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Apoptotic mechanisms during competition of ribosomal protein mutant cells: roles of the initiator caspases Dronc and Dream/Strica

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Heterozygosity for mutations in ribosomal protein genes frequently leads to a dominant phenotype of retarded growth and small adult bristles in *Drosophila* (the Minute phenotype). Cells with Minute genotypes are subject to cell competition, characterized by their selective apoptosis and removal in mosaic tissues that contain wild-type cells. Competitive apoptosis was found to depend on the pro-apoptotic *reaper*, *grim* and *head involution defective* genes but was independent of p53. *Rp*/+ cells are protected by anti-apoptotic baculovirus p35 expression but lacked the usual hallmarks of 'undead' cells. They lacked Dronc activity, and neither expression of dominant-negative Dronc nor *dronc* knockdown by dsRNA prevented competitive apoptosis, which also continued in *dronc* null mutant cells or in the absence of the initiator caspases *dredd* and *dream/strica*. Only simultaneous knockdown of *dronc* and *dream/strica* by dsRNA was sufficient to protect *Rp*/+ cells from competition. By contrast, *Rp/Rp* cells were also protected by baculovirus p35, but *Rp/Rp* death was *dronc*-dependent, and undead *Rp/Rp* cells exhibited typical *dronc*-dependent expression of Wingless. Independence of p53 and unusual dependence on Dream/Strica distinguish competitive cell death from noncompetitive apoptosis of *Rp/Rp* cells and from many other examples of cell death.
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In *Drosophila*, heterozygous mutation of many ribosomal protein gene loci leads to the dominant 'Minute' phenotype, named for its small thin bristles.[1,2] Minute animals show a dominant developmental delay. In addition, Minute (that is, *Rp*/+) cells tend to be lost from mosaics that contain wild-type cells, making it difficult for clones of *Rp*/+ genotypes to survive and contribute to the adult.[3–7] Such conditional cell viability that depends on a heterotypic cellular environment is termed 'cell competition'.[4]

Competition of *Rp*/+ clones is suppressed by equalizing growth rates through starvation[8] or nonmosaic mutation of a second *Rp* locus.[4] Hyperplastic clones that express higher levels of *myc*[9,10] or lower levels of the Salvador-Hippo-Warts pathway tumor suppressors out-compete nearby wild-type cells, that is, they are 'super-competitors'.[7,11] Competition based on *c-myc* also occurs in mouse embryogenesis.[12] Differential growth is not always sufficient to cause cell competition, as cells growing rapidly due to elevated CyclinD/Cdk4 activity or higher activity of the insulin/IGF pathway are not super-competitive.[9] Differences in Jak/Stat signaling, Wg signaling and cell adhesion are also reported to generate cell competition.[13–15] These findings suggest that cell competition arises from specific interactions between cells, rather than as a general consequence of differential growth.

Apoptotic cell death is a fundamental part of cell competition. Elimination of *Rp*/+ clones is delayed by expression of the caspase inhibitor baculovirus p35.[5] Apoptosis of *Rp*/+ cells also occurs when clones of wild-type cells arise in *Rp*/+ backgrounds, predominantly among *Rp*/+ cells nearby wild-type cells.[6,16] As expected, such apoptosis is prevented by expression of baculovirus p35 or DIAP1.[6,16,17]

Cell competition has been hypothesized to contribute to human cancer, because most tumors have an altered genotype, and because many genes implicated in cell competition are homologs of oncogenes and tumor suppressors.[18–21] Cell competition may contribute to homeostasis of organ growth[4,9] and to antitumor surveillance.[22–26]

Cell competition may be a means to eliminate certain categories of aneuploid cells.[27,28] Seventy-nine ribosomal protein genes, sixty-six of which are haploinsufficient Minute loci, are distributed throughout the *Drosophila* genome.[2] Copy number changes to parts of the genome are likely to perturb relative dose of *Rp*/+ genes, and those that reduce *Rp* gene dose could be subject to cell competition. This suggests cell competition can eliminate some aneuploid cells even after DNA damage responses have ceased.[27–29]

In humans, heterozygosity for multiple different *Rp* mutations causes Diamond Blackfan Anemia.[30] Accumulation of ribosomal assembly intermediates or of unassembled ribosomal proteins in these genotypes activates p53, for example

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through the binding of the p53 ubiquitin ligase Mdm2 by RpL11 or RpL5.[31] The p53 pathway leads to cell cycle arrest and/or apoptosis,[32] and loss of hematopoietic stem cells causes anemia. Diamond Blackfan Anemia is a condition of non-mosaic individuals, so its relationship to cell competition is unclear.

The uncertain nature of the cell interactions that trigger competition might be illuminated if the initiation of competitive apoptosis was understood. The *Drosophila* genome encodes three potential initiator caspases that might be activated through long prodomains, and four effector caspase zymogens lacking prodomains that are activated by initiator caspases and by one another.[33] Here, the p53 and initiator caspase requirements for competitive cell death of *Rp/+* cells were determined. Whereas Dronc is the initiator caspase for most apoptosis in *Drosophila*,[34–37] we found that competitive cell death could occur without *dronc* or p53. Experiments that eliminated multiple initiator caspases simultaneously demonstrated that competitive apoptosis of *Rp/+* cells required Dronc and Dream/Strica redundantly, a difference from most other apoptotic genotypes in *Drosophila*, for example, *Rp/Rp* cells generated in these experiments died in a Dronc-dependent manner.

Results

Cell competition depended on Reaper, Hid and Grim.
Cells dying during cell competition are positive in TUNEL, and immunoreactive to anti-active caspase antibodies. Elimination of *RpL19/+* or *RpS3/+* clones is delayed by p35 expression,[5,6] and elimination of *RpS17/+* clones is delayed by p35 expression or DIAP1 expression.[17] These findings establish that cell competition removes cells by caspase-dependent programmed cell death.

The pro-apoptotic proteins Hid, Grim and Reaper antagonize DIAP1 so that their expression releases caspase activity from negative regulation.[38,39] The role of *rpr*, *grim* and *hid* in competitive cell death was evaluated using a deficiency, Df(3L)H99, which removes all three genes.[33,40] When clones of *RpL36/+* cells are generated in a wild-type background, making use of an *RpL36+* transgene linked to FRT80B, these cells are eliminated by cell competition[7] (Figures 1a and b). By contrast, clones of *RpL36/+*; Df(3L)H99/Df(3L)H99 cells survived in the imaginal discs and adult eye (Figures 1c and e). These findings indicated that cell competition required one or more of Reaper, Hid and Grim.

To examine expression levels, *β*-galactosidase reporters for *hid* and *rpr* were examined. When clones of wild-type cells were induced in *RpS3/+* animals, *hid-LacZ* expression was elevated in the *RpS3/+* cells (Figure 1g). This applied to all *RpS3/+* cells, however, not only cells dying through cell competition (Figure 1g). When clones of wild-type cells were induced in *RpS18/+* animals, *rpr-LacZ* expression was elevated in all the *RpS18/+* cells (Figure 1h).

Competitive cell death occurred independently of p53. A
cis-regulatory element mediates stress-induced Reaper and Hid expression and apoptosis during embryogenesis, and in response to gamma-irradiation via p53.[41] To examine

cis-regulation of *rpr* and *hid* transcription in cell competition, we made use of the IRER (irradiation-responsive enhancer region) deficiency.[41] In contrast to deletion of *rpr*, *grim* and *hid* (Figures 1d and e) loss of the IRER *cis*-regulatory region did not permit the survival of *RpL36/+* (Figure 1f). Therefore, competitive cell death did not depend on transcriptional induction through the IRER *cis*-regulatory region.

To evaluate the contribution of *p53* itself to cell competition, clones of wild-type cells were induced in *RpS18/+* animals that also lacked *p53*. Apoptosis of *RpS18/+* cells was not lowered significantly by *p53* mutation (Figure 2). *p53* null alleles also failed to rescue clones of *RpL36/+* cells from competitive elimination during development (Figures 2d–f).

Because p53 has a role in the growth stimulation of super-competitor cells overexpressing Myc,[42] we examined the effect of *p53* homozygosity in wild-type cells competing with *RpS3/+* cells. *RpS3/+* cells in contact with wild-type or with *p53* mutant cells died with equal frequency, and *p53* mutations did not significantly affect growth of wild-type cells in this context (Figures 2g–i). There was a trend to lower levels of apoptosis within *p53* mutant cells themselves compared with wild-type cells, however, which could indicate that the occasional death of wild-type cells was *p53*-dependent (Figure 2g).

