

The *Drosophila* caspases *Strica* and *Dronc* function redundantly in programmed cell death during oogenesis

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Programmed cell death (PCD) in the *Drosophila* ovary occurs either during mid-oogenesis, resulting in degeneration of the entire egg chamber or during late oogenesis, to facilitate the development of the oocyte. PCD during oogenesis is regulated by mechanisms different from those that control cell death in other *Drosophila* tissues. We have analyzed the role of caspases in PCD of the female germline by examining caspase mutants and overexpressing caspase inhibitors. Imprecise *P*-element excision was used to generate mutants of the initiator caspase *strica*. While null mutants of *strica* or another initiator caspase, *dronc*, display no ovary phenotype, we find that *strica* exhibits redundancy with *dronc*, during both mid- and late oogenesis. Ovaries of double mutants contain defective mid-stage egg chambers similar to those reported previously in *dcp-1* mutants, and mature egg chambers with persisting nurse cell nuclei. In addition, the effector caspases *drice* and *dcp-1* also display redundant functions during late oogenesis, resulting in persisting nurse cell nuclei. These findings indicate that caspases are required for nurse cell death during mid-oogenesis, and participate in developmental nurse cell death during late oogenesis. This reveals a novel pathway of cell death in the ovary that utilizes *strica*, *dronc*, *dcp-1* and *drice*, and importantly illustrates strong redundancy among the caspases.

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The *Drosophila* caspases include three initiators with long prodomains – *dronc* (*Drosophila* *Nedd-2-like* caspase), *dredd* (*Death-related ced-3/Nedd2-like*) and *strica*, and four effectors – *dcp-1* (*death caspase-1*), *drice* (*Drosophila ice*), *damm* and *decay*.¹ Mutations have been reported in *dronc*, *dredd*, *decay*, *dcp-1* and *drice*.^{1–10} Embryos maternally and zygotically mutant for *dronc* display head involution defects and a significant decrease in apoptotic cells.^{4,5} Mosaics and escapers exhibit defects in eyes, wings, spermatid individualization and programmed cell death (PCD) of larval salivary glands.^{4–7,11} *dredd*, however, functions primarily in the immune response.² *drice* mutant embryos show decreased PCD, as well as defects in eye, arista and wing development.^{9,10} *dcp-1* is required for germline PCD in mid-oogenesis.³ While some adults contain extra bristles¹² and there is reduced apoptosis in eye discs,⁸ mutants show no other defects in PCD.

Caspases have been shown to display redundancy, with one caspase compensating for loss of another. For example, single mutations in mammalian caspases-3 and -7 resulted in

healthy mice, whereas double knockouts displayed cardiac defects and died shortly after birth.¹³ In the *Drosophila* eye, RNA interference (RNAi) depletion of *drice* and *dcp-1* suppressed *head involution defective* (*hid*)-induced death better than removal of either caspase alone.¹⁴ *dcp-1*; *drice* double mutants have extra arista branches and decreased levels of embryonic apoptosis, compared to single mutants.^{9,10} The failure of *dronc* mutants to display a complete loss of PCD raises the possibility of redundancy with another caspase.⁵ Clues toward the identity of this caspase may come from RNAi studies showing that *dronc* and *strica* suppressed *hid*-induced PCD better than single RNAi depletions.¹⁴

PCD in the *Drosophila* ovary is less well understood than in other tissues. Individual egg chambers are composed of an oocyte and 15 germline-derived nurse cells, surrounded by approximately 650 somatic follicle cells.¹⁵ Egg chambers proceed through 14 stages (st) of development, and toward the end of oogenesis transport (dump) their cytoplasmic contents into the growing oocyte. After dumping, fragmented

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Abbreviations: bp, basepairs; *dark*, *Drosophila* *Apaf-1-related killer* (Flybase: *Ark*); *Df*, deficiency; *DN*, dominant negative; *dronc*, *Drosophila* *Nedd-2-like* caspase (Flybase: *Nc*); *diap1*, *Drosophila* *inhibitor of apoptosis protein 1* (Flybase: *thread*); *dcp-1*, *death caspase-1*; *drice*, *Drosophila* *Ice*; *dredd*, *Death-related ced-3/Nedd2-like*; *hid*, *head involution defective* (Flybase: *Wrinkled*); *GLC*, germline clone; *NGT*, *nanos-Gal4-tubulin*; *PCD*, programmed cell death; *RNAi*, RNA interference; *RT-PCR*, reverse transcriptase polymerase chain reaction; *st*, stage; *strica*, *serine/threonine rich caspase* (Flybase: *dream*)

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and condensed nurse cell nuclear remnants are phagocytosed by the surrounding follicle cells.¹⁶

In addition to developmental nurse cell death during late oogenesis, entire egg chambers can undergo degeneration during mid-oogenesis in response to limited nutrients, certain chemicals, defects in hormone signaling, or poor environmental conditions.¹⁷ In contrast to developmental nurse cell PCD which occurs normally during the production of mature egg chambers, PCD during mid-oogenesis is thought to be the outcome of a checkpoint, where there is a final opportunity to eliminate defective egg chambers before vitellogenesis.¹⁷

PCD during oogenesis appears to be regulated differently than cell death in other *Drosophila* tissues. Reaper, Hid and Grim, which are required for almost all embryonic PCD,¹ are not required for nurse cell death.¹⁸ Although caspases are expressed at high levels just before nurse cell death,^{19–23} studies utilizing caspase reporters and inhibitors suggest that they do not play a role in developmental PCD.^{24,25} Caspase-3 activity is extremely high throughout egg chambers undergoing mid-oogenesis degeneration.²⁴ We have previously shown that the effector caspase *dcp-1* is essential for germline PCD in mid-oogenesis.³ Starved *dcp-1* mutants show st7–8 egg chambers with uncondensed nurse cells and a lack of follicle cells,³ suggesting that follicle cells died but nurse cells persisted. Overexpression of *Drosophila* inhibitor of apoptosis protein 1 (*diap1*) or the baculovirus caspase inhibitor *p35* results in mid-oogenesis defects similar to *dcp-1* mutants,^{24,25} supporting a requirement for caspases during mid-oogenesis PCD.

Here, we further analyze the role of caspases and their inhibitors in *Drosophila* oogenesis. Neither *dronc* nor *dredd* mutants were found to disrupt PCD in the ovary at either mid- or late oogenesis. Examination of a third initiator caspase, *strica*, was performed by creating deletions in the gene. Whereas small deletions in *strica* resulted in moderate defects during oogenesis, a deletion of the entire gene resulted in only a few abnormal egg chambers. Double mutants removing *strica* and *dronc* demonstrated a high frequency of egg chambers defective in both mid- and late oogenesis. Additionally, *dcp-1* and *drice* act redundantly during late oogenesis. Contrary to previous reports, our findings indicate that caspases do participate in developmental nurse cell death. We have revealed a novel pathway of PCD in the ovary that utilizes *strica*, *dronc*, *dcp-1* and *drice*, and illustrates significant redundancy among the caspases.

