supplemented media was added to each well. At 24-hour intervals, aliquots of 100-200 $\mu$ L were

removed after scraping the well bottoms and assayed for cell survival.

We titrated DIAP1 dsRNA with a range of concentrations between 0-20 $\mu$ g RNA per cell well to evaluate transfection efficiency and determine the amount of RNA that produced half-maximal cell death, which was used for double transfections in which both a caspase and DIAP1 were knocked down. To control for the possibility that dsRNA addition by itself could induce cell death, the total amount of dsRNA added to cell wells was kept constant within experiments by adding dsRNA targeting GFP (green fluorescent protein, a gene native to jellyfish that is not present in *Drosophila*). Because caspase half-lives were potentially longer than that of DIAP1, double transfections were performed on two separate days. Cells were transfected with 10 $\mu$ g of caspase dsRNA first, because protection from DIAP1-induced apoptosis requires thorough degradation of preexisting caspase. A total of 10 $\mu$ g of caspase dsRNA and 5 $\mu$ g of DIAP1 dsRNA (for double knockdowns) or 5 $\mu$ g GFP dsRNA (for single knockdowns) was added at least 48 and ideally 72 hours later; the additional caspase was intended to reinforce the suppression of caspase expression. The gap between transfections ensured that the caspase was sufficiently degraded before apoptosis induction and that the cells had recovered from the stress of serum-starvation before being transfected again. Each experiment included at least one and ideally three control wells containing cells transfected only with GFP dsRNA, and between one and three wells containing cells transfected with DIAP1 dsRNA on the second day. These controls allowed us to establish growth rates over time for normal and apoptotic cells.

## **Caspase Activity Assays and UV-Induced Apoptosis**

In preparation for caspase activity assays, it was useful to induce apoptosis more quickly and efficiently than by DIAP1 knockdown. Healthy cells or caspase single knockdowns were irradiated with ultraviolet light of 254nm wavelength at  $4.8 \times 10^6 \mu\text{J}/\text{min}$  for 12.2 minutes (adapted from Wright et al. 2005). Cells were incubated for 24 hours before being harvested and counted.

Cell lysis and caspase activity assays using the BIOMOL QuantiPak Kit were performed according to the manufacturer's instructions. Briefly, cells were pelleted, washed with PBS, and resuspended to a concentration of approximately  $2 \times 10^5$  cells/ $\mu\text{L}$  in the recommended lysis buffer (50mM HEPES pH 7.4,

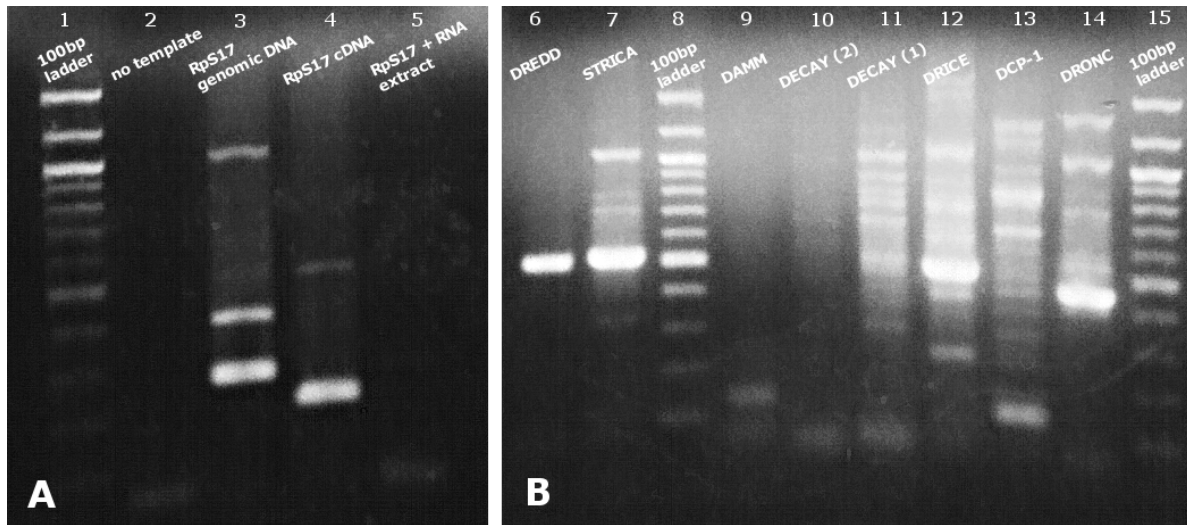
0.1% CHAPS, 1mM DTT, 0.1mM EDTA). After a 5-minute incubation period on ice, cells were centrifuged again at 4°C and the cytosolic-extract-containing supernatant was used for assay preparation.