Baculovirus p35 allows *Rp/Rp* cells to survive and divide.
The caspase inhibitor baculovirus p35 blocks apoptosis, but does not inhibit Dronc, the main initiator caspase for developmental cell death.[43–45] Dronc substrate cleavage in cells protected by p35 is detected by a polyclonal antiserum raised against mouse Caspase-3 called 'CM1'.[46,47] Rescued cells with persistent Dronc activity are termed 'undead',[48,49] and map locations of developmental cell death signaling.[46,50]

To map Dronc-dependent apoptosis in cell competition, p35 was expressed in the posterior compartment of *Rp/+* wing discs, and wild-type clones induced. Such p35 expression blocked competitive cell death completely, as measured by TUNEL (Figure 3a).[6] In the anterior compartments (lacking p35 expression) CM1 labeled single *Rp/+* cells at boundaries with wild-type clones, as described previously.[6] TUNEL labeling confirmed that most of these cells were apoptotic (Figures 3a and b). By contrast to the anterior compartments, CM1 did not label single posterior cells but clusters of cells, typically numbering about 10 cells and near to +/+ clones (Figure 3a). Most clusters were not of the *Rp/+* genotype. Rather, *β*-galactosidase labeling indicated the undead cell clusters were the *β*-gal *Rp/β*-gal *Rp* genotype arising as the reciprocal recombinant to +/+ cells (Figures 3a and c). Rescue by p35 indicated that the death of *Rp/Rp* cells was caspase-dependent, at least for mutations in *RpS3*.

CM1 labeling of *Rp/+* cells expressing p35 was rare (Figure 3a). No undead *Rp/+* cells were seen at boundaries between wild-type clones and *Rp/+* cells. CM1 labeling was sometimes observed, however, in p35-expressing *Rp/+* cells away from boundaries with +/+ clones (Figure 3d). These clusters were much less common than *Rp/Rp* clusters. This suggests that *Rp/+* cells protected from competitive cell death by p35 expressed little Dronc activity, less than undead *Rp/Rp* cells.
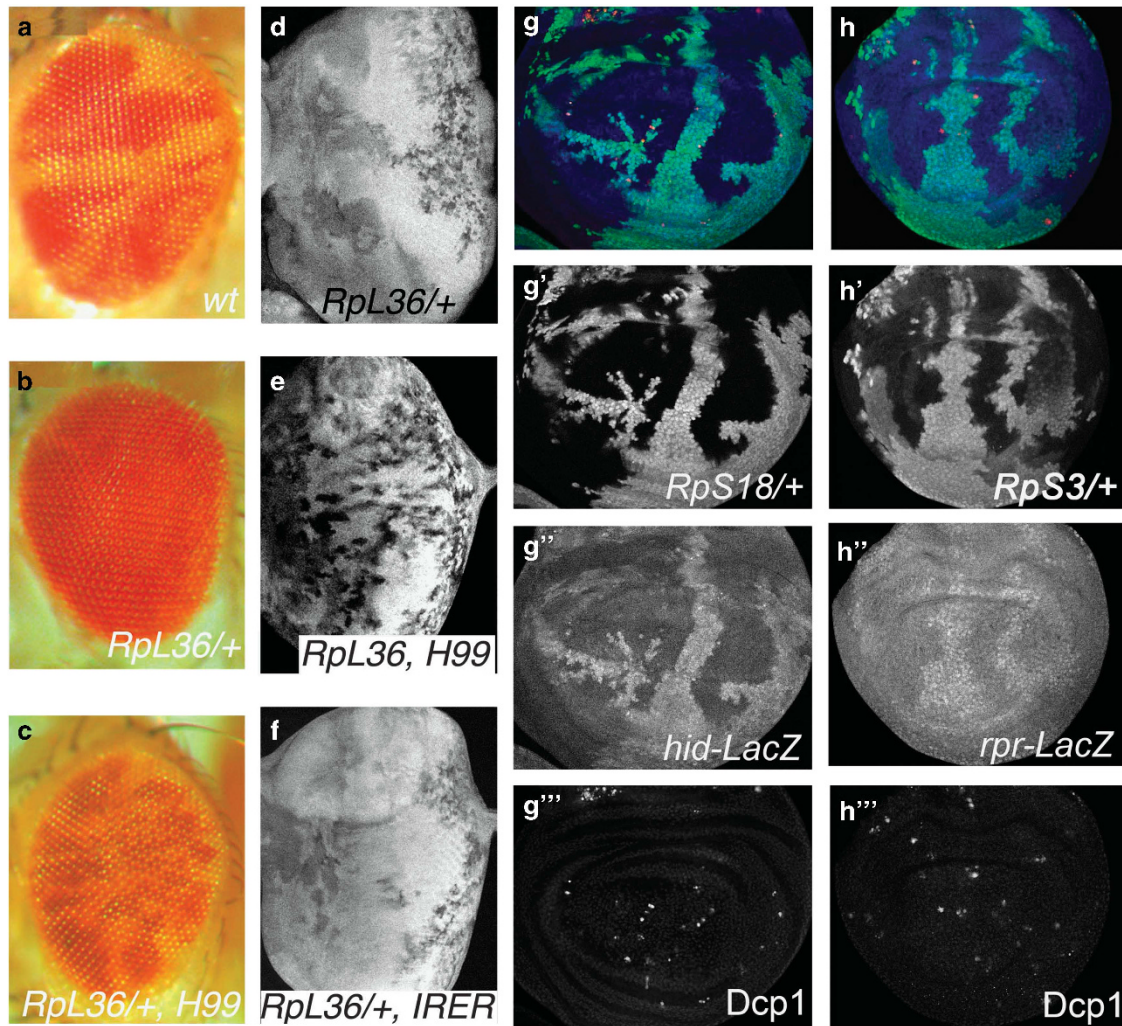
Figure 1 Cell competition depends on Reaper, Hid and Grim. (**a**) Genotype: *y w p[eyFLP ry⁺]; FRT80B/FRT80B p[w⁺]*. Recombinant *y w p[eyFLP ry⁺]; FRT80B/FRT80B* cells are unpigmented (white); recombinant *y w p[eyFLP ry⁺]; FRT80B p[w⁺]/FRT80B p[w⁺]* cells are pigmented (red). (**b**) Genotype: *w RpL36/y w p[eyFLP ry⁺]; p[RpL36⁺ w⁺] FRT80B/ FRT80B*. Cell competition causes elimination of unpigmented *Rp/+* eye cells of the recombinant genotype *w RpL36/y w p[eyFLP ry⁺]; FRT80B/FRT80B*. (**c**) Genotype: *w RpL36/y w p [eyFLP ry⁺]; p[RpL36⁺ w⁺] FRT80B/Df(3L)H99 FRT80B. Rp/+* cells of genotype *w RpL36/y w p[eyFLP ry⁺]*; Df(3L)H99 FRT80B survive in the adult eye. This indicates that genes deleted by Df(3L)H99 must be present for competitive elimination of *Rp/+* cells. (**d**) Genotype: *w RpL36/y w p[eyFLP ry⁺]; p[RpL36⁺ w⁺] p[arm-LacZ w⁺] FRT80B/FRT80B*. Few *RpL36/+* cells (black, unlabeled for β-galactosidase) remain in the eye imaginal disc, because of competition with neighboring cells that carry the *RpL36⁺* transgene linked to *p[arm-LacZ w⁺]*. (**e**) Genotype: *w RpL36/y w p[eyFLP ry⁺]; p[RpL36⁺ w⁺] p[arm-LacZ w⁺] FRT80B/Df(3L)H99 FRT80B*. Many *RpL36/+; Df(3L)H99* clones (black, unlabeled for β-galactosidase) occur in a mosaic eye imaginal disc, showing that genes deleted by Df(3L)H99 must be present for competitive elimination of *Rp/+* cells. (**f**) Genotype: *w RpL36/y w p [eyFLP ry⁺]; p[RpL36⁺ w⁺] p[arm-LacZ w⁺] FRT80B/Df(3L)IRER FRT80B*. Like the control shown in **d**, few *RpL36/+* cells survive in the eye disc (black, unlabeled for β-galactosidase). Unlike *Df(3L)H99*, the regulatory region deletion *Df(3L)IRER* did not rescue *RpL36/+* cells from competition. (**g**) Genotype: *hsFlp; FRT42 ubi-GFP M(2)56f/FRT42; hid²⁰⁻¹⁰-LacZ/+*. GFP (green: g′), β-galactosidase (blue: g′′), and anti-Dcp1 (red: g′′′). *Hid-LacZ* expression is elevated in all the *M(2)56f/+* wing disc cells. It is sometimes higher still in those undergoing apoptosis, but not consistently. Clones homozygous for the *FRT42 ubi-GFP M(2)56f* chromosome should be generated in these experiments, but none were seen, reflecting cell lethality of the *M(2)56f* mutation. (**h**) Genotype: *hsFlp; rpr^XRE^-LacZ/+; FRT82 ubi-GFP M(3)95A/FRT82* labeled for GFP (green: h′), β-galactosidase (blue: h′′), and anti-Dcp1 (red: h′′′). *Rpr-LacZ* expression is elevated in all the *M(3)95A/+* wing disc cells, and is not higher still in those undergoing apoptosis. Clones homozygous for the *FRT82 ubi-GFP M(3)95A* chromosome should be generated in these experiments, but none were seen, reflecting cell lethality of the *M(3)95A* mutation

Dronc activity promotes Wg expression in undead *Rp/Rp* cells but not in competed *Rp/+* cells. Ectopic expression of Wingless proteins is a Dronc function that occurs in the presence of p35.[48,51,52] The p35-expressing *Rp/+* cells did not express ectopic Wg at boundaries with +/+ clones, although normal, developmentally localized Wg expression was readily detected (Figure 4a). In contrast, undead *Rp/Rp* cells expressed Wg, which was abolished by co-expressing Dronc-DN along with p35 (Figures 4a and b).