Results

Dynamic regulation of DIAP1 during oogenesis. To investigate the regulation of caspase activity in oogenesis, we examined the expression of the caspase inhibitor DIAP1. Immunostaining of wild-type ovarioles with anti-DIAP1 revealed that DIAP1 expression was high from the germarium until st7. It then decreased during st7–8 before increasing again at st9–11 (Figure 1a). Expression decreased again at st11, and localization of DIAP1 shifted from nuclear to cytoplasmic (Figure 1b and c). Nuclear localization in nurse cells has also been observed with a different DIAP1 antibody (K White, personal communication). These results

are consistent with previous findings on *diap1* transcript levels by *in situ* hybridization.¹⁸ Interestingly, these stage-specific decreases in *diap1* expression coincide with the stages when PCD occurs.

To further examine regulation by DIAP1, we compared DIAP1 and active caspase-3 staining. In healthy egg chambers at mid-oogenesis, active caspase-3 and DIAP1 stained reciprocally. DIAP1 was nuclear, while caspase-3 activity was found at low levels in the cytoplasm (Figure 1d–g, arrowheads). However, a startling contrast was observed in degenerating egg chambers, where very low levels of DIAP1 were found to be solely cytoplasmic and excluded from nurse cell nuclei (Figure 1d and e). Conversely, active caspase-3 in degenerating egg chambers displayed intense staining throughout the egg chambers (Figure 1f and g, arrows).

Overexpression of *diap1* or *p35* inhibits germline PCD during mid- and late oogenesis.

We also investigated the role of caspases in oogenesis by overexpressing *diap1* in the germline with the *UASp/nanos-Gal4:VP16* system.²⁶ As expected, egg chambers from *UASp-diap1; nanos-Gal4:VP16* transgenic flies displayed a significant increase in DIAP1 expression relative to controls (Figure 1a and h, i). Interestingly, overexpression lines showed a dramatic decrease in DIAP1 at st7–8 in healthy egg chambers, similar to wild-type flies (Figure 1a and h, i, arrows). *nanos-Gal4:VP16* can drive expression of *UASp-lacZ* during st7–8 (Figure 1j)²⁶; thus the lack of DIAP1 overexpression driven by Gal4 at this stage indicates that downregulation of DIAP1 is post-transcriptional. This may occur through the autoubiquitination of DIAP1 via its RING domain acting as a ubiquitin ligase.²⁷

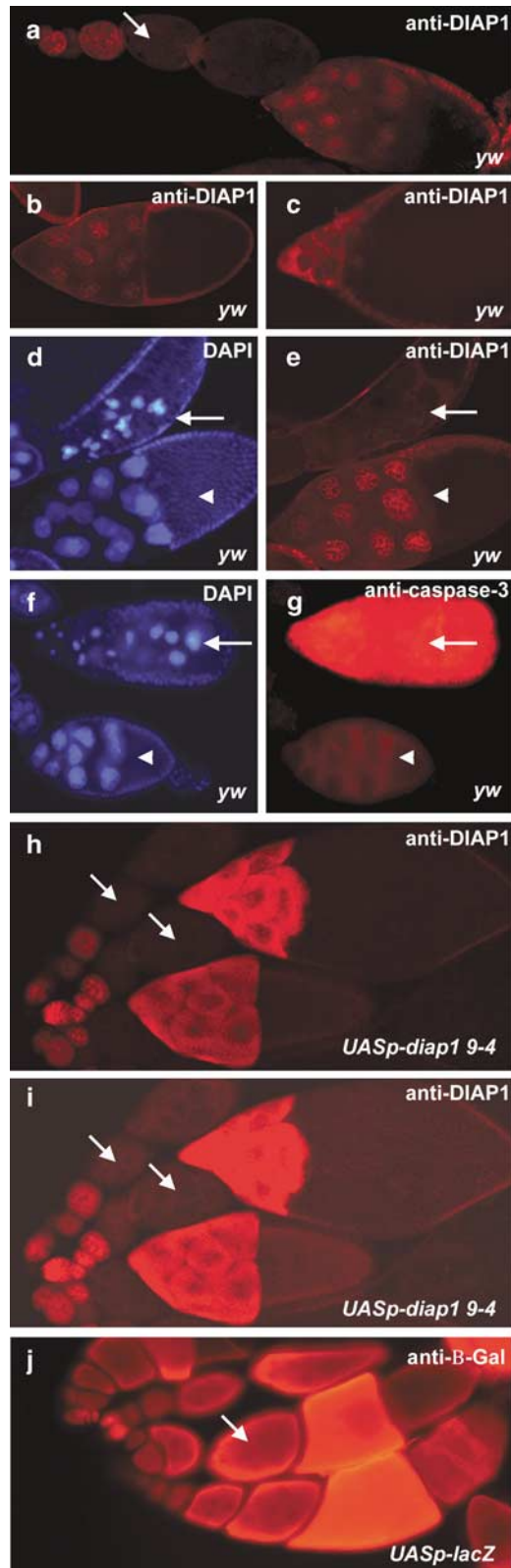
Overexpression of *diap1* or *p35* resulted in defective egg chambers during mid-oogenesis (Figure 2b) as reported previously,^{24,25} resembling *dcp-1* mutants.³ The overexpression lines displayed 93–100% defective mid-stage egg chambers, while control ovaries contained only 2% defective egg chambers (Table 1).

In *diap1* and *p35* overexpression lines, cytoplasmic transport occurred normally, indicating that caspases do not play a major role in dumping. However, *UASp-diap1* and *UASp-p35* ovaries exhibited persisting nurse cell nuclei in up to 21% of st14 egg chambers (Figure 2d, Table 1), suggesting that caspases are involved in the final stages of nurse cell PCD. Combined overexpression of *diap1* and *p35* resulted in 31% persisting nuclei (Table 1). This increased percentage in the double transgenic lines may reflect an additive effect of inhibiting two separate pathways, suggesting that *p35* and *diap1* can inhibit different caspases. Overexpression of *diap1* in the ovary did not appear to affect embryo viability but egg production was reduced in older females (data not shown).

dronc and *dredd* are not required for PCD during oogenesis.

We wished to determine which caspases were required for PCD in oogenesis. Previous studies have demonstrated that *dronc* is required for most PCDs at multiple developmental stages.^{4,5} Furthermore, mutations in *dronc* suppressed degeneration of the ovary induced by a partial loss of *diap1*.⁵ Based on these findings, *dronc* was a strong candidate to play a role in PCD during oogenesis.

dronc^{l24} and *dronc*^{l29} are both semi-lethal nonsense mutations.⁵ Surviving flies displayed extra aristal branches on the posterior axis in comparison to wild type (Figure 3a



and b), demonstrating a requirement for *dronc* in antennal development. This phenotype is similar to that seen in *hid* mutants,²⁸ suggesting that *dronc* acts downstream of *hid* in the arista.