Each extract was assayed for activity against the substrates DEVD-pNA, IETD-pNA, and YVAD-pNA. The *p*-nitroaniline absorbs light only when cleaved from the peptide, providing a colorimetric assay for caspase activity against each substrate. Assays were carried out in 100 $\mu$ L volume consisting of 80 $\mu$ L fresh assay buffer (50mM HEPES pH 7.4, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA, 10% glycerol), 10 $\mu$ L peptide substrate, and 10 $\mu$ L cell extract. Absorbance readings at 405nm were taken at 5 and 30 minutes after reaction initiation, although the 30-minute readings proved unreliable (possibly due to degradation of the free *p*-nitroaniline).

## Results

### Expression Profiling

We performed RT-PCR on total RNA extracts from non-apoptotic cells to determine which of the seven *Drosophila* caspases were expressed in S2 cells. The resulting PCR products were run on 1% ethidium bromide agarose gels and visualized by UV irradiation as pictured in Figure 2. The same primers were also used in PCR against genomic DNA to confirm successful amplification of each target (data not shown). Although the PCR procedure tended to produce a significant amount of background priming, probably due to the large number of cycles, the most prominent band in each lane corresponds to the correct size for the transcript in question. Only DEWAY lacks a clear band at its expected size, and this result is maintained when an alternate set of primers is used (lane 10). We concluded that six of the seven caspases - all but DEWAY - are expressed in S2 cells; therefore, our RNAi experiments did not include attempts to transfect with DEWAY dsRNA.



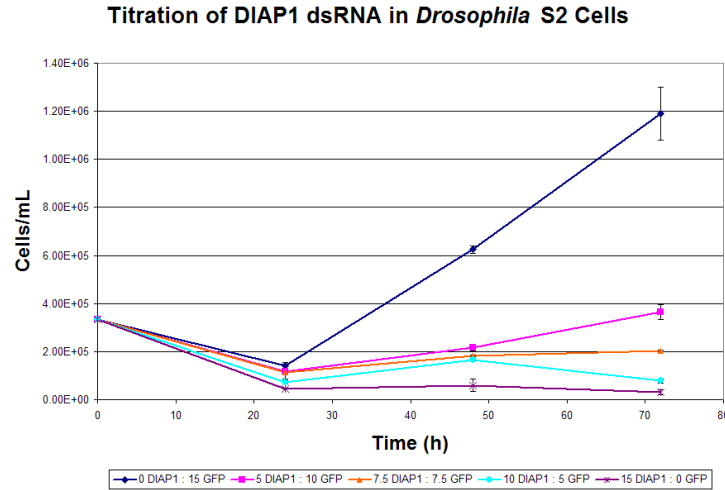
**Figure 2: Caspase expression profiling of *Drosophila* S2 cells.** (A) The lowest bright band in the ladder is 500bp. RpS17 primers are controls that amplify a segment of constitutively expressed ribosomal RNA at expected sizes of 270bp for genomic DNA and 211 bp for cDNA. The absence of a 270bp product in the cDNA or RNA extract lanes (4 and 5) reveals minimal DNA contamination. (B) Clear bands for DREDD, STRICA, DAMM, DRICE, DCP-1, and DRONC appear at the expected sizes of 504, 518, 139, 506, 132, and 504bp respectively (lanes 6,7,9,12,13,14). Lane 11, DEWAY(1), shows an absence of a strong band at the expected size of 325bp using the primers described in Table 2; DEWAY(2) primers (lane 10) were borrowed from Jay Blumling and also show no product.

## Identifying Downstream Caspases

Before initiating double knockdown experiments, we titrated DIAP1 to determine the amount of dsRNA needed for a half-maximal apoptotic effect. DIAP1 concentrations ranging from 0 $\mu$ g to 20 $\mu$ g per well (equivalent to 0-20 $\mu$ g per 1 million cells at time of plating) were tested, with GFP added to equalize the total amount of RNA to 20 $\mu$ g per well. The growth curves are shown in Figure 3. The half-maximal value was 5-7.5 $\mu$ g.