Ectopic Wg expression from undead cells promotes growth.[48,51,52] We and others have reported that posterior compartments are not larger when p35 is expressed during cell competition.[6,9] To re-examine this, we varied the number of *Rp/Rp* twin spots. A 10-min heat shock induced 10 recombination events per compartment, on average, with no detectable effect on wing size (Figure 4c). By contrast, 60-min heat shock resulted in 30 undead *Rp/Rp* clones in the posterior compartment, on average (Figure 4d), and a significant increase in the

size of posterior compartments, detectable in the adult wings (Figures 4e and f). As no Wg protein was detected in *Rp*/+ cells, we attribute such ectopic growth to the undead *Rp*/*Rp* cells.

Dronc is not required for death of competed *Rp*/+ cells. As Dronc activity was not detectable in *Rp*/+ cells, its role was tested genetically. Clones of +/+ cells were induced in *Rp*/+ wing discs, where Dronc-DN expression was targeted to posterior compartments. Cell death was similar in the posterior compartments expressing Dronc-DN to the anterior compartments (Figure 5a), suggesting that Dronc was not essential for competitive cell death. A second approach used *dronc* null mutations.[9] Homozygous mutant clones grew like their wild-type twins in non-Minute backgrounds (Figures 5b–d). When mitotic recombination was used to generate clones of *RpL36*/+ cells in a wild-type background, such clones were outcompeted and not recovered (Figure 5e). *RpL36*/+ clones were still eliminated when homozygous for *dronc* null mutations, indicating that *dronc* was not crucial for their competition (Figures 5f and g).

Dronc participates in cell death of *Rp*/*Rp* cells. We also assessed *dronc* in undead *Rp*/*Rp* cells (Figure 3). Dronc-DN abolished CM1 labeling of *Rp*/*Rp* cells expressing p35, indicating Dronc activity in undead *Rp*/*Rp* cells (Figure 5h). These results also confirmed that Dronc-DN expression reduced Dronc activity *in vivo*, in the same preparations where *Rp*/+ cells were studied. When Dronc-DN was expressed without p35, *Rp*/*Rp* cells were still observed (Figure 5a). The protection afforded by DroncDN was incomplete in two respects: clones of *Rp*/*Rp* cells expressing Dronc-DN were smaller and less abundant than clusters of *Rp*/*Rp* cells expressing p35 (Figures 5a and 3a); some *Rp*/*Rp* cells expressing Dronc-DN were labeled by CM1 and showed the condensed morphology typical of apoptotic cells (Figure 5a, inset arrow). Taken together, the findings suggest that Dronc contributed to *Rp*/*Rp* cell death, but that some apoptosis continued in the presence of Dronc-DN.

Cell death of competed *Rp*/+ cells is independent of Dredd and Dream/strica. The other potential apical caspases Dream/strica and Dredd might be responsible for Dronc-independent competitive cell death. Both are inhibited by p35, consistent with the fact that p35 prevents both death and CM1 labeling of *Rp*/+ cells.

The role of Dream/strica was examined using a deletion in this gene, by inducing +/+ clones in *Rp*/+ animals that were *dream*[4] homozygous. Competitive cell death occurred at rates indistinguishable between *dream*[4] homozygotes and controls (Figures 6a, b, e and f). Therefore, Dream/strica was dispensable for competitive cell death. As *Rp*/*Rp* clones were not seen, Dream/strica may be dispensable for death of these cells also (Figure 6).

The role of Dredd was examined using a null allele, by inducing +/+ clones in *Rp*/+ animals that were *dredd*[B118] homozygous. Competitive cell death occurred at rates indistinguishable between *dredd*[B118] homozygotes and controls (Figures 6c, d, g and h). No *Rp*/*Rp* clones were seen. Therefore, *dredd* also appeared dispensable for competitive cell death and for death of *Rp*/*Rp* cells.

Competitive cell death depends redundantly on Dream/strica and Dronc. Although not individually required, initiator caspases might act redundantly in competitive apoptosis of *Rp*/+ cells. Although the *dronc*, *dream* and *dredd* triple mutant genotype has been constructed previously,[53] severe viability and fertility effects were encountered when combining caspase mutations with Minute backgrounds. An alternative inducible RNAi approach was undertaken (Figure 7). When dsRNA for *dronc* was expressed in *Rp*/+ posterior compartments, competitive cell death occurred at boundaries with +/+ clones (Figure 7a), like the results with Dronc-DN (Figure 5a). The ~25% reduction compared with the control anterior compartments was not significant statistically (Figure 7d). Interestingly, dsRNA for *dronc* reduced apoptosis of *Rp*/+ cells that were *not* adjacent to wild-type clones. This was obvious in wing discs that lacked any wild-type clones, where *dronc* knockdown reduced apoptosis in posterior compartments (supplementary Figure 1). This finding suggested that some *Rp*/+ cells undergo *dronc*-dependent apoptosis independently of cell competition, and also that the dsRNA was effective. When dsRNA for *dream* was expressed in *Rp*/+ posterior compartments, cell death continued at boundaries with +/+ clones (Figure 7b), at rates not significantly different from controls (Figures 7b and e), similar to the results obtained using *dream* mutants (Figures 6a, b, e and f). By contrast, expressing dsRNAs for both *dronc* and *dream* simultaneously was sufficient to eliminate cell death almost completely in *Rp*/+ posterior compartments containing +/+ clones (Figures 7c and f).

Caspase knockdown could also protect *Rp*/*Rp* clones (Supplementary Figure 2). When dsRNA for *dronc* was expressed, *Rp*/*Rp* clones survived (Supplementary Figure 2A), like when Dronc-DN was expressed (Figure 5a). No *Rp*/*Rp* clones survived when dsRNA for *dream* was expressed (Supplementary Figure 2B), like the results with *dream* mutants (Figure 6b). *Rp*/*Rp* clones did survive when both *dronc* and *dream* were knocked down (Supplementary Figure 2C),

Taken together, the findings indicate that competitive cell death of *Rp*/+ cells depended redundantly on Dronc and Dream initiator caspases, whereas death of *Rp*/*Rp* cells depended mainly on Dronc.

Discussion

Rp/+ cells in competition with wild-type cells undergo programmed cell death. Regardless of whether *Rp*/+ clones are in a wild-type background, or wild-type clones are in a *Rp*/+ background, cell death occurs that can be prevented by the caspase inhibitors baculovirus p35 and DIAP1, or by removing the pro-apoptotic genes *grim*, *reaper* and *hid*[5,6,16,54] (Figure 1). We show that competitive cell death involves less Dronc activity than other forms of apoptosis, and can be initiated by Dream/Strica.

Apoptosis of *Rp*/*Rp* cells. Unexpectedly, we found that *Rp*/*Rp* cells die through apoptosis, and therefore can survive and divide in the presence of baculovirus p35. In our experiments, cells homozygous for *M(3)95A*, a mutation

thought to correspond to *RpS3*,[2] were protected by p35, Dronc-DN or dsRNA for *dronc* (Figures 3a, c, 4b, c and 5a and Supplementary Figure 2). The findings are not specific to this mutation: p35 protected clones homozygous for the *M(3)i*[55] mutation corresponding to *RpS17* (see Figures 2f and 4d of Martin *et al*[17]).