Despite the requirement for *dronc* in other tissues, only minor defects during oogenesis were seen in either the few homozygous survivors or in germline clones (GLCs; Figure 3c and d). *dronc*^{l29} GLC ovaries displayed slight defects in mid-oogenesis PCD, where some egg chambers showed condensed nurse cell nuclei but no somatic follicle cells (Figure 3e). Normally the dying nurse cells are removed before the follicle cells die. Slight defects were also seen in

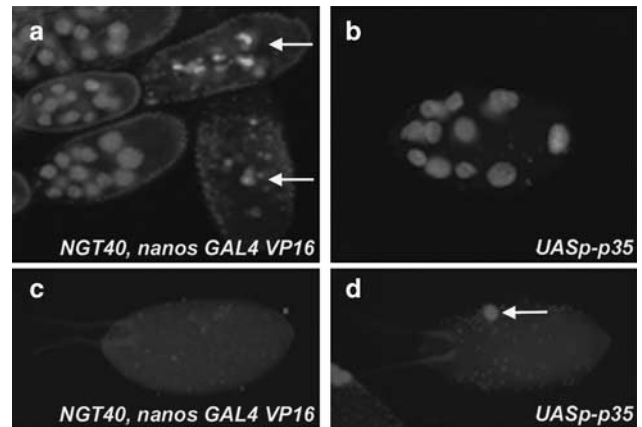


Figure 2 Overexpression of *diap1* and *p35* inhibits mid-oogenesis nurse cell death, and partially disrupts late-oogenesis nurse cell death. (a) *NGT40; nanos-Gal4:VP16* control ovaries contain degenerating egg chambers characterized by condensed nurse cell nuclei at st7–9 (arrows). (b) *UASp-p35* ovaries display defective egg chambers during mid-oogenesis, characterized by a lack of follicle cells but containing large, uncondensed nurse cell nuclei. (c) *NGT40; nanos-Gal4:VP16* egg chambers undergo condensation and phagocytosis of nurse cell nuclei by st14. (d) Approximately 20% of *UASp-p35* st14 egg chambers show uncondensed, persisting nurse cell nuclei (arrow). Egg chambers are stained with DAPI

Figure 1 DIAP1 expression and localization during oogenesis. (a–g) Wild-type egg chambers. (a) Anti-DIAP1 staining (red) is high from the germarium to st7 and at st9. DIAP1 expression is low during st7–8 (arrow). (b) DIAP1 is localized to the nurse cell nuclei before st11. (c) A st12 egg chamber demonstrating that DIAP1 becomes cytoplasmic. (d) 4',6 diamidino-2-phenylindole (DAPI) staining of a st8 degenerating egg chamber (arrow) and a healthy st10 egg chamber (arrowhead). (e) The same egg chambers as in (d), stained with anti-DIAP1. The degenerating egg chamber (arrow) displays no nuclear and very little cytoplasmic staining of DIAP1, while the healthy egg chamber (arrowhead) shows high DIAP1 staining in the nurse cell nuclei. (f) DAPI staining of additional degenerating (arrow) and healthy (arrowhead) mid-stage egg chambers. (g) The egg chambers in (f) stained with anti-active caspase-3 (red) show a pattern of staining reciprocal to DIAP1. High levels of active caspase-3 are seen in the degenerating chamber (g, arrow), while staining is present only at very low levels in the cytoplasm of a healthy egg chamber (g, arrowhead). (h) *NGT/UASp-diap1 9-4; nosGAL4VP16* ovarioles showed higher and mostly cytoplasmic anti-DIAP1 staining at st10 compared to wild type. They also demonstrated a drastic reduction in staining during mid-oogenesis (arrows), similar to wild-type ovaries (a). (i) The same image as (h) with brightness enhanced in Adobe Photoshop. (j) *NGT/UASp-lacZ; nosGAL4VP16* ovarioles stained with anti-β-galactosidase showing expression during st7–10. Arrow indicates mid-stage egg chamber. All images except (d, f and j) were visualized on the confocal microscope, while (d, f and j) were taken on a conventional fluorescent microscope

Table 1 Phenotypic quantification of *diap1* and *p35* over-expression

Transgene(s)	Gal4 copy#	Mid-stage egg chambers			Stage 14 egg chambers		
		% Degenerating	% pwop ^a	n ^b	% Normal	% Persisting nuclei	n ^c
<i>UASp-p35</i>	1	7	93	90	85	15	443
<i>UASp-p35</i>	2	ND	ND	ND	81	19	150
<i>UASp-p35</i>	3	ND	ND	ND	75	25	57
<i>UASp-p35^d</i>	2	ND	ND	ND	90	10 ^e	61
<i>UASp-diap1 9-4</i>	2	7	93	48	78	22	209
<i>UASp-diap1 15a</i>	1–2	7	93	106	95	5 ^e	512
<i>UASp-diap1 9-4; UASp-p35</i>	1	ND	ND	ND	86	14	84
<i>UASp-diap1 9-4; UASp-p35</i>	2	0	100	103	69	31	107
<i>UASp-diap1 15a; UASp-p35</i>	1	1	99	104	85	15	109
<i>UASp-diap1 15a; UASp-p35</i>	2	0	100	136	71	29	125
Control	3–4	98	2	53	95	5	362

Flies carried one copy of each UAS transgene and varying numbers of the GAL4 driver as indicated (*NGT40* and/or *Nanos-GAL4:VP16*). ^apwop (peas without pods) refers to abnormal mid-stage egg chambers with large, intact nurse cell nuclei but lacking follicle cells. ^bn is the total number of degenerating and pwop egg chambers scored. ^cn is the total number of stage 14 egg chambers scored. ^dFlies in this experiment carried two copies of *UASp-p35*. ^eAll phenotypes were statistically significant ($P < 0.05$, χ^2 -analysis) except for these

