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Cell Competition Time Line: Winners Kill Losers, which Are Extruded and Engulfed by Hemocytes

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SUMMARY

Cell competition is a mechanism that eliminates slow dividing cells from a growing population. It is believed that the genes *wasp*, *psr*, and *draper* are active in the cells that win the competition ("winner cells") and that they are essential in the winner cells for the induction of apoptosis and for the elimination of the "loser cells." Here, we show that lack of those genes in winner cells appears to be dispensable for cell-competition-induced apoptosis and during *dmyc*-induced supercompetition. Moreover, winner clones do not need those genes in order to preserve their growth advantage. Finally, we find that most of the clearance of the apoptotic debris is not performed by winners but by recruited hemocytes, which are required for the removal of the apoptotic corpses at the very end. Therefore, engulfment is a consequence—not a cause—of loser cells' death.

INTRODUCTION

Multicellular animals possess genes and mechanisms that recognize and eliminate less-adapted or weaker cells of developmental primordia, ensuring that viable but suboptimal cells do not accumulate during development or aging.

How groups of cells compare their relative fitness levels and decide which cells will remain in the tissue ("winner cells") and which cells will die ("loser cells") is becoming increasingly clear. Recent work in the *Drosophila* wing imaginal discs has shown that it proceeds through a multistep process.

First, an insult, for example, a mutation, increases or decreases the fitness of a particular cell within the imaginal disc epithelium. Because many insults can negatively affect "cellular fitness," mutations connected to cell competition usually modify the normal physiology, proliferation, or metabolic rate of cells. Best understood among the mutations that decrease cell fitness are those that reduce protein synthesis rates, such as heterozygous mutations in ribosomal protein genes called *Minutes* (Morata and Ripoll, 1975; Moreno et al., 2002a), mutations in genes that affect cell growth and proliferation, such as *Drosophila myc* (*dmyc*) (de la Cova et al., 2004; Moreno and Basler, 2004), but also mutations that affect cell signaling pathways required for growth and survival, such as the Dpp/BMP/TGFβ pathway (Moreno et al., 2002a).

All those mutations share the characteristic that they are required for optimal functioning of the cells, but a reduction-of-function does not directly trigger autonomous cell death (Moreno et al., 2002a). The mutant cells are still functional and are only culled by programmed cell death (Moreno et al., 2002a) if "fitter" cells are present that can replace them (Morata and Ripoll, 1975; Moreno et al., 2002a; Simpson, 1979).

In a second step after the initial insult, expression of different isoforms of the cell membrane protein Flower (Fwe) label cells as winners or losers (Rhiner et al., 2010). Interestingly, this gene has been recently implicated in the susceptibility to skin papilloma in mice (Petrova et al., 2012), supporting the connection between supercompetition and cancer development. In addition, loser cells also upregulate the secreted protein dSPARC (Portela et al., 2010), which acts as a self-protecting signal and has also been implicated in cancer (Arnold and Brekken, 2009; Brekken et al., 2003; Clark and Sage, 2008; Framson and Sage, 2004; Petrova et al., 2011; Sangaletti et al., 2003).

All the changes described above occur in the loser cells. The only genes that have been shown to function in winner cells and are believed to be absolutely required for cell competition are the genes *draper*, *wasp*, and *psr* (Baker and Li, 2008; Li and Baker, 2007). The gene *draper* encodes for a homolog of the CED-1 phagocytic receptor in *C. elegans* (Freeman et al., 2003; Zhou et al., 2001), which has been implicated in the phagocytosis of apoptotic cells by cultured *Drosophila* hemocytes (Manaka et al., 2004), in axon remodelling and engulfment by glial cells (Awasaki et al., 2006; MacDonald et al., 2006). *wasp* is an actin regulator that has been shown to act in the phagocytosis of *Staphylococcus aureus* in *Drosophila* (Pearson et al., 2003). Finally, the phosphatidylserine receptor gene named *psr* (Fadok et al., 2000), despite the fact that it has been proposed in some studies to have a role in engulfment in mammals (Fadok et al., 1992; Martin et al., 1995; van den Eijnde et al., 1998; Verhoven et al., 1995), encodes for a nuclear protein in *Drosophila* without a specific role in engulfment (Cikala et al., 2004; Cui et al., 2004; Krieser et al., 2007; Mitchell et al., 2006). A cell competition study with these genes proposed that +/+ winner

cells eat their way through mosaic compartments, consuming *M*/+ loser cells along the way (Li and Baker, 2007). Also, dMyc-overexpressing cells were thought to eliminate *wt* loser cells by active engulfment during supercompetition (Li and Baker, 2007).