We next performed a series of double knockdown experiments using each caspase in conjunction with DIAP1 to evaluate whether inhibiting the expression of the target caspase rescued cells from apoptotic death. Initially, we used 7.5 $\mu$ g of DIAP1 dsRNA along with each caspase; however, this caused all the double knockdowns to die at rates approximately equal to the DIAP1 control (data not shown). When the amount of DIAP1 RNA was reduced to 5 $\mu$ g, we obtained the results displayed in Figure 4.



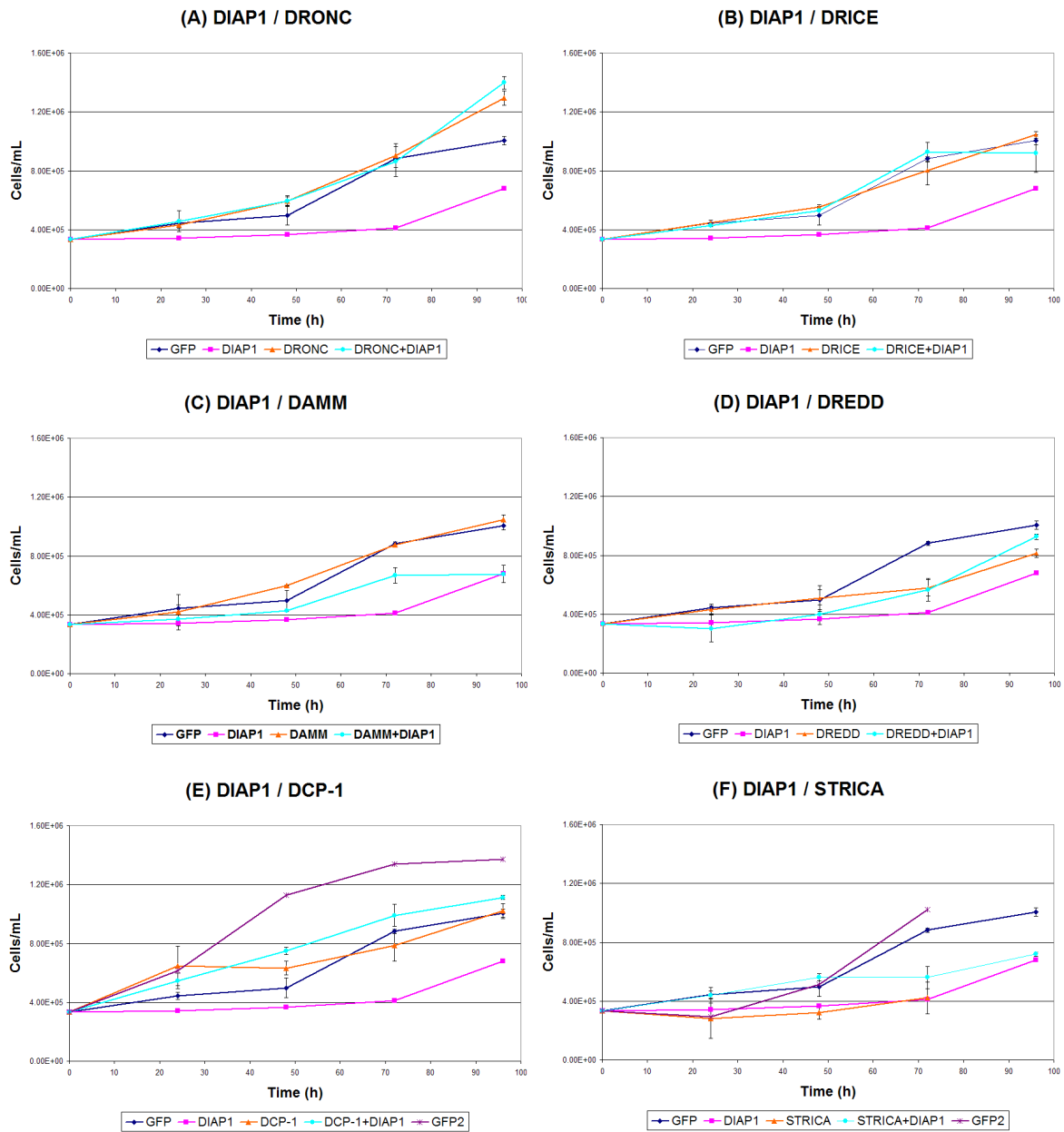


**Figure 3: DIAP1 RNAi titration.** DIAP1 dsRNA concentrations ranging from 0-20 $\mu$ g per well and equalized with GFP dsRNA to a consistent total of 20 $\mu$ g RNA added reveal a half-maximal value of about 5-7.5 $\mu$ g. Data points represent the average of two experiments and error bars indicate the range.

DRONC knockdowns appeared to completely rescue cells from DIAP1-induced cell death (Figure 4A), and in fact both the DRONC single knockdown and the DRONC/DIAP1 double knockdown proliferated slightly more than cells transfected with GFP, suggesting that suppression of DRONC decreases the basal rate of cell death in otherwise healthy cell populations. DCP-1 and DRICE knockdowns conferred almost total resistance (Figure 4B,E). DAMM knockdowns (Figure 4C) appeared to slightly suppress the apoptotic phenotype at 72 hours, but this effect was all but eliminated by 96 hours, suggesting that knocking down DAMM has at most a small protective effect. The results for DREDD and STRICA are more difficult to interpret; the DREDD/DIAP1 double knockdown actually