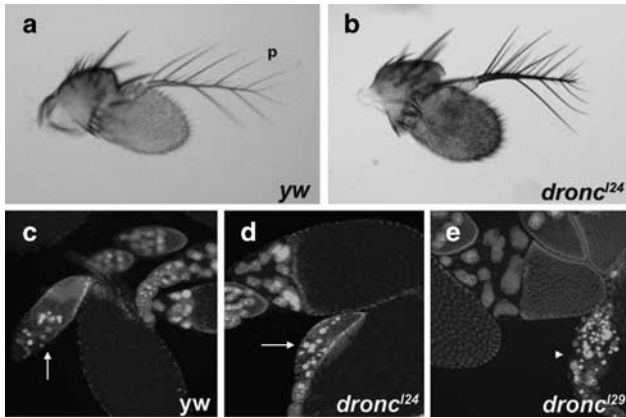


Figure 3 Mutations in *dronc* result in increased number of aristal branches but do not have a major effect on oogenesis. (a) *yellow white (yw)* flies display aristae with approximately five posterior (p) branches. (b) *dronc¹²⁴* homozygous flies contain approximately 6–8 extra branches on the posterior. In the ovary of both healthy *yw* (c) and *dronc¹²⁴* (d) flies, there are occasional degenerating egg chambers (arrows) seen at mid-oogenesis. (e) In *dronc¹²⁹* ovaries, both normally degenerating egg chambers (not shown) and abnormal egg chambers (arrowhead) at mid-oogenesis are occasionally seen, characterized by condensed nurse cells and few or no follicle cells. (c–e) were stained with DAPI

late oogenesis, as 10% of *dronc* GLC st14 egg chambers contained persisting nurse cell nuclei. The lack of a major phenotype, however, indicates that *dronc* is not required for PCD in oogenesis. Consistent with this, we found no phenotype with germline expression of dominant negative (DN) *dronc* constructs (data not shown). However, the possibility of redundancy between *dronc* and another caspase(s) still existed.

Given the absence of any significant oogenesis defects in *dronc* mutants, we analyzed a possible role for another initiator caspase, *dredd*. *dredd^{B118}* flies are viable,² and examination of their ovaries revealed no abnormal phenotype during oogenesis. This suggests that *dredd*, on its own, has no significant role in oogenesis. To determine if *dredd* shares a redundant role with *dronc*, *dredd^{B118}; UASp-droncDN/nanos-*

Gal4:VP16 flies were generated. Again, no ovary defects were observed (data not shown) indicating that *dronc* and *dredd* do not have overlapping functions. Furthermore, *dredd^{B118}dronc* GLC were examined and no defects in mid-oogenesis were observed (data not shown). This suggests that either an initiator caspase may not be required during oogenesis, or the third initiator, *strica*,²² is the key to transducing the apoptotic signal.

Generation of *strica* alleles through imprecise P-element excision. To generate mutations in *strica*, imprecise P-element excision was performed. We used the P-element insertion, *P{XP}d06491*, which was located 11 bp (basepairs) upstream of the predicted start of *strica* transcription (Figure 4a). We identified four of 295 excision lines that carried deletions in *strica* (Figure 4). While the first three deletion alleles still leave part of the coding region, *strica⁴* is a 2.63 kb deletion removing 355 bp upstream of the P-element insertion site, the entire 527aa coding region and the all of the transcribed region except the final 70 bp (Figure 4a). All alleles of *strica* are viable and fertile, and do not appear to disrupt the flanking genes.

strica mRNA levels in adult flies were examined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR), and confirmed that *strica⁴* flies are null. Furthermore, *strica²* and *strica³* were each found to express approximately 2.2-fold less *strica* mRNA than control (Figure 4b). Decreased *strica* expression was also seen in *P{XP}d06491* flies (Figure 4b). It is unclear whether the mRNA expressed in *strica²* and *strica³* flies is translated. There are several potential translational start sites that could put the truncated proteins into their normal frame. The similarity in phenotype to *P{XP}d06491* (see below) suggests that these alleles have some residual function.

***strica* participates during germline PCD in mid-oogenesis.** To determine whether *strica* plays a role in oogenesis, ovaries from *strica* mutants were examined. The original insertion line, *P{XP}d06491*, and two partial deletion alleles, *strica²* and *strica³*, showed clear defects in

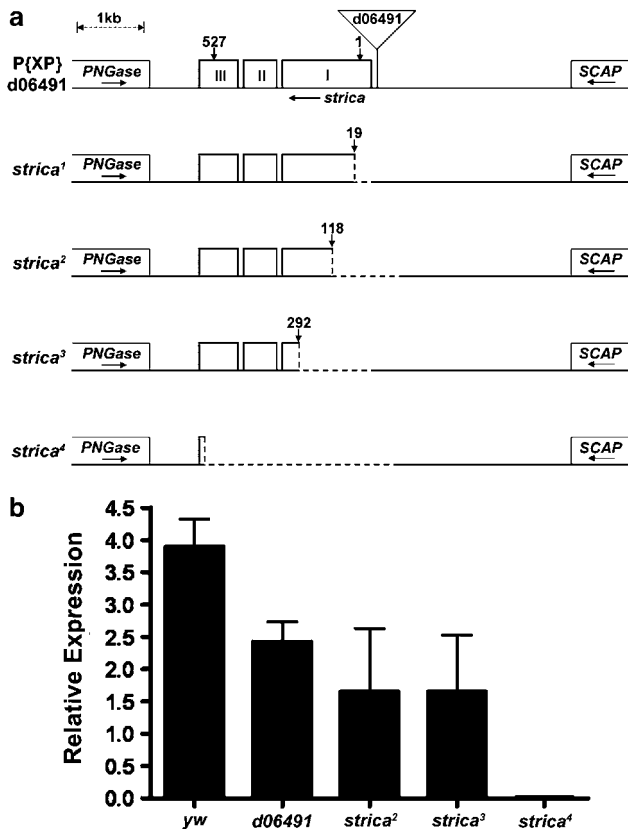


Figure 4 Genomic organization of *strica*. (a) The genomic organization at the *strica* locus for the *P*-element insertion line, P{XP}d06491, and the four deletion lines. The three exons of *strica* are labeled I, II and III, and the first and last (527) amino acid positions are indicated. Deletions are indicated by dotted lines. Everything is drawn to scale except for the size of the *P*-element. *strica*¹ is a 230 bp deletion removing the region from the *P*-element insertion site through the first 18aa. *strica*² is a 1084 bp deletion removing the first 118aa and 549 bp upstream of the *P*-element insertion, while *strica*³ is a 1047 bp deletion removing the region from the *P*-element insertion site to the first 291aa. *strica*⁴ is a 2.63 kb deletion removing 355 bp upstream of the *P*-element insertion site, the entire 527aa coding region and all of the transcribed region except the final 70 bp. (b) Quantitative real time RT-PCR of *strica* mutants. Each bar represents the average of three separate biological replicates (RNA isolations). Expression levels are shown as mean normalized mRNA expression values, taking into account primer efficiency. Error bars represent the standard error of measurement (S.E.M.)

mid-oogenesis. Approximately 30% of dying mid-stage egg chambers in *strica*² and *strica*³ (Figure 5b and f) showed defects in germline PCD, resembling *dcp-1*^{Prev1} mutants.³ Additionally, a small percentage of partially defective egg chambers resembling those seen in *dronc*¹²⁹ were found (Figure 5c and f). Surprisingly, no abnormal mid-stage egg chambers were seen in ovaries of *strica*⁴, the null allele (Figure 5f). The phenotypes observed in the partial deletion alleles are not due to a background mutation on the starting chromosome, as precise excision lines did not show the defective phenotypes. Additionally, *strica*⁴ in trans to a deletion of the region (*Df(2R)nap9*) (*Df*, deficiency) also appeared similar to wild type, while *strica*²/*Df(2R)nap9* and *strica*³/*Df(2R)nap9* displayed approximately 35% abnormal egg chambers (Figure 5f; data not shown). Furthermore, the presence of abnormal mid-stage egg chambers in *strica*²/

*strica*³ (Figure 5f) and P{XP}d06491 (not shown) ovaries suggest that partial loss of *strica* is responsible for the mid-oogenesis defects. However, the possibility of truncated proteins suggests that they could be neomorphic or antimorphic alleles.

strica alleles that displayed abnormal phenotypes during mid-oogenesis also exhibited minor abnormalities during late oogenesis. While no significant defects were seen in cytoplasmic transport, *strica*² displayed 12% of st14 egg chambers with persisting nurse cell nuclei (Figure 5e). Conversely, just as *strica*⁴ showed no significant defects during mid-oogenesis, 98% of st14 egg chambers in the *strica*⁴ null mutant appeared normal.