Similar conclusions were drawn in a second study examining cells mutant for tumor suppressor genes, such as *scrib* or *dlg.* Such cells lose their epithelial integrity and are eliminated by surrounding wild-type tissue, which seemed to activate the ELMO/Mbc-mediated pathway, thereby culling mutant cells by engulfment (Ohsawa et al., 2011). This interpretation was based on experiments where RNAi, against *elmo* or *mbc*, suppressed elimination of *scrib* mutant clones (Ohsawa et al., 2011). However, whether the elimination of *scrib* mutant clones is related genetically to cell competition is still unclear. For example, *eiger*, the *Drosophila* homolog of the Tumor Necrosis Factor (Igaki et al., 2002; Moreno et al., 2002a, 2002b) is required for the elimination and apparent engulfment of *scrib* mutant cells, whereas it seems dispensable for the elimination of *M*/+ losers by +/+ cells (Ohsawa et al., 2011).

Here, we find that lack of *psr*, *wasp*, and *draper* in winner cells appears to be dispensable for cell competition-induced apoptosis and does not impair the growth of winner cells. In addition, we show that hemocytes are the cells required to remove the apoptotic corpses and the cellular debris after apoptosis has been fully executed.

In summary, (1) our results show that corpse engulfment is not a decision-making step in cell competition-induced apoptosis but rather one that follows death of cells, and (2) our results agree with previous reports (Franc et al., 1999a) showing that most of corpse engulfment is performed by the professional phagocytes, rather than by epithelial cells.

RESULTS

draper, *wasp*, and *psr* Are Not Essential for the Elimination of Loser Cells during Supercompetition

The genes *draper* (*drpr*), *wasp*, and *psr* have been shown to play a role in winner cells to kill and engulf loser cells in different cell competition scenarios (Li and Baker, 2007).

We have tested the relevance of these genes in a dmyc-supercompetitor assay because this is a well-established competition scenario in which cells expressing higher levels of dMyc under the *tubulin* promoter (*tub* > *dmyc*) can outcompete surrounding wild-type cells, which in this case, act as loser cells (de la Cova et al., 2004; Moreno and Basler, 2004). dMyc-overexpressing cells that are able to outcompete and eliminate wild-type cells have been called supercompetitors (Moreno and Basler, 2004). FRT-FLP technique was used to generate *wt* loser cells next to dMyc-overexpressing supercompetitor cells (Figure 1A), both in a wild-type (*wt*) background (Figures 1E–1G) and in a *drpr*−/− background (Figures 1H–1J; *drpr*−/− background was verified by RT-PCR, Figure S1A; antibody staining, Figures S1E–S1G; and active Caspase-3 staining, Figures S1J and S1K) in wing imaginal discs of *Drosophila melanogaster*. A time-course experiment was performed to determine whether *drpr* was essential for the elimination of loser cells. Statistical analysis showed that *wt* loser cells disappeared with similar kinetics from the tissue, both in *wt* and *drpr*−/− backgrounds, without any significant difference (Figure 1B). We then conducted a second experiment of supercompetition, generating *tub* > *dmyc* clones in a *wt* background. To knockdown the expression of *drpr* in imaginal disc cells, a specific *UAS-drpr RNAi* was activated in the posterior compartment under the *engrailed* > *Gal4* (*GFP*) driver. *tub* > *dmyc* clones in the anterior (with Drpr) and posterior (reduced Drpr) compartment were compared, and no differences regarding the induction of apoptosis (considering both apical Caspase and basal apoptotic debris) or the expansion of the dMyc-overexpressing clones by cell competition were found (Figures S1C, S1D, S1H and, S1I).

Apoptotic Corpses Accumulate basally in the Absence of Functional *draper*

The final outcome of cell competition is the apoptosis of the loser cells (Moreno et al., 2002a; Rhiner et al., 2010). Thus, Caspase-3 activation, a marker of apoptosis, can be used to detect the death of loser cells in cell competition (Moreno and Basler, 2004). The remaining cell corpses tend to be extruded basally and are finally cleared from the tissue (Moreno and Basler, 2004). We observed that loser cells in the *drpr*−/− background activated Caspase-3 normally but such apoptotic cells accumulated basally in *drpr*−/− wing imaginal discs at 48 hr after clone induction (ACI) (Figures 1K–1M), compared to *wt* discs at the same time point where corpse removal is functional (Figures 1N–1P), suggesting that engulfment is only required for the clearance of already dead cells (cellular debris full of active Caspase-3 called apoptotic corpses, located basally, and therefore outside the normal plane of the epithelium). TUNEL staining was performed as another way to confirm the elimination of loser cells, showing comparable results (Figures 1C and 1D).