It is doubtful that cells divide in the complete absence of essential ribosomal proteins. Even if these alleles are null, presumably each clone of *Rp/Rp* cells begins as a recombinant cell that inherits ribosomes from the *Rp/+* mother cell. Our results suggest that as ribosome numbers diminish with time and growth, apoptosis is triggered before
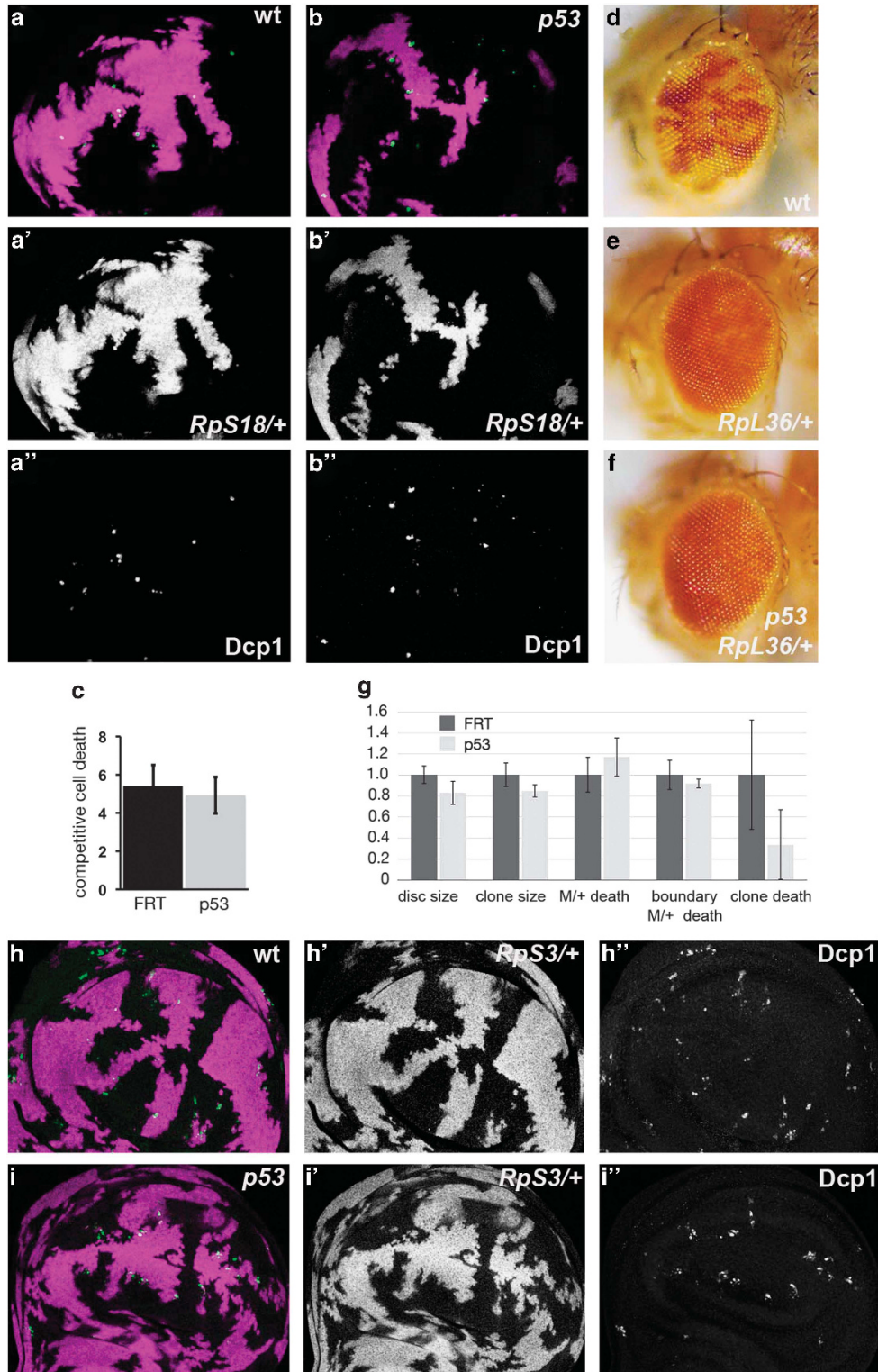
growth becomes impossible, and *Rp/Rp* cells can undergo a few more divisions if they are protected from apoptosis. Apoptosis of *Rp/Rp* cells provides a useful contrast with competitive death of *Rp/+* cells within the same preparations.

Apoptotic *Rp/+* cells lack *dronc* activity. When p35 protects cells from effector caspases, persistent Dronc activity marks the 'undead' cells.[46,51,52] Undead *Rp/Rp* cells were labeled by CM1 and expressed Wg. They lost these undead markers when Dronc-DN was expressed along with p35, confirming Dronc activity (Figures 3, 4 and 5h). By contrast, *Rp/+* cells protected by p35 were unlabeled by CM1 antibodies or for Wg (Figures 3 and 4). Therefore, competitive death of *Rp/+* cells seems to involve little Dronc activity. Competitive death is initiated either not by Dronc, or by Dronc activity at low levels. The Dronc independence of competitive apoptosis precluded using undead cells to map competitive cell death signals, which has been possible for developmental apoptosis.[46]

Different Dronc requirements in competitive and noncompetitive apoptosis. The different levels of Dronc activity in *Rp/+* and *Rp/Rp* cells mirrored different *dronc* requirements. Expression of Dronc-DN protected *Rp/Rp* cells, albeit less effectively than baculovirus p35 (Figure 5a), as did dsRNA for *dronc* (Supplementary Figure 2). Dronc-DN may be less effective than p35 because Dronc-DN does not inhibit Dronc activity completely. Alternatively, if *Rp/Rp* cells are subject to competition with *Rp/+* cells in addition to cell-autonomous cell death, *Rp/Rp* cells that were protected from apoptosis by Dronc-DN would still be subject to Dronc-independent competition with neighboring *Rp/+* cells, and for this reason would survive less well than *Rp/Rp* cells protected by p35.

By contrast, *Rp/+* clones were eliminated in the absence of *dronc* (Figures 5e–g) or in the presence of Dronc-DN or dsRNA for *dronc* (Figures 5 and 7). Dronc-DN and dsRNA for *dronc* may retain some activity, and clones of cells homozygous for *dronc* null alleles may inherit Dronc protein or RNA, but as these reagents effectively blocked dronc-dependent processes in *Rp/Rp* cells (Figures 3, 4, 5 and Supplementary Figure 2), competitive death of *Rp/+* cells must require less Dronc activity than is normal in other forms of apoptosis, such as that of *Rp/Rp* cells.

Competitive apoptosis depends redundantly on Dronc and Dream/Strica. The remaining initiator caspases Dredd and Dream/strica were also dispensable for competitive cell death (Figure 6). Effector caspases, which are clearly both activated and required, must either be activated by multiple initiator caspases redundantly, or by novel mechanisms. It proved possible to express dsRNA for both *dronc* and *dream* in competing cells, and this blocked competitive cell death almost completely, indicating that Dronc and Dream/Strica acted redundantly to initiate competitive cell death (Figures 7c and f). It was notable that dsRNA for *dronc* alone led to some reduction in cell death, although it was not significant statistically in our study (Figures 7a and d). This may indicate that *dream* does not substitute completely for *dronc*. However, reduced cell death of *Rp/+* cells even in *Rp/+* imaginal discs that lack wild-type clones indicates that *Rp/+* cells were subject to a background of dronc-dependent cell death not due to cell competition (Supplementary Figure 1). Alone, dsRNA for dream/strica had no effect (Figures 7b and e). Overall, these data indicate that *dronc* and *dream* could each initiate competitive cell death, rendering competitive cell death less dependent on *dronc* than most apoptosis in *Drosophila*.

Another group concluded that competitive death of *RpS17/+* cells is *dronc*-dependent, and that p35 expression during cell competition leads to undead *Rp/+* cells. Their study, however, did not differentiate the apoptosis of *Rp/Rp* cells from *Rp/+* cells, or note the presence of undead *Rp/Rp* in the presence of p35.[17] In addition, the *M(3)i*[55] chromosome that is mutant for *RpS17* may exhibit a higher background of dronc-dependent, noncompetitive *Rp/+* cell death than the *M(3)95A* chromosome used here (our unpublished observations, and compare Figure 3a of this work with Figure 4d from Martin *et al*[17]). It is important to distinguish these dronc-dependent death mechanisms from cell competition.

Role of pro-apoptotic genes in cell competition. Dronc is commonly activated in the apoptosome by expression of the