proliferated more by 96 hours than the DREDD single knockdown, although both proliferated more than DIAP1 alone (Figure 4D). From this latter observation we suggest that DREDD knockdowns as well may have a small protective effect. Finally, STRICA single knockdowns seemed to inhibit cell proliferation by themselves (Figure 4F).

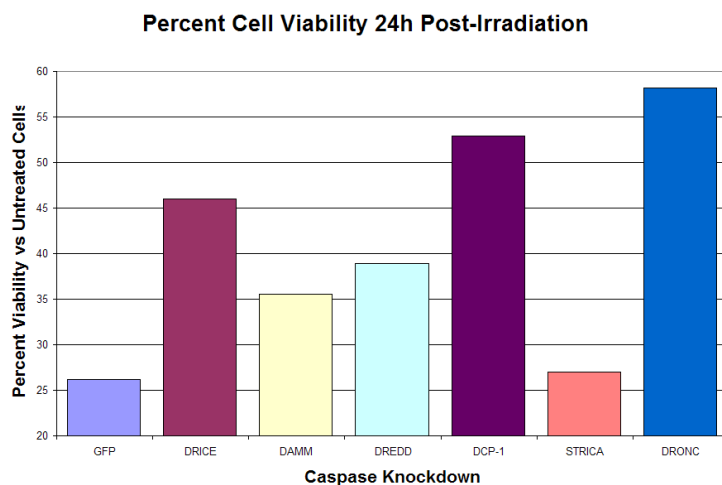
## Examining Caspase Activity

Biochemical assays of caspase activity in cell lysates complemented our transfection results and provided information about feedback effects in the caspase activation pathway. Because caspases are not active



**Figure 4: Double transfections of DIAP1 and expressed caspases.** With the exception of the double knockdowns in (E) and (F), which were done in triplicate, each data point represents the average of two wells and error bars represent the range. Note that the error in DIAP1 measurements is very small. On each graph is displayed a DIAP1 transfection, a caspase single knockdown, a caspase/DIAP1 double knockdown, and a GFP control. (A), DRONC; (B), DRICE; (C), DAMM; (D), DREDD; (E), DCP-1; (F), STRICA. In (E) and (F), single and double knockdowns were transfected at different times with cells at different ages. In (E), GFP, DIAP1, and DCP-1 were performed at the same time, and GFP2 represents a single GFP control well transfected at the same time and with the same cells as DCP-1/DIAP1. In (F), GFP, DIAP1, and STRICA/DIAP1 were transfected at the same time, and GFP2 represents a single GFP control well transfected at the same time and with the same cells as the STRICA single knockdown.