To differentiate between developmental and cell competition-induced apoptosis, apoptotic corpses were quantified in *wt* (*drpr*+) *discs and in drpr*−/− wing discs in the presence or absence of cell competition (Figures 1Q–1S). As expected, there was an increase of Caspase-3-positive cells in *drpr*−/− discs compared with *drpr*+ discs and a further increase (by 20%) in *drpr*−/− discs under cell competition.

These results suggested that *drpr* is necessary to remove the apoptotic debris produced by cell competition but not for the extrusion of the dead cells outside the plane of the epithelium.

wasp and *psr* Homozygous Mutant Larvae Show a Developmental Delay

The experiments with *drpr* described above were straightforwardly interpreted because we found that the *drpr* mutation does not induce any detectable growth defect during larval development. In particular, we perceived no delays in the development of *wt* larvae compared to *drpr* mutants (Figure S1B) and no differences in wing imaginal disc size at comparable stages throughout development (Figures S1J and S1K). However, when the same experiment was done with *wasp* and *psr* null mutants, a defect in growth was observed for these two mutants (Figure S1B), which made time-course comparisons complicated. Nevertheless, active Caspase-3 staining showed that loser clones were dying in both mutant backgrounds, similar to the *wt* situation (Figures 1T–1V).

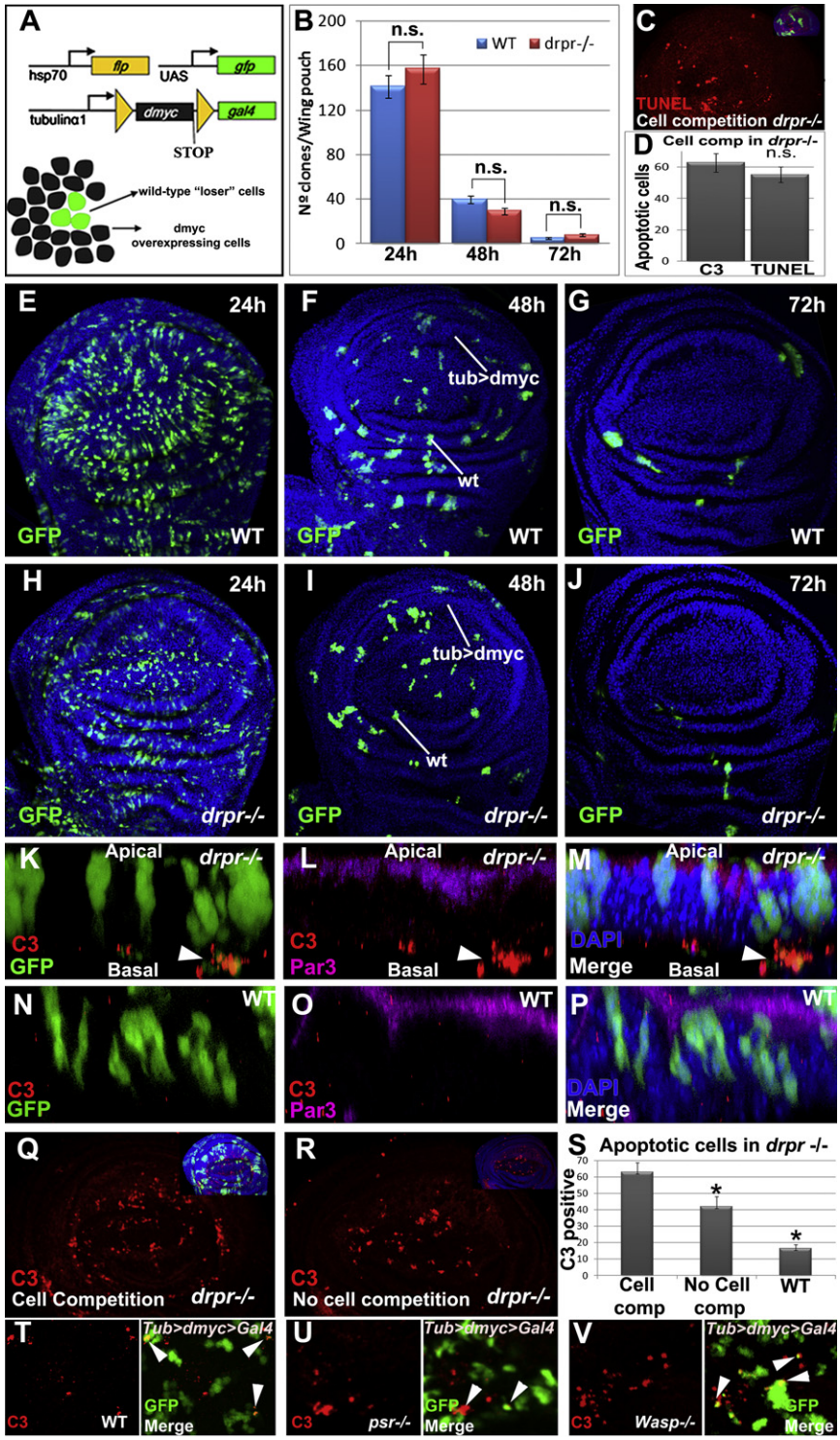


Figure 1. *draper* Is Not Essential for the Elimination of Loser Cells during Supercompetition