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Figure 2 Competitive apoptosis in the absence of p53. (**a** and **b**) Wing imaginal discs labeled for the *RpS18* chromosome in magenta (anti-GFP, a′, b′, h′, i′) and apoptotic cells in green (anti-active Dcp1, a″, b″, h″, i″). Clones of wild-type cells (lacking magenta labeling) have been induced by heat-shock-induced mitotic recombination. In (**a**) apoptotic cells *RpS18/+* occur at interfaces between wild-type and *RpS18/+* cells. Genotype: *y w hsF; FRT42D/FRT42D p[Ubi-GFP] M(2)56F.* (**b**) In the absence of p53, competitive apoptosis continues at the interfaces between wild-type and *RpS18/+* cells. Genotype: *y w hsF; FRT42D/FRT42D p[Ubi-GFP] M(2)56F; p53^{5A-1-4}/p53^{11-1B-1}. RpS18/RpS18* cells should be generated as reciprocal recombinants in these experiments, but such clones were never seen, indicating that this genotype was cell-lethal in the presence or absence of p53. (**c**) Quantification of *RpS18/+* cell death rates at interfaces with wild-type cells, normalized for the length of boundaries. (**d**) A mosaic eye from genotype *y w p[eyFLP ry^+]; FRT82/FRT82 p[w^+].* The adult eye predominantly contains two recombinant genotypes. The *y w p[eyFLP ry^+]; FRT82/FRT82* cells are unpigmented (white). The *y w p[eyFLP ry^+]; FRT82 p[w^+]/FRT82 p[w^+]* cells are pigmented (red). (**e**) A mosaic eye from genotype *w RpL36/y w p[eyFLP ry^+]; p[RpL36^+ w^+] FRT82/FRT82* Cell competition causes elimination of unpigmented *Rp/+* eye cells of the recombinant genotype *w RpL36/y w p[eyFLP ry^+]; FRT82/FRT82.* (**f**) A mosaic eye from genotype *w RpL36/y w p[eyFLP ry^+]; p[RpL36^+ w^+] FRT82/FRT82 p53^{5A-1}.* Cell competition causes elimination of unpigmented recombinant *Rp/+* eye cells, even though they are homozygous for the *p53* null allele. (**g**) Properties of *p53^{5A-1}* homozygous clones induced in hsFlp; FRT82 M95A armLacZ/FRT82 p53^{5A-1} wing imaginal discs, induced at 92 ± 4 h AEL at 25 °C, normalized to data from control clones induced in parallel in hsFlp; FRT82 M95A armLacZ/FRT82 wing discs. Clones lacking *p53* did not significantly affect size of the wing discs, clone size, frequency of cell death among M95A/+ cells or frequency of competitive M95A/+ cell death along clone boundaries. Data represent the mean and S.D. from three separate experiments (significant differences were not seen in any of the three individual experiments). Measurements of cell death rate within the wild-type and *p53* homozygous clones themselves exhibit high S.E.s because of the small absolute numbers of non-Minute cells that die, often zero or one per wing disc, a rate more than 20 times lower than the death of M95A/+ cells. (**h**) Wing imaginal disc labeled for the *M95A/+* genotype (anti-β-galactosidase: magenta) and for apoptotic cells (anti-Dcp1: green). Most dying *M95A/+* cells are at borders between wild-type clones and *M95A/+* cells. (**i**) Wing imaginal disc labeled for the *M95A/+* genotype (anti-β-galactosidase: magenta) and for apoptotic cells (anti-Dcp1: green). Most dying *M95A/+* cells are at borders between *p53^{5A-1}* homozygous clones and *M95A/p53^{5A-1}* cells
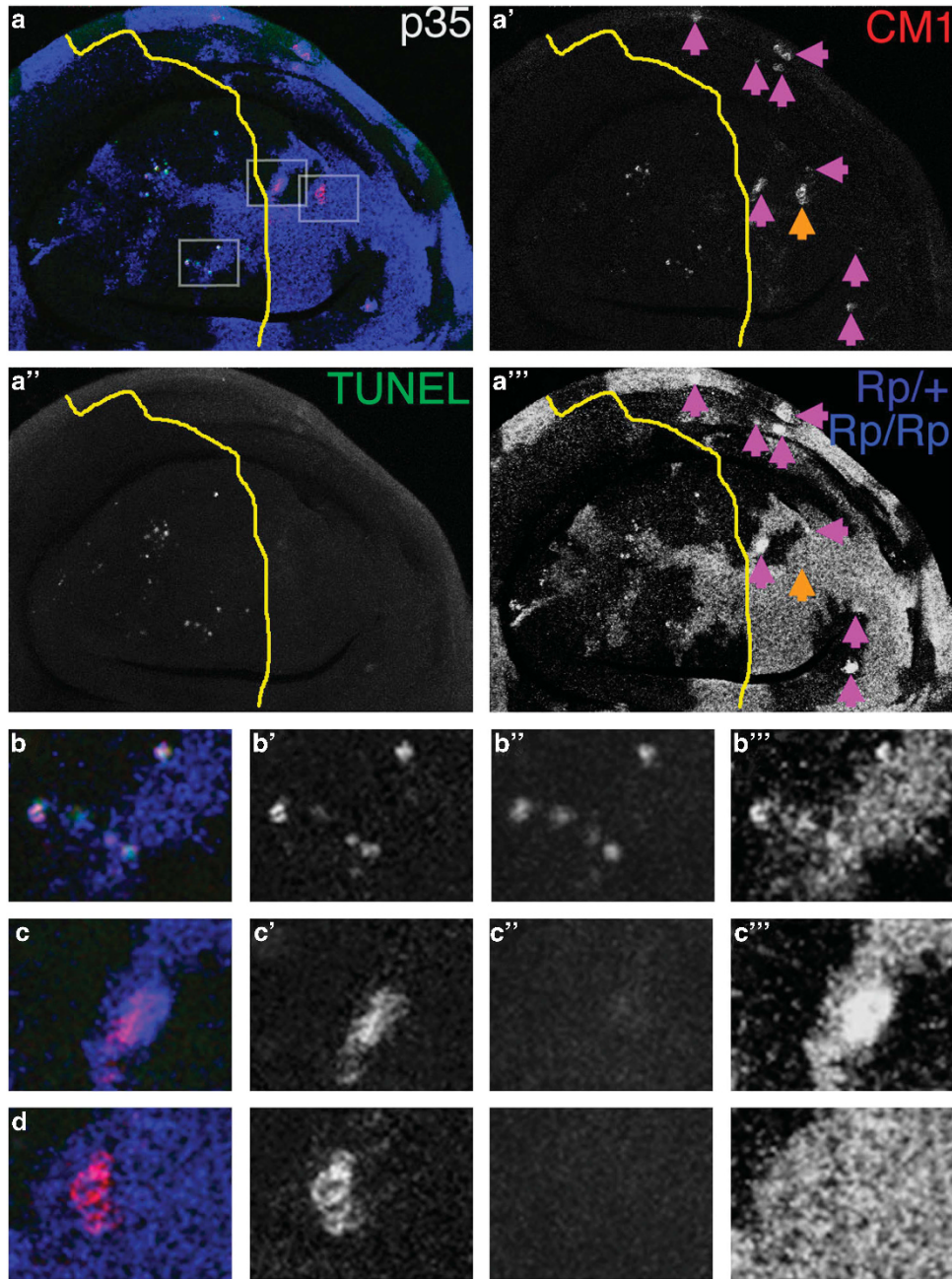
Figure 3 Protection of *Rp* mutant cells by Baculovirus p35. (**a**) Wing imaginal disc labeled for the *M(3)95A* chromosome in blue (a′, anti-*β*-galactosidase labeling), apoptosis (a″, TUNEL in green) and caspase cleavage (a‴, CM1 antibody in red). Only occasionally were cells observed labeled by CM1 but not TUNEL or *vice versa*. In anterior compartments, most dying cells are coincidentally labeled by TUNEL and CM1, and represent *Rp*/+ cells with wild-type neighbors. In posterior compartments, baculovirus p35 completely suppressed cell death as detected by TUNEL. Caspase activation occurred in clusters of undead cells. Nearly all such undead cell clusters corresponded to the *Rp*/*Rp* genotype (magenta arrows); clusters of *Rp*/+ undead cells were less common (orange arrow), and also seen in *Rp*/+ discs without recombinant clones (data not shown). Three rectangles indicated regions enlarged in **b–d**. (**b**) Enlargement from **a**, indicating the general coincidence between TUNEL (b″) and CM1 (b′) labels, and the location of most dying cells to the boundaries of the *Rp*/+ territory (b‴) with wild-type cells. Note that apoptotic cells can often be recognized from the appearance of their *β*-galactosidase immunoreactivity, presumably a consequence of the concentration of cellular contents that is a defining morphological feature of apoptosis. A very few cells with such an appearance lacked either TUNEL or anti-caspase labeling, suggesting that some apoptotic cells may not be labeled by either method (data not shown). (**c**) Enlargement from **a**, showing a cluster of undead *Rp*/*Rp* cells labeled for caspase activation (CM1, c′). c″, c‴ definitions as for panel **b**. (**d**) Enlargement from **a**, showing a cluster of undead *Rp*/+ cells labeled for caspase activation (CM1). Genotype: *y w hsF; en-GAL4 UAS-GFP/UAS-p35; FRT82B/FRT82B p[arm-LacZ] M(3)95A*