Despite the phenotype of *strica*² in oogenesis, we failed to see any defects in PCD during embryogenesis in *strica*² and *strica*⁴ using acridine orange to reveal apoptotic cells or a *slit-lacZ* enhancer trap which labels midline glia (data not shown). A recent study utilizing RNAi depletion has suggested that *strica* is required for the death of larval salivary glands.¹⁴ However, we did not observe any significant degree of persisting salivary glands in *strica*⁴ mutants. Additionally, *strica* mutants had minimal defects in the pattern of lattice cell apoptosis in the pupal retina (C Brachmann, personal communication).

***strica* and *dronc* display redundant functions during mid- and late oogenesis.**

The lack of any clear phenotypes in *strica*⁴ compared to *strica*² and *strica*³ suggested that *strica* could share a redundant function with another caspase that could compensate when *strica* is completely deleted. *dronc* was a strong candidate to display redundancy with *strica* based on its critical role in many other tissues that undergo PCD. To determine whether *strica* displays redundancy with *dronc*, we generated double mutants null for both caspases. *strica*⁴; *dronc*¹²⁴ and *strica*⁴; *dronc*¹²⁹ GLC females were fertile but their ovaries contained 31 and 46% of mid-stage egg chambers with missing follicle cells, but large surviving nurse cells (Figure 6b and f) resembling *dcp-1* mutants.³ Many of these contained excessive numbers of nurse cells (Figure 6c). Among st6 egg chambers, we found that 18% ($n = 117$) contained excessive nurse cells, indicating a possible defect in PCD in the germarium.^{17,29} Double mutants also showed an increase in mid-stage egg chambers with condensed nurse cell nuclei but lacking follicle cells (Figure 6f). Such phenotypes were not seen at any significant level in *strica*⁴, *dronc*¹²⁴ or *dronc*¹²⁹ alone (Figure 6f). Furthermore, no significant phenotypes were seen in *strica*²/*strica*⁴; *dronc*¹²⁹ GLCs (Figure 6f), suggesting that residual Strica activity in the *strica*² allele is sufficient for normal PCD. Egg chambers from *strica*⁴; *dronc* GLCs that degenerated normally also showed normal levels of activated caspase-3 staining (data not shown), suggesting that downstream caspases could still be activated in the absence of *dronc* and *strica*.

In late oogenesis, *strica*⁴; *dronc* GLC ovaries contained 16–21% st14 egg chambers with persisting nurse cell nuclei (Figures 6e and 7). Persisting nurse cell nuclei were not observed at any significant level in *strica*⁴ alone, and in only 10% of *dronc*¹²⁴ or *dronc*¹²⁹ st14 egg chambers (Figure 7). To verify that these phenotypes were due to the combined loss of

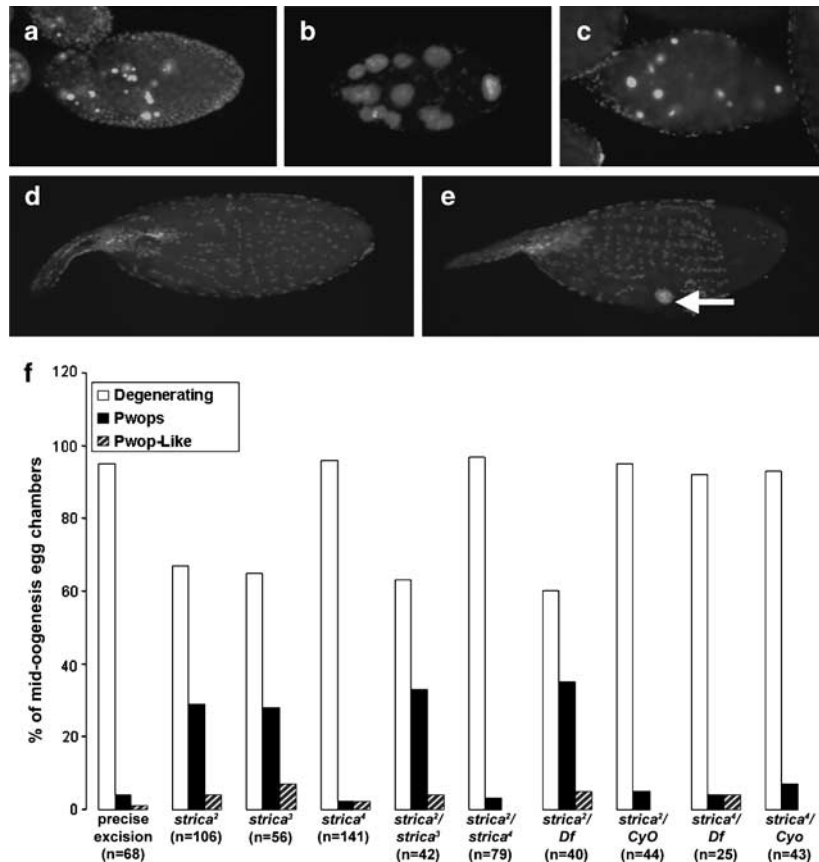


Figure 5 *strica* mutants show defects in PCD. (a–e) Egg chambers from *strica*² flies stained with DAPI. (a) Dying mid-stage egg chambers from *strica*² flies displayed phenotypes including (a) normal degenerating egg chambers, (b) defective mid-stage egg chambers characterized by large intact nuclei but a loss of follicle cells and (c) egg chambers with condensed nurse cell nuclei with a loss of follicle cells. During late oogenesis, *strica*² flies exhibited both (d) st14 egg chambers that developed normally, with no remaining nurse cell nuclei and (e) st14 egg chambers with persisting nurse cell nuclei (arrow). (f) The percentage of mid-stage egg chambers degenerating or failing to die normally was quantified in both *strica* mutants and flies that underwent precise excision of d06491. Pwop (peas without pods) refers to the egg chamber seen in (b), while pwop-like refers to that seen in (c)

strica and *dronc*, double mutants carrying a *dronc*⁺ genomic rescue transgene were generated. These flies showed completely normal degeneration during both mid- and late oogenesis (Figure 6f; data not shown).