(A) Flip-out technique used to generate GFP-marked wild-type (*wt*) cells in a dMyc-overexpressing background by combining three transgenes (*hs-Flp; tub > dmyc > Gal4/Cyo; UAS-GFP* flies). Heat shock (hs) promotes the expression of the Flipase (Flp) enzyme in a random subset of cells leading to excision of a dmyc cassette flanked by FRT sites in the *tub > dmyc > Gal4* transgene. Upon removal of the dmyc cassette (including the stop codon), the *Gal4* gene is placed under the control of the *tubulin* promoter and subsequently activates *UAS-GFP* expression. This supercompetitor assay is depicted on the left at the cellular level. Clones of GFP marked *wt* cells (green) are generated in a *tub > dmyc* background (black).

(B) Quantification of remaining loser clones in the supercompetitor assay at different time points after clone induction (ACI). Between 24 and 43 wing discs were measured for each genotype. Error bars represent SEM. There are no statistical differences ($p > 0.01$) according to a Student's t test (n.s., not significant).

(C) TUNEL staining (red) of a wing imaginal disc under cell competition in a *drpr−/−* background.

(D) Quantification of the number of apoptotic cells (considering both apical Caspase and basally accumulated apoptotic debris) in a *tub > dmyc drpr−/−* background comparing active Caspase-3 and TUNEL stainings. Error bars represent SEM. There are no statistical differences ($p > 0.01$) according to a Student's t test (n.s., not significant).

(E–J) Loser *wt* clones (GFP, green) are eliminated by cell competition in a *tub > dmyc* background (E–G) as in a *tub > dmyc drpr−/−* background (H–J). DAPI is shown in blue.

(K–P) Transversal cut of a wing imaginal disc showing loser *wt* clones (GFP, green) basally extruded in a *tub > dmyc drpr−/−* background at 48 hr ACI (K–M) compared with the *tub > dmyc* background at the same time point (N–P). Active Caspase-3 is shown in red. The apical marker Par3 is shown in magenta and DAPI in blue.