pro-apoptotic genes *rpr*, *grim* or *hid*, which lead to the degradation of anti-apoptotic DIAP1 protein.[55] Indeed, *rpr* and *hid* transcription was elevated in *Rp*/+ cells, and deleting *rpr*, *grim* and *hid* prevented cell competition (Figure 1). These data fall short of proof that elevated *rpr*, *grim* or *hid* expression initiate competitive cell death. Transcriptional reporters for *hid* and *rpr* were elevated in all *Rp*/+ cells, most of which were not apoptotic (Figure 1). As *Rp*/+ cells do not
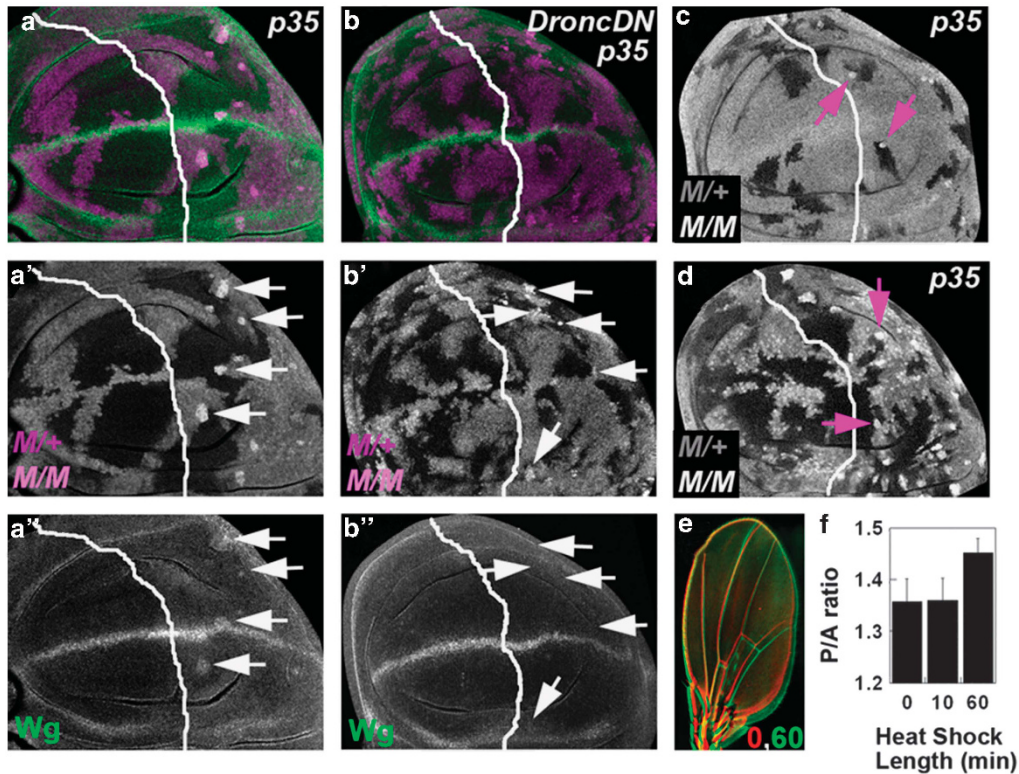
Figure 4 Characterization of undead *Rp/Rp* cells. (**a**) Wing imaginal disc labeled for genotype (magenta, a′) and Wingless protein (Green, a″). Arrows point to examples of undead *Rp/Rp* clones in the p35-expressing posterior compartment. Many express ectopic Wg protein. Ectopic Wg was not seen in cells of other genotypes. (**b**) Co-expression of baculovirus p35 and Dronc-DN, labeled for genotype (magenta, b′) and Wingless protein (Green, b″). Arrows indicate undead *M/M* clones. Ectopic Wg expression is suppressed in the presence of Dronc-DN. Genotype: *y w hsF; en-GAL4 UAS-GFP/UAS-p35 UAS-Dronc^{CARD}; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**c**) Wing imaginal disc containing wild-type and *M/M* clones induced after 10-min heat shock. Arrows indicate examples of undead *M/M* clones in the posterior compartment. (**d**) Wing imaginal disc containing wild-type and *M/M* clones induced after 60-min heat shock. Arrows indicate examples of undead *M/M* clones in the posterior compartment. Compared with **c**, more clones are induced by the extended heat shock. (**e**) Overlay of wings without heat shock (red) and subject to 60-min heat shock (green), illustrating increased posterior compartment size following heat shock. Genotype: *y w hsF; en-GAL4 UAS-GFP/UAS-p35; FRT82B/FRT82B p[arm-LacZ] M(3)95A*, except for **b**. (**f**) Quantification of results shown in **e**. Ten-minute heat shock induction of clones did not significantly change posterior compartment size in comparison with the anterior, whereas 60-min heat shock did

transcribe higher levels of DIAP1,[56] elevated *rpr* or *hid* may sensitize *Rp/+* cells to cell death without being the specific trigger. Elevating DIAP1 after *rpr*, *grim* and *hid* deletion might de-sensitize *Rp/+* cells, rather than block a specific pathway. An interesting possibility is that *Rp/+* cells elevate a nonautonomous survival factor. Such a factor would be present at lower levels near to wild-type cells, potentially providing a trigger for competitive cell death at clone boundaries. Such a scenario remains hypothetical until the factor is identified.

Competitive apoptosis is independent of p53. Transcription of *rpr* and *hid* in response to stress or gamma-irradiation depends on the IRER enhancer.[41] Unlike some other examples of apoptosis, competitive cell death did not depend on this enhancer, ruling out at least one mechanism by which changes in pro-apoptotic gene expression levels could lead to cell competition. The IRER enhancer, as well as the transcription of *hid* and *rpr*, are transcriptional targets of p53, and p53 is activated by Rp mutations in vertebrates.[31] Cell competition continued in the absence of p53, however (Figure 2). In addition, *Rp/+* cells did not express ectopic

Wg in the presence of p35 (Figure 4a), which would be expected if p53 was activated.[57]

It is significant that competitive cell death in *Drosophila* continued in the absence of p53 (Figure 2) for two reasons. First, it indicated that cell competition was not mediated by the vertebrate nucleolar stress pathway, whereby certain ribosomal proteins stabilize p53 and trigger death of hematopoietic stem cells.[31] Accordingly, the MDM2 ubiquitin ligase that is targeted by vertebrate ribosomal proteins is not encoded in the *Drosophila* genome. It remains possible that other mechanisms of nucleolar stress occur in *Drosophila*. Secondly, it has been suggested that cell competition could be a mechanism to remove aneuploid cells.[27,28] As there is p53-independent removal of aneuploid cells,[27,28] our findings are consistent with cell competition as the mechanism.

Dream/Strica in competitive cell death. It could be proposed that expression levels determine the contributions of Dronc and Dream/strica to cell death. In this view, Dream/strica levels are inadequate to initiate developmental cell death, but higher levels of Dream/strica proteins in *Rp/+* cells become sufficient to initiate competitive apoptosis in the absence of Dronc. There is evidence against this simple
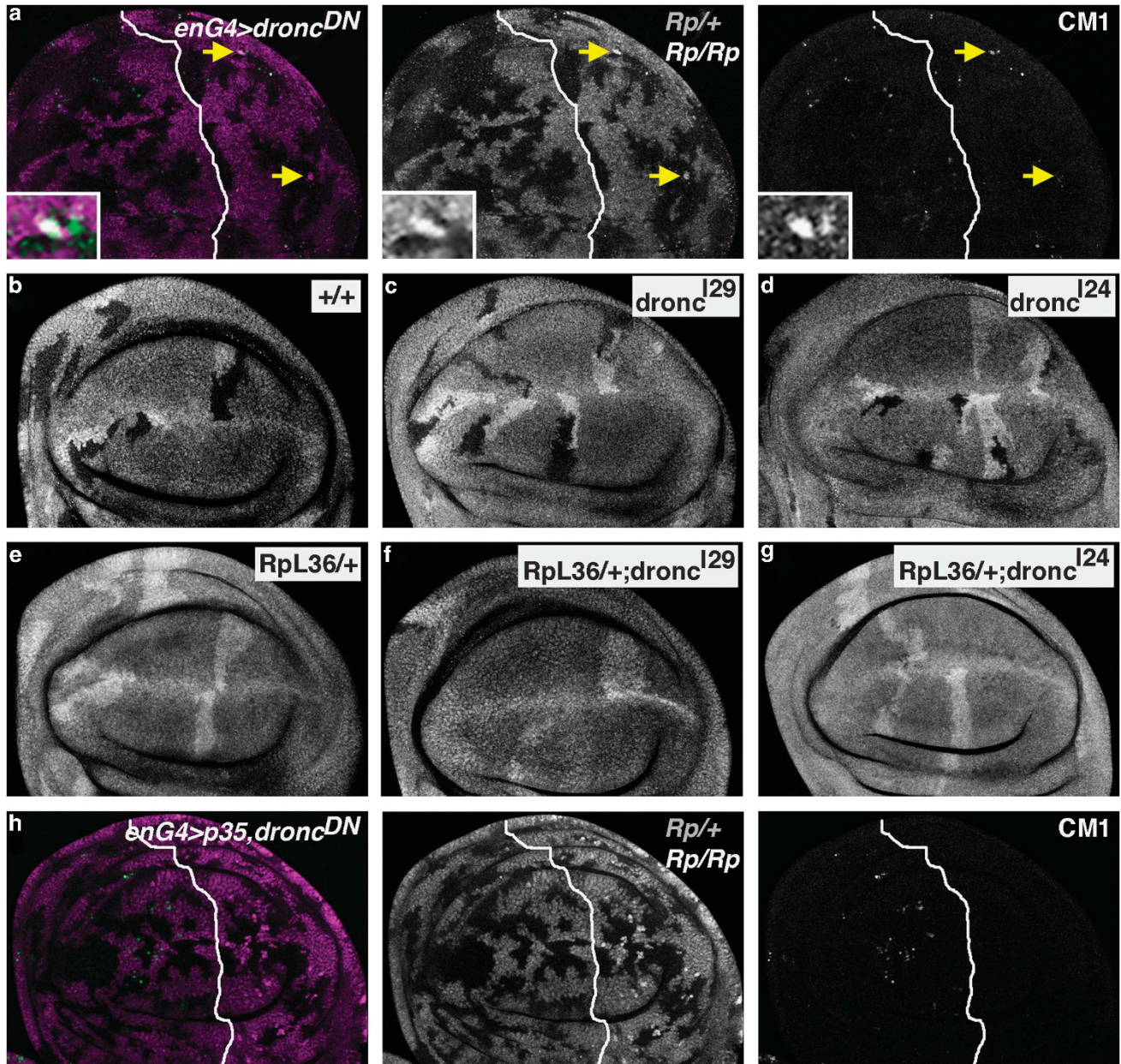
Figure 5 Requirements for *dronc* in Minute apoptosis. (**a**) Dronc-DN expression in posterior compartments did not suppress competitive death of *Rp*/+ cells. *Rp*/*Rp* cells were seen in the posterior compartments expressing Dronc-DN (arrows). However, some *Rp*/*Rp* clones were labeled by CM1 and appeared to contain apoptotic cells (arrows). (**b**) Clones of FRT80B control cells grew comparably to their twin spots. (**c**) Clones of *dronc*^{I29} homozygous cells grew comparably to their twin spots. (**d**) Clones of *dronc*^{I24} homozygous cells grew comparably to their twin spots. (**e**) Clones heterozygous for *RpL36* were not recovered after mitotic recombination. (**f**) No *RpL36*/+ clones homozygous for *dronc*^{I24} were recovered. (**g**) No *RpL36*/+ clones homozygous for *dronc*^{I94} were recovered. (**h**) When baculovirus p35 and Dronc-DN were co-expressed in posterior compartments, *Rp*/*Rp* clones lacked the CM1 labeling typical of 'undead' cells. Genotypes: (**a**) *y w hsF; en-GAL4 UAS-GFP/UAS-Dronc*^{CARD}; *FRT82B/FRT82B p[arm-LacZ] M(3) 95A*. (**b**) *y w hsF; FRT80B/p{arm-LacZ} FRT80B*. (**c**) *y w hsF; dronc*^{I29} *FRT80B/p{arm-LacZ} FRT80B*. (**d**) *y w hsF; dronc*^{I24} *FRT80B/p{arm-LacZ} FRT80B*. (**e**) *y w hsF/w M(1)Bld; FRT80B/p[arm-LacZ] p[w*$^+$ *RpL36*$^+$*] FRT80B*. (**f**) *y w hsF/w M(1)Bld; dronc*^{I24} *FRT80B/p[arm-LacZ] p[w*$^+$ *RpL36*$^+$*] FRT80B*. (**g**) *y w hsF/w M(1)Bld; dronc*^{I29} *FRT80B/p[arm-LacZ] p[w*$^+$ *RpL36*$^+$*] FRT80B*. (**h**) *y w hsF; en-GAL4 UAS-GFP/UAS-p35 UAS-Dronc*^{CARD}; *FRT82B/FRT82B p[arm-LacZ] M(3)95A*