To determine if *dredd* also acted redundantly with *strica*, we examined *dredd*^{B118}*strica*⁴ double mutants and found that they did not display any defects during oogenesis (data not shown). Additionally, *dredd*^{B118}; *strica*⁴; *dronc*^{I29} GLCs were generated and phenotypes were not more severe than *strica*⁴; *dronc*^{I29} GLCs alone (data not shown).

***dcp-1* and *drice* display redundancy during late oogenesis.** Because *dronc* and *strica* demonstrated redundancy during oogenesis, we hypothesized that other caspases may also display overlapping functions in nurse cell death. Recent studies have shown *dcp-1* and *drice* to be redundant in the embryo and arista,^{9,10} implicating them as good candidates for redundancy in the ovary as well. *dcp-1*^{Prev1} mutants display 10% persisting nuclei during late oogenesis, while *drice*¹⁷ mutants show only 5% persisting nuclei. However, *dcp-1*^{Prev1}*drice*¹⁷ double-mutant GLCs displayed 21% persisting nurse cell nuclei (Figure 7). Thus,

dcp-1 and *drice* also appear to play redundant functions during late oogenesis.

Discussion

Our previous findings with *dcp-1* and overexpression of *diap1* indicated that caspases are required for PCD during mid-oogenesis.^{3,24} This was supported by a subsequent study overexpressing *p35* in the female germline.²⁵ However, the identity of the initiator caspase(s) responsible for *dcp-1* activity was unclear, as was the role of caspases during late oogenesis. Here we have shown that the ovary utilizes a novel pathway of caspase activation during mid- and late oogenesis that depends largely on redundancy among caspases.

We examined the three initiator caspases, with no significant defects seen in *dronc* or *dredd* mutants. The lack of a phenotype in *dronc* mutants was particularly surprising, considering its importance in most tissues and its ability to suppress the ovarian degeneration caused by loss of *diap1*.^{4–8,14} Partial deletion *strica* mutants contained abnormal egg chambers during mid-oogenesis, characterized by absent or degenerating follicle cells and intact nurse cells, similar to those seen in *dcp-1* mutants.³ This indicated a role

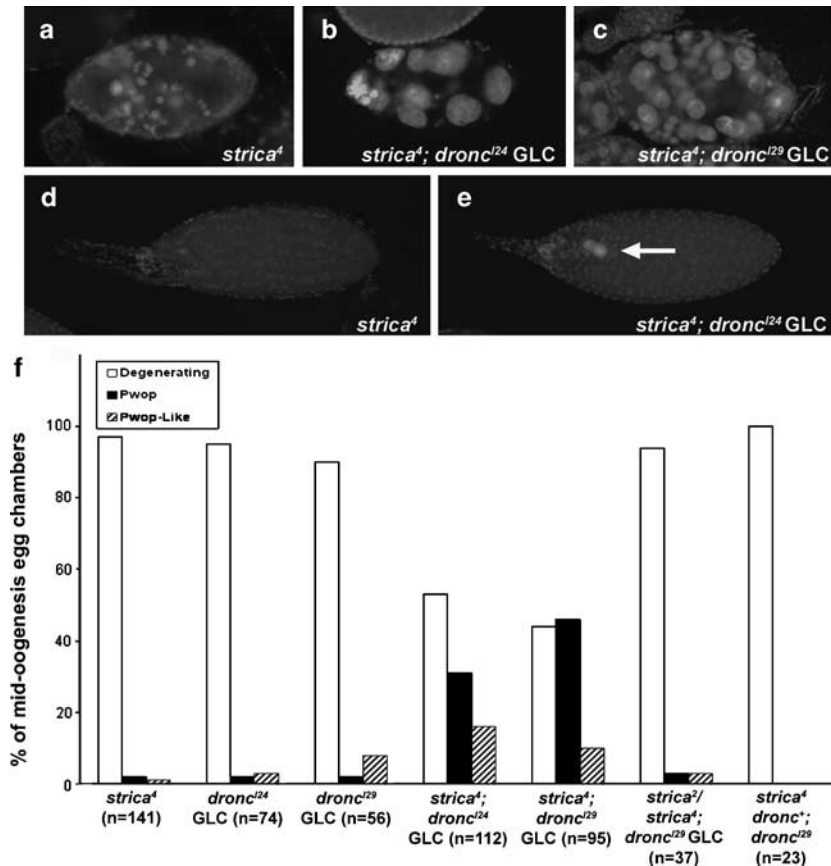


Figure 6 *strica* and *dronc* act redundantly during PCD in oogenesis. (a–e) Egg chambers stained with DAPI. (a) Dying mid-stage egg chamber from *strica*⁴ flies showing normal degeneration of the nurse cells before death of the follicle cells. (b and c) Defective mid-stage egg chambers from *strica*⁴; *dronc*²⁴ or *129 GLCs characterized by large intact nurse cell nuclei but a lack of follicle cells. These egg chambers occasionally contained more than 15 nurse cell nuclei, as seen in (c). (d) A *strica*⁴ st14 egg chamber developed normally, with no remaining nurse cell nuclei. (e) A *strica*⁴; *dronc*²⁴ or *129 GLC st14 egg chamber displays persisting nurse cell nuclei (arrow). (f) The percentage of mid-stage egg chambers degenerating or failing to die normally was quantified in both single *strica* and *dronc* mutants, and GLCs removing both caspases. Pwop (peas without pods) refers to the egg chamber seen in (b and c), while pwop-like refers to the phenotype shown previously in Figure 5c**

for *strica* as an initiator caspase participating in the regulation of cell death during mid-oogenesis. Interestingly, *strica*⁴, a null allele of *strica*, did not display any defects during mid-oogenesis. We hypothesized that this may be due to another caspase compensating for the absence of *strica*.

dronc was a strong candidate to exhibit redundancy with *strica* based on the central role of *dronc* in most PCD. Additionally, a previous study showed that *strica* and *dronc* display redundancy in the eye.¹⁴ Indeed, *strica*; *dronc* double mutants contained a high percentage of defective mid-stage egg chambers. Leulier et al.¹⁴ have previously proposed that Strica acts in a pathway parallel to a Dronc-Drice cascade. Consistent with this model, we found that *strica*; *drice* double mutants also produced egg chambers defective in PCD in mid-oogenesis (data not shown). Because *strica*; *dronc* double mutants do not display complete inhibition of mid-oogenesis PCD, as is seen in *dcp-1*^{Prev1} and overexpression of the caspase inhibitors, a third caspase may act redundantly with *strica* and *dronc*. We tested whether *dredd* might perform this role, but triple mutant GLCs did not show a stronger phenotype than *strica*; *dronc* GLCs. Alternatively, *dcp-1* may be activated through a novel mechanism of caspase

activation. The drastic downregulation of DIAP1 activity during mid-oogenesis indicates that lower initial levels of caspase activity may be sufficient to initiate the degeneration of an egg chamber. Such low levels of caspase activity would be inhibited at other stages by the higher level of DIAP1.