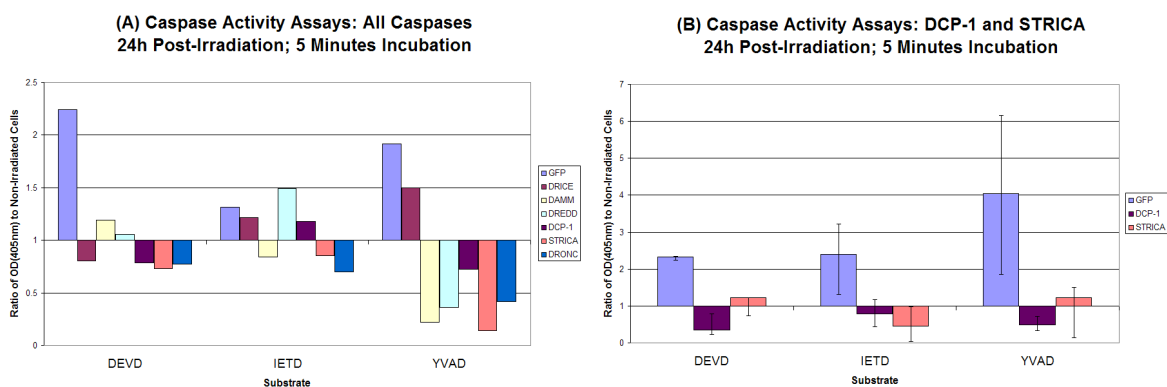
in healthy cells, measurements of caspase activity had to be made on apoptotic populations; however, as described previously, some caspase knockdowns confer near-complete resistance to apoptosis induced by DIAP1. Singly transfected cells in which caspases had been knocked down were therefore exposed to UV light as described (see Materials and Methods), which is a harsher treatment than transfection with half-maximal DIAP1. Percent cell viability 24h post-irradiation is shown in Figure 5. Consistent with our transfection results, DRONC, DRICE, and DCP-1 knockdowns confer greater resistance to apoptosis than do DAMM or DREDD knockdowns, although all five display some suppression of the apoptotic phenotype. STRICA knockdowns fare no better than GFP-transfected irradiated cells, but this is not surprising, since STRICA single knockdowns seem to kill cells on their own. UV-irradiated single knockdowns were not tested.



**Figure 5: Percent cell viability at 24 hours post-irradiation.** Data points display the number of cells in UV-treated caspase knockdown or GFP-transfected populations as a percentage of non-irradiated controls incubated under the same conditions.

Cell lysates were made 24h post-irradiation and assayed with the peptide substrates DEVD, IETD, and YVAD. Results are presented in Figure 6 as the ratio of the optical density of each lysate relative to un-irradiated controls. Because the substrate peptides are coupled to a *p*-nitroaniline ring that is colored only when free in solution, higher optical density relative to untreated controls implies higher levels of caspase activity. As expected, GFP-transfected control cells showed increased activity toward DEVD, the canonical caspase substrate, as well as toward the DRONC-specific substrate IETD. YVAD was expected to be inert,

as it is reported to be in apoptotic mammalian cells (BIOMOL QuantiPak Product Data Sheet); however, cleavage of YVAD is significantly upregulated in apoptotic cells. Figure 6 displays results after a 5-minute incubation at room temperature; data was also obtained after a 30-minute incubation period (not shown), but a decline in optical density in some samples between 5 and 30 minutes led to concerns about *p*-nitroaniline degradation on this timescale.



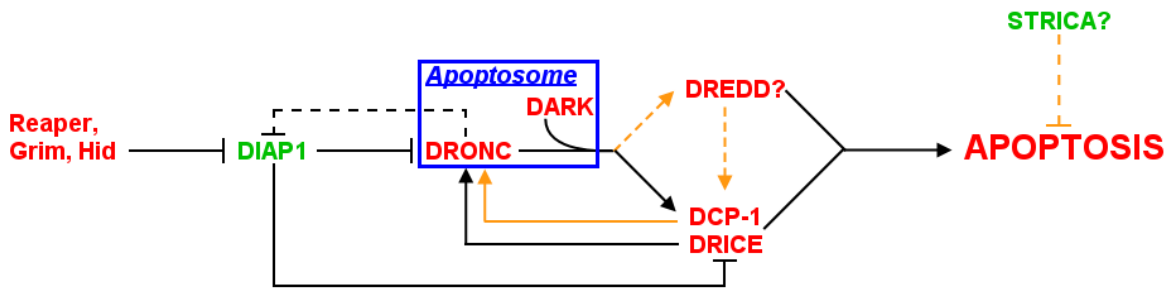
**Figure 6: Caspase activity assays of UV-treated caspase single knockdowns.** Data is presented as the ratio of the  $OD_{405nm}$  of each knockdown or of GFP-transfected cells to the  $OD_{405nm}$  of non-irradiated control cells incubated under the same conditions. **(A)** All caspases. Cells were irradiated as described in Materials and Methods and lysed 24 hours post-irradiation. Each data point represents a single assay. **(B)** DCP-1 and STRICA. Cells were prepared as in **(A)**, but since these caspase knockdowns were assayed as part of two experiments, data points displayed here represent the average of two assays, and error bars represent the range. Note the high variability in the YVAD measurements.