model, however, because the lack of undead *Rp*/+ cells in the presence of baculovirus p35 indicated that Dronc activity was unusually low in competitive apoptosis. In addition, apoptosis of *Rp*/*Rp* cells, as well as noncompetitive apoptosis of *Rp*/+ cells, were both *dronc*-dependent but independent of *dream/strica.* The fact that *Rp*/+ cells are capable of *dronc*-dependent and *dronc/dream*-dependent cell death processes

is difficult to explain unless competitive cell death specifically activates Dream/strica in addition to Dronc.

Little is yet known concerning the molecular mechanisms of Dream/strica activation. Dream/strica differs from other initiator caspases in that its extensive prodomain is serine/threonine-rich and lacks a caspase recruitment domain or death effector domain, suggesting that it could be activated by
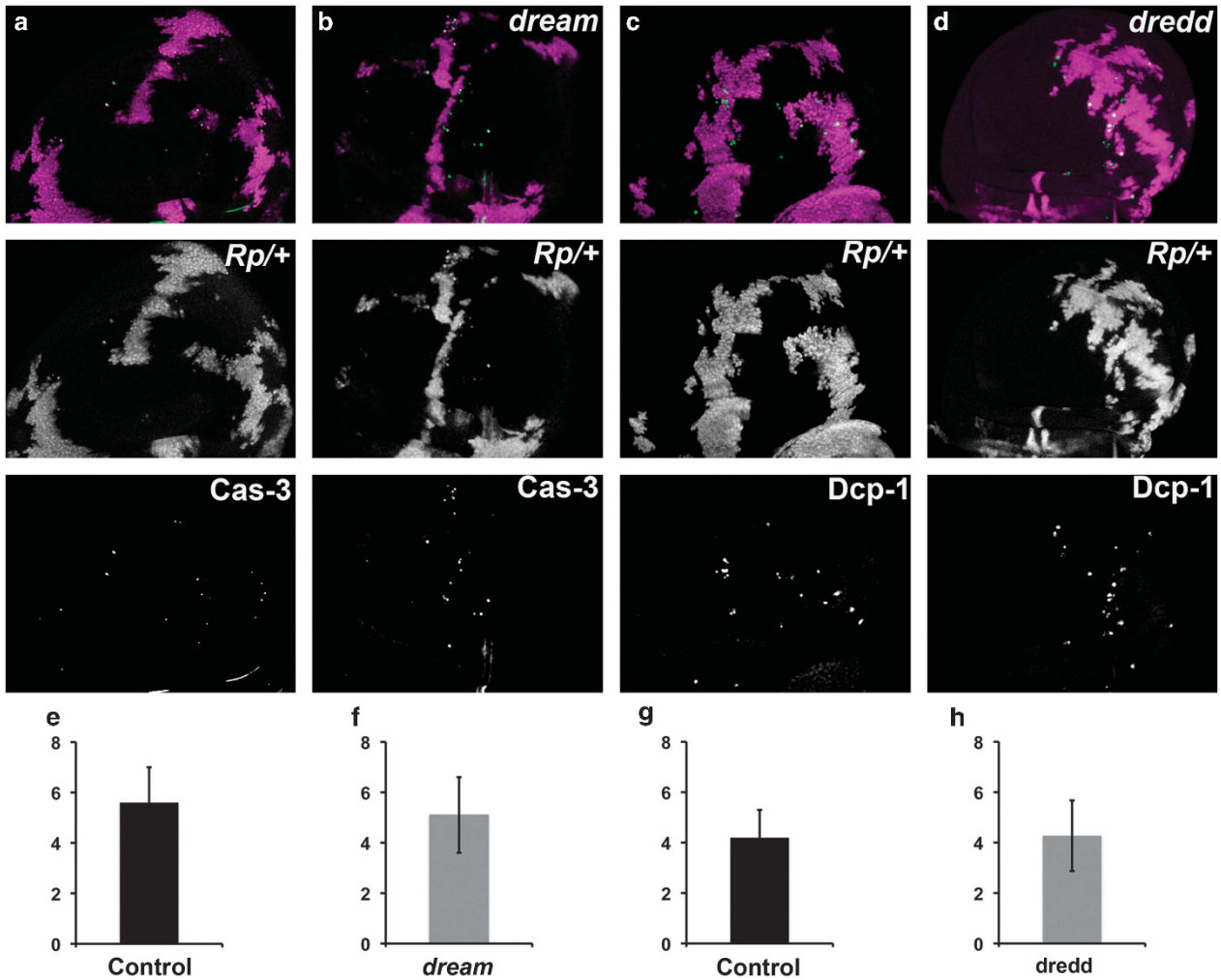
Figure 6 Dream/Strica and Dredd are dispensable for competitive death of *Rp/+* cells. (**a**) Clones of wild-type cells (unlabeled, black) in *RpS3/+* background (magenta). Cell death labeled by activated caspase-3 labeling (green). Genotype: *y w hsF; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**b**) Clones of non-Minute cells (unlabeled, black) in *RpS3/+* background (magenta) induced in animals homozygous for a *dream* null allele. Cell death labeled by activated caspase-3 labeling (green). Genotype: *y w hsF; dream⁴/dream⁴; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**c**) Clones of non-Minute cells (unlabeled, black) in *RpS18/+* background (magenta). Cell death labeled by activated Dcp-1 labeling (green). Genotype: *y w hsF; FRT42D/FRT42D p[Ubi-GFP] M(2)56F*. (**d**) Clones of wild type cells in *RpS18/+* background in animals homozygous for a *dredd* null allele. Cell death labeled by activated Dcp-1 labeling (green). Genotype: *dredd^B118 hsflp; FRT42D/FRT42D p[Ubi-GFP] M(2)56F*. (**e–h**) Quantification of *Rp/+* cell death at clone boundaries for genotypes shown in **a–d**. For the *y* axis, cell death rates quantified per micron of boundary between genotypes

a distinct mechanism.[58] The protein interacts with both DIAP1 and DIAP2,[58] and some studies suggest that Dream/strica accelerates aspects of *dronc*-dependent apoptosis. Dream/strica and Dronc are also required redundantly for nurse cell death during oogenesis and the programmed elimination of certain peptidergic neurons from the CNS during pupariation.[53,59] Further characterization of mechanisms of Dream/Strica activation may be revealing concerning the induction of competitive apoptosis by interactions between wild-type and *Rp/+* cells.