During late oogenesis, nurse cell nuclei normally degenerate and are removed from the egg chamber by st14. However, we found persisting nurse cell nuclei in several caspase mutant combinations. Importantly, we have found redundancy in late oogenesis between *strica* and *dronc*, and between *dcp-1* and *drice*. *strica*⁴; *dronc* GLC ovaries contained 16–21% of st14 egg chambers with persisting nurse cell nuclei, while *dcp-1*^{Prev1} *drice*¹⁷ GLC ovaries contained 21%. Persisting nuclei were observed at much lower frequencies in single mutants of *strica*, *dronc*, *dcp-1* and *drice* (Figure 7), whereas comparable levels were found when *diap1* and *p35* were overexpressed. Defective PCD in late oogenesis was likely not detected in previous studies with the inhibitors,^{24,25} because persisting nuclei only become apparent with increased Gal4 copy number.

Unlike mid-oogenesis, we found that caspase inhibition was able to disrupt developmental PCD in a maximum of 31%

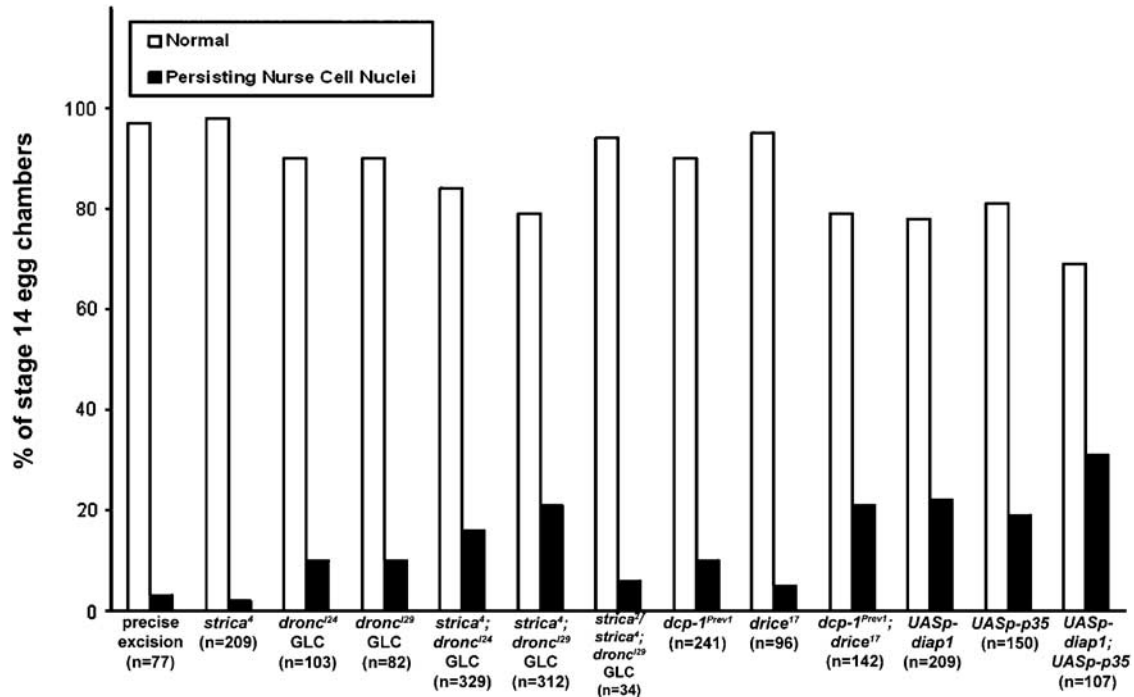


Figure 7 Caspases display redundant functions during late oogenesis. The percentage of st14 egg chambers appearing normal or containing persisting nurse cell nuclei was quantified in both caspase mutants and flies overexpressing caspase inhibitors

of st14 egg chambers. It is possible that a third caspase that is p35 and DIAP1 resistant may also be involved. Alternatively, late oogenesis may be regulated by both apoptotic and non-apoptotic forms of cell death. Redundancy between apoptosis and other forms of cell death has been demonstrated in the mammalian death receptor pathway, which normally leads to apoptosis through caspase-8. Cell death still takes place in certain cell types following inhibition of caspase-8, through a switch to either autophagy or necrosis.³⁰ Persisting st14 nuclei have also been reported in mutants lacking the transmembrane protein Spinster.³¹ Cell death induced by the human ortholog to Spinster, Hspin1, appears to include the formation of autophagic vacuoles.³² While autophagic vacuoles have been reported previously in nurse cells,³³ a recent study using GFP fusion proteins failed to see any evidence of such vacuoles.²⁵ Further study of autophagy in a background where caspases are inhibited is warranted.

Overall, our findings demonstrate that caspases are required for mid-oogenesis PCD, and play a partial role in late oogenesis PCD. During mid-oogenesis, *dronc*, *strica* and potentially another factor act redundantly, likely by activating *dcp-1*. This may occur through Strica and Dronc binding a common adaptor molecule such as Dark (*Drosophila Apaf-1-related killer*). Thus in the absence of Strica, increased Dronc binding to the adaptor may take place. Interestingly, *dark* GLCs show a phenotype similar to *strica*; *dronc* double mutants in late oogenesis but have normal PCD in mid-oogenesis (BP Bass and KM, unpublished), suggesting there could be a distinct adaptor that functions in mid-oogenesis. Alternatively, redundancy may occur through upstream components that normally initiate PCD via divergent Strica and Dronc pathways. In this model, upon disruption of Strica,

signaling through Dronc is upregulated. Although the effect on late oogenesis is not as strong as it is in mid-oogenesis, it makes economical sense that the ovary utilizes a similar mechanism of caspase activation at both stages, in which *dronc* and *strica* appear to activate *dcp-1*, *drice* and perhaps other effector caspases. Future studies may reveal a caspase-independent pathway that likely plays a role in developmental PCD in the ovary.