The caspase knockdowns all display cleavage behavior that is very distinct from that of GFP-transfected irradiated cells. Although we did not obtain sufficient RNA to perform RT-PCR on the cells in our transfection experiments, we can reasonably expect that this substantial difference in activity toward caspase substrates reflects appreciable knockdown of the target caspase in each case.

## Discussion

Although we embarked on this research project with the stated goal of identifying which of the expressed caspases are required for apoptosis in S2 cells, the data suggest that apoptosis induction is more subtle than is implied a binary classification of “required” or “redundant.” DRONC, DRICE, and DCP-1 knockdowns largely or totally suppress apoptosis induced by transfection with half-maximal amounts of DIAP1, and

reduce apoptosis induced by UV irradiation by about 40-50% . Since UV light is known to activate the pro-apoptotic signaling peptide Reaper and the apoptosome component DARK (Zhou and Steller, 2003), as well as inducing the processing of DRONC to its active form (Muro et al., 2002), it is reasonable to assume that UV irradiation triggers apoptosis through the DIAP1 pathway. Our data imply that, under the extremely severe cellular stress induced by UV irradiation, a moderate degree of caspase-dependent apoptosis can occur even in when these “required” caspases are severely depleted. Since we were not able to isolate enough RNA for RT-PCR, we cannot determine how much of each caspase transcript remained after RNAi or whether this amount was likely to have been sufficient to induce death under severe stress. Figure 7 suggests a model of the apoptosis pathway modified to account for our observations; interactions that are suggested by our transfection and activity assay data are shown in orange.



**Figure 7: The revised *Drosophila* apoptosis pathway.** Interactions that were suggested by our data are indicated by orange lines. Originally adapted from Richardson and Kumar, 2002.

In the cases of DRONC, DRICE, and DCP-1, the caspase activity assays are consistent with expectations and suggest positive-feedback interactions between the three molecules. All three knockdowns significantly decrease DEVD cleavage to levels below those of non-irradiated cells, which was expected because the effector caspases DRICE and DCP-1 preferentially cleave DEVD, and DRONC’s main role in apoptosis induction is to activate the effector caspases. Similarly, DRONC knockdowns have the lowest IETD cleavage of any lysate tested, and DRONC is the only *Drosophila* caspase known to cleave IETD. This latter fact implies that, unless other caspases have previously unknown activity toward IETD, the remaining knockdowns exert their effects on IETD cleavage via an effect on DRONC. Since DRICE is known to participate in a positive-feedback loop by which it activates DRONC (Yan et al., 2004), and DCP-1 is generally closely related

to DRICE (Kumar and Doumanis, 2000), these two effector caspases' effects on IETD cleavage is readily explicable. In fact, one question that arises from the data is why these knockdowns do not have an even stronger negative effect.

The behavior of DRONC, DRICE, and DCP-1 knockdowns under pro-apoptotic conditions was relatively predictable based on the *Drosophila* apoptosis literature, which focuses mainly on these three caspases in characterizing the activation of the apoptotic pathway. Fewer studies have characterized DREDD, DAMM, and STRICA, and our data on them is more equivocal. DREDD seems to confer mild resistance to DIAP1-induced apoptosis. In the case of DAMM, partial rescue from apoptosis appears to have occurred at 72 hours, but the effect is reversed at 96 hours (Figure 4C). It is not clear without additional replicates which of the two datapoints more accurately reflects the behavior of the cell populations. Finally, the data on STRICA is difficult to interpret. We have consistently observed that STRICA single knockdowns kill cells to about the same extent as DIAP1, while STRICA/DIAP1 double knockdowns proliferate somewhat more. This is in contrast to the observations of Doumanis et al. (2001), where overexpression of STRICA in *Drosophila* SL2 cells was found to induce apoptosis. Since caspases are almost always pro-apoptotic, it is not clear why suppression of STRICA should promote cell death. Fruitful further exploration of this phenomenon would include DNA extraction to distinguish between apoptotic and necrotic death, as well as additional replicates of STRICA single knockdowns with a new preparation of dsRNA to control for the remote possibility of cytotoxic contamination.