Materials and Methods

Fly strains. Genotypes of each experiment are described in figure legends. The following genetic strains were used: *Df(3L)H99*;[3] *FRT80B*;[60] *M(1)Bld*; *p{w⁺, rpL36⁺}*;[7] *DF(3L)IRER*;[41] *p53⁵ᴬ⁻¹⁻⁴*; and *p53^{11-1B-1}*,[61] *en-Gal4 UAS-GFP*;[62] *UAS-p35*;[43]

arm-LacZ;[63] *ubi-GFP*,[64] *UAS-DroncDN*;[45] *dronc^{I24}*; *dronc^{I29}*,[37] *dream⁴* and *dredd^{B118}*,[53] *hid^{20-10}-LacZ* and *rpr^{XRE}-LacZ*;[65] *UAS-dsRNA^{dronc}* (VDRC100424) and *UAS-dsRNA^{dream}* (VDRC22594) *UAS-dcr2* (VDRC60007);[66] for M(2)56F (a mutation of *RpS18*), *M(3)i^{55}* (a mutation of *RpS17*) and M(3)95A (a mutation of *RpS3*) see Marygold et al.[2]

Mosaics. Mosaic clones were generated by the FRT-FLP technique.[67] Flies were maintained at 25 °C on standard media unless otherwise noted. Unless otherwise specified, for Minute genotypes 37 °C heat shock was administered for 60 min at 72 ± 12 or 84 ± 12 h after egg laying and imaginal discs were dissected 72 h later; other genotypes were heat-shocked 60 ± 12 h after egg laying and imaginal discs dissected 60 h later.

Immunohistochemistry. Antibody and TUNEL labelings were performed as described.[68] Confocal images were recorded using Biorad Radiance (Hercules, CA, USA) 2000 and Leica (Wetzlar, Germany) SP2 systems and processed using ImageJ (rsb.info.nih.gov) and Photoshop (Adobe Systems Inc., San Jose, CA,
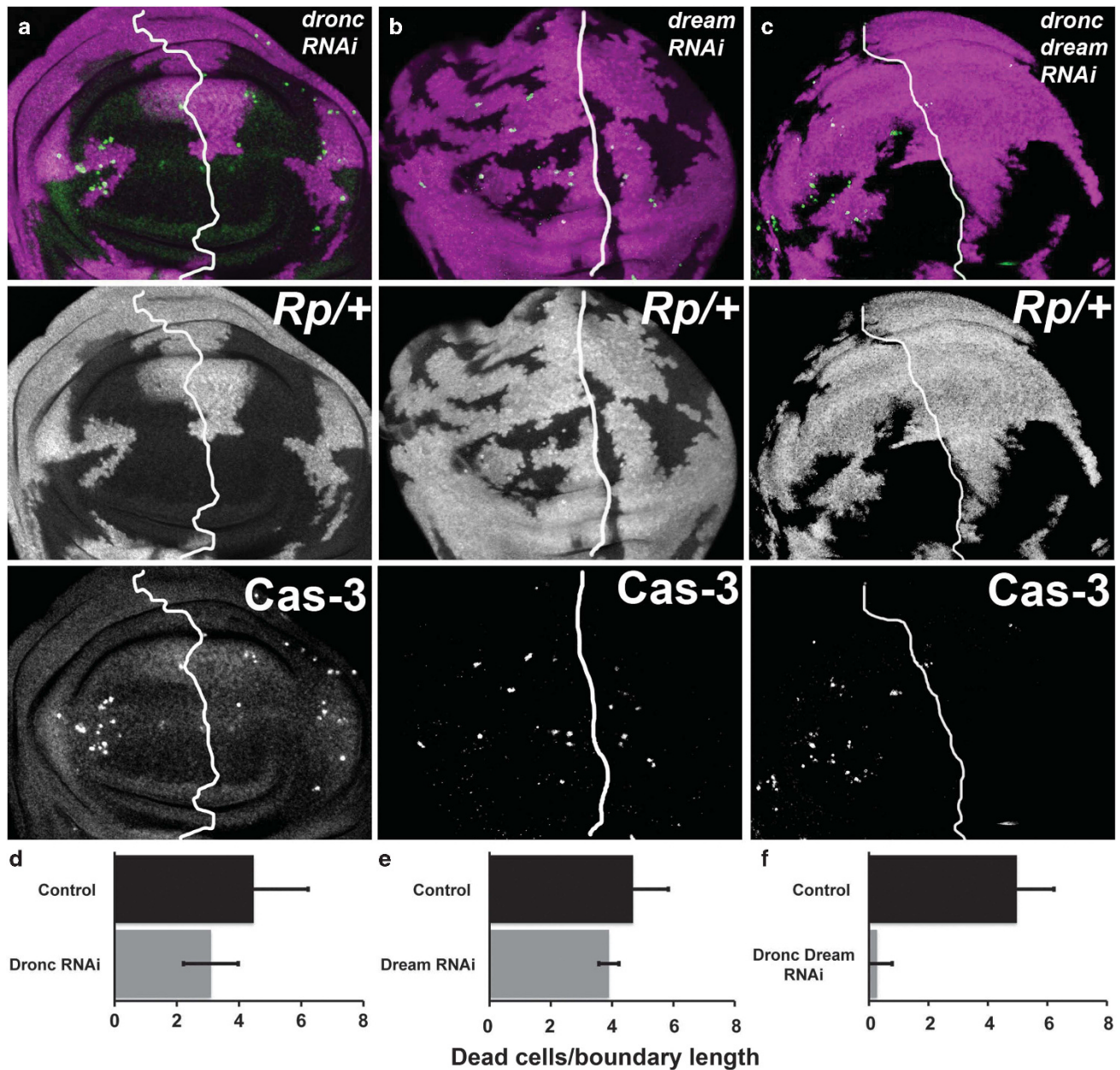
Figure 7 Competitive death of *Rp*/+ cells depends on both Dronc and Dream/Strica. Third instar wing imaginal discs where +/+ cell clones are unlabelled (black), *Rp*/+ cells are labeled for β-galactosidase expression (magenta), cell death labeled in green and the compartmental boundary is marked in white line. Co-expression of dronc and dream specific dsRNA in the posterior compartment blocks cell death of *Rp*/+ cells due to competition but dsRNA against dronc or dream alone could not block it. (**a**) Clones of-wild type cells (unlabeled, black) in *RpS3*/+ background (magenta). Cell death labeled by activated caspase-3 labeling (green). Posterior compartment expression of dsRNA for *dronc* made little difference. Genotype: *y w hsF/UAS-Dicer-2; en-GAL4 UAS-GFP/UAS-Dronc^RNAi; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**b**) Clones of wild type cells (unlabeled, black) in *RpS3*/+ background (magenta). Cell death labeled by activated caspase-3 labeling (green). Posterior compartment expression of dsRNA for *dream/strica* made no difference. Genotype: *y w hsF/UAS-Dicer-2; en-GAL4 UAS-GFP/UAS-Dream^RNAi; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**c**) Clones of wild type cells (unlabeled, black) in *RpS3*/+ background (magenta). Cell death labeled by activated caspase-3 labeling (green). Posterior compartment co-expression of dsRNAs for *dronc* and *dream/strica* abolished competitive apoptosis. Genotype: *y w hsF/UAS-Dicer-2; en-GAL4 UAS-GFP/UAS-Dronc^RNAi -UAS-Dream^RNAi; FRT82B/FRT82B p[arm-LacZ] M(3)95A*. (**d–f**) Quantified data corresponding to **a–c**. Differences were not significant in **d** and **e** ($P > 0.05$), but highly significant for **f** ($P < 0.01$)

USA). Primary antibodies are as follows: mouse anti-β-galactosidase (mAb40-1a) from DSHB; rabbit anti-activated caspase 3 (CM1 from Idun Pharmaceuticals, San Diego, CA, USA);[69] rabbit anti-activated-caspase 3 (Cell Signaling Technology, Danvers, MA, USA); rabbit anti-activated DCP1 (Cell Signaling Technology); rat anti-GFP (Nacalai Tesque Inc., Kyoto, Japan); rabbit anti-GFP (Invitrogen,

ThermoFischer Scientific, Waltham, MA, USA). Labeling for anti-active Dcp1 is similar to CM1, but does not persist in the presence of p35 (our unpublished observations).

Secondary antibodies were multilabeling antibodies from Jackson Immuno-Research Laboratories (West Grove, PA, USA).

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