Materials and Methods

Fly strains and crosses. *Yellow white* flies were used as a wild-type control. P{XP}d06491 used in the creation of *strica* alleles was obtained from the Exelixis *Drosophila* stock collection at Harvard. *UASp-diap1*, *UASp-p35*, *UASp-lacZ* and *UASp-droncDN* were crossed to the *nanos-Gal4-tubulin* (*NGT40*); *nanos-Gal4:VP16* driver. *NGT40*; *nanos-Gal4:VP16* flies were obtained from Rachel Cox³⁴ and *UASp-lacZ* flies were from Pernille Rorth.²³ *drice*¹⁷ (strong loss-of-function), *dronc*²⁴ (null) and *dronc*²⁹ (null) flies were received from Andreas Bergmann,^{5,10} *dredd*^{B118} (null) and *dronc*⁺ transgenic flies were obtained from John Abrams.^{2,4} *dcp-1*^{Prev1} (null) mutants and *UASp-diap1* lines were generated in our laboratory.^{3,24} All other lines were obtained from the Bloomington stock center. Fly lines were maintained on a standard cornmeal, agar, molasses and yeast food and raised at 25°C. Because of the reduced viability of *dronc* mutants, GLCs were generated as described previously using the *FLP/FRT/ovo*^D system.^{35,36} To generate double mutant GLCs, *strica*⁴/*CyO*; *dronc* *FRT/TM6B* females were crossed to *HSlip*; *strica*⁴/*CyO*; *ovo*^D*FRT/MKRS* males and progeny were heat shocked twice at 37°C for 1 h during the third instar larval stage. The GLC-carrying *strica*⁴/*strica*⁴; *ovo*^D *FRT/dronc* *FRT* females were collected and conditioned as described below. For triple mutant GLCs, the *dredd*^{B118} mutation was crossed into the *strica*⁴/*CyO*; *dronc*¹²⁹*FRT/TM6B* stock or recombined onto the *HSlip* chromosome (BP Bass, unpublished). *dredd*^{B118}; *strica*⁴/*CyO*; *dronc*¹²⁹*FRT/TM6B* females were crossed to *dredd*^{B118} *HSlip*; *strica*⁴/*CyO*; *ovo*^D*FRT/MKRS* males and treated as described above. The *dronc*⁺ genomic transgene was recombined onto the *strica*⁴ chromosome and crossed into the *dronc*¹²⁹*FRT/TM6B* background. To confirm that the stocks were correct, the *strica*⁴ and *dredd*^{B118} mutations were verified by PCR and DNA sequencing, respectively. The presence of the *dronc*

mutation was visualized by its pupal lethal phenotype (either on its own or outcrossed to another *dronc* allele).

Transgenic flies. Three dominant-negative *dronc* constructs, *pro-dronc C>A*, ΔN *dronc C>A* and *CARD-only* (gifts from Pascal Meier, Institute of Cancer Research, London), were subcloned from *pUAS* into *pUASp* via *EcoRI* and *XbaI* sites in both vectors to yield *UASp-droncDN* constructs. For generating the *UASp-p35* construct, the pSH36-1 vector (a gift from S Shaham, the Rockefeller University) was digested with *BamHI* and *EagI*, and the fragment containing the p35 coding region was ligated into the *BamHI* and *NotI* sites of a modified version of the *UASp* vector, *pUASp-2* (a gift from A Hudson, Yale University School of Medicine). Standard *Drosophila* techniques were used to generate transgenic lines from these constructs.

Imprecise P-element excision. *P{XP}d06491* homozygous flies were crossed to *Sp/CyO*; *Sb 2-3/TM6* to mobilize the *P*-element. Single *d06491*, *w⁺/CyO*; *Sb 12-3/+* males were crossed to *CyO/Gla* females. Single male progeny of the genotype *d06491 revertant (w⁻)/CyO* were then crossed to *CyO/Gla* females to create stable lines. Genomic DNA was amplified from single flies as described.³⁷ Revertant homozygotes were screened by PCR for deletions first using primers GGATCGGTACCAGAAGAAA and GAGTGGTCGTCGTAGTGGT flanking the insertion site, which produced a 720 bp product in wild-type flies. If no PCR product resulted, PCR reactions were performed with primers TTGTGGCGTTAAAGTAGGC and CAATCCGACCCAGACTAA, which resulted in a 2.7 kb band in wild-type flies, or primers CTCGTTAAGCCGGTGTTC and CAATTGCTGGGACGATTTT, which resulted in a 4.3 kb product in wild-type flies. Both the 2.7 and 4.3 kb PCR reactions were performed with Platinum Taq Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA).

Aristal dissections and mounting. Antennae were dissected in isopropanol and transferred to a slide. Following the evaporation of isopropanol, a 1:1 ratio of Canadian Balsam and methylsalicylate (Sigma, Highland, IL, USA) was added to mount aristae. The tissue was covered with a coverslip and examined under the light microscope.

RNA isolation and RT-PCR. Isolation of RNA was performed using TRIZOL (Invitrogen, Carlsbad, CA, USA). For each sample, RNA was isolated from 15 male flies homogenized in 1 ml of TRIZOL Reagent. cDNA synthesis was performed with the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) using a random hexamer pd(N)₆ primer. cDNA was made from three different RNA preparations.

To determine relative levels of cDNA, quantitative real-time PCR was performed. A 30 μ l reaction consisted of 15 μ l of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA), 3 μ l of each primer, 1 μ l of the cDNA reaction and 8 μ l of water. *strica* primers ACGAATTCGAAAGGGAAGT and TCGTCCAGGGAGTAGTCGTC flanking the first intron were used at 5 pmol/ μ l. Control *lamin* primers GATGATATCAAGCGTCTCTG and GCCTGGTTGATTTGTTGTC were used at 3 pmol/ μ l. Samples were divided into triplicates and run on a 7900HT Sequence Detection System (Applied Biosystems). A dilution set was also performed to take into account primer efficiency. Samples were run using the cycle 50°C at 2 min, 95°C at 10 min (40 \times (95°C at 15 s, 60°C at 1 min)), 60°C at 1 min and 95°C at 15 s. Analysis of real-time PCR was performed using Q-Gene (Biotechniques), to obtain the mean normalized expression levels.³⁸ Creation of graphs and statistical analysis were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

Ovary dissections and antibody staining. Flies were conditioned on yeast paste for 4–6 days before dissection, and ovaries were dissected in *Drosophila* Ringers. Egg chambers were stained as described previously.³⁹ To quantify persisting nurse cell nuclei, only st14 egg chambers with fully formed dorsal appendages were counted. Degenerating mid-stage egg chambers occurred spontaneously or were induced by nutrient deprivation as described.²⁴ Primary antibodies included rabbit anti-cleaved caspase-3 diluted 1:100 (Cell Signaling, Danvers, MA, USA), mouse anti- β -galactosidase diluted 1:400 (Promega) and mouse anti-DIAP1 diluted 1:300 (a gift from Bruce Hay). Secondary antibodies included Cy-3-conjugated goat anti-mouse or goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:200. Samples were mounted in Vectashield with 4',6 diamidino-2-phenylindole (Vector Labs, Burlingame, CA, USA). Samples were viewed using an Olympus (Melville, NY,

USA) BX60 fluorescent microscope and digital camera (Olympus model U-TV0.5XC). Images were taken using MagnaFIRE SP and processed in Adobe Photoshop. Confocal images were taken on an Olympus Fluoview confocal microscope, and images were processed in Adobe Photoshop.

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