The behavior of DREDD, DAMM, and STRICA knockdowns in activity assays is similarly perplexing. The significant decrease in DEVD cleavage in DREDD knockdowns lends credence to the hypothesis that it plays a role in DRONC-induced activation of downstream effectors (Richardson and Kumar, 2002). However, our data shows that the DREDD knockdown actually increases IETD cleavage relative to GFP-transfected irradiated cells. The reason for this is not readily apparent, although it is not known whether this is a reliably reproducible effect. Of all the caspase knockdowns, DAMM has the smallest effect on DEVD cleavage, although DAMM knockdowns are still deficient relative to GFP-transfected apoptotic cells. DAMM also greatly decreases IETD cleavage, although it has not been reported to interact with DRONC. Finally,

STRICA is again a puzzle, since STRICA knockdowns eliminate both DEVD and IETD cleavage, yet purified STRICA does not cleave any known caspase substrate (Doumanis et al., 2001).

The ability of GFP-transfected irradiated cells to cleave the substrate YVAD was also quite surprising, since YVAD was expected to be inert. It is included in the assay kit as an inert control that is not cleaved in mammalian cells, and has also been used for the same purpose in studies of *Drosophila* caspases (e.g., Hawkins et al., 2000). In our hands YVAD provided the least consistent results of the three substrates, although this effect could be due to the incidental fact that it was always assayed last. Furthermore, all caspase knockdowns dramatically decreased YVAD cleavage, although DRICE knockdowns seemed to have the least effect. Results for STRICA proved inconsistent, with one assay showing activity close to that of GFP-transfected cells, and another showing a substantial decrease in activity. Because so many caspases eliminated YVAD activity, it is impossible to suggest that a particular caspase is responsible. YVAD cleavage may even be due to an entirely unrelated protease. Kondo et al. (1997) report YVAD cleavage activity in S2 cells during Fas-induced, but not DIAP1-induced, apoptosis; however, they were not able to isolate and characterize the protein or proteins responsible, and there seems to have been little followup on this result.

More detailed data could be obtained from caspase activity assays performed under more optimal conditions. Monitoring the reaction at higher time resolution, rather than at discrete and widely disparate timepoints, would provide key data on the kinetics of the reactions catalyzed by *Drosophila* caspases. The kinetics of mammalian caspases have been extensively characterized using this method, so its application to *Drosophila* caspases would be useful. A full kinetic study could also examine in more detail whether the free *p*-nitroaniline molecule is degraded by *Drosophila* cell lysates. Additionally, comparison of an irradiated caspase/DIAP1 double knockdown to the corresponding irradiated single knockdown would provide more detailed information about whether and to what extent basal substrate cleavage is perturbed by the suppression of caspase expression. Finally, we had limited amounts of time and limited numbers of cells to work with, so only DCP-1 and STRICA assays were repeated more than once; one of our two attempts to perform these assays on all six caspase knockdowns yielded too few cells to obtain significant absorbance readings. In the cases of DCP-1 and STRICA the two experiments gave very similar results for both DEVD and IETD,

with more variability in the YVAD data.

Overall, we find strong experimental evidence for the hypothesis that DRONC, DRICE, and DCP-1 are required for DIAP1-mediated apoptosis in *Drosophila* S2 cells. Caspase activity assays further suggest that both DRICE and DCP-1 activate DRONC in a positive-feedback loop in the course of caspase activation. We also find clear evidence that DEWAY is not expressed in S2 cells; because DEWAY is reported to be an effector caspase, it may be redundant with DRICE and DCP-1. DREDD and DAMM do not seem to be required for apoptosis, but suppressing their expression does yield a small protective effect against apoptotic cell death. The effects of STRICA on apoptosis remain difficult to discern.

## Acknowledgements

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