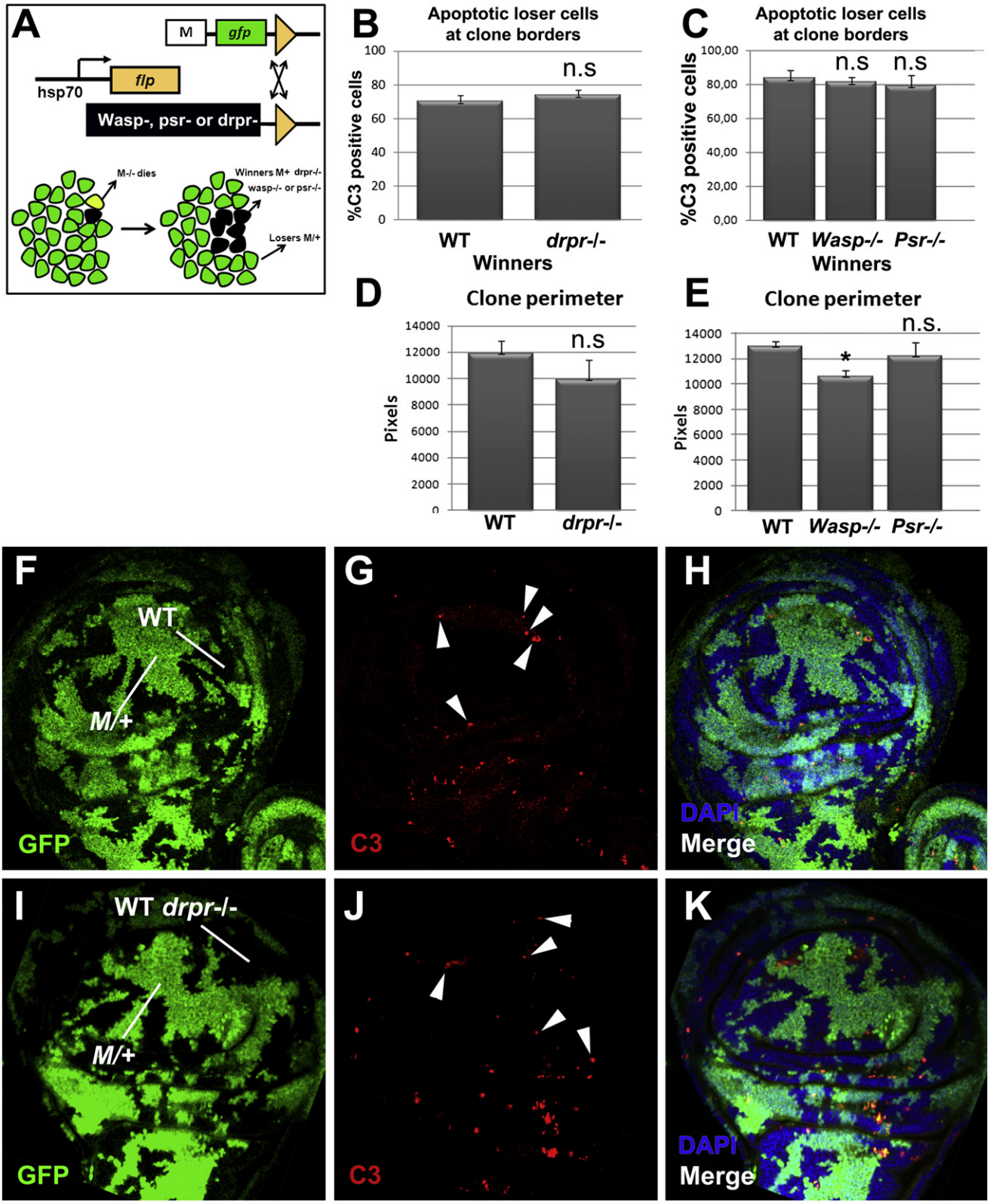
(Q) Wing imaginal disc showing Caspase-3-positive cells (image shows a maximal projection of the wing imaginal disc, thus including apical Caspase and basally accumulated apoptotic debris) in a *drpr−/−* background under cell competition.

(R) Compared with the same mutant background as (Q) but without competition.

(S) The number of Caspase-3-positive cells in (Q) appeared to be statistically higher than in (R) (p value = 0.0168) and higher than in a control situation (WT bar p value = 0.0001), according to a Student's t test. Error bars represent SEM.

(T–V) Caspase-3 staining of loser clones (GFP, green) dying in a *tub > dmyc* background as in a *tub > dmyc psr−/−* and *tub > dmyc wasp−/−* backgrounds at 48 hr ACI. Active Caspase-3 is shown in red.

See also Figure S1.


A
M
gfp
hsp70
flp
Wasp-, psr- or drpr-
M-/- dies
Winners M+ drpr-/- wasp-/- or psr-/-
Losers M/+
B
Apoptotic loser cells at clone borders
%C3 positive cells
100
80
60
40
20
0
n.s
WT
drpr-/-
Winners
C
Apoptotic loser cells at clone borders
%C3 positive cells
100,00
80,00
60,00
40,00
20,00
0,00
n.s
n.s
WT
Wasp-/-
Psr-/-
Winners
D
Clone perimeter
Pixels
14000
12000
10000
8000
6000
4000
2000
0
n.s
WT
drpr-/-
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Clone perimeter
Pixels
14000
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4000
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WT drpr-/-
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DAPI
Merge

We therefore decided to analyze those genes in a cell competition scenario using the *Minute* (*M*) technique (Morata and Ripoll, 1975; Simpson, 1979), where the larval background is only heterozygous for the mutations (see below).

M/+ Loser Cells Are Still Outcompeted by +/+ Cells in the Absence of *drpr, wasp*, or *psr*

Next, we used the *Minute* (*M*) technique (Morata and Ripoll, 1975; Simpson, 1979) to confront *M*/+ loser cells (heterozygously mutant for a ribosomal protein gene) with winner +/+ cells, either homozygously mutant for *drpr*, *wasp*, or *psr* (Figure 2A) or classical winner +/+ cells (Figures 2B–2K and 3A–3L).

We measured the number of active Caspase-3-positive cells among all *M*/+ loser cells and specifically among the population of *M*/+ loser cells next to the clone of +/+ winner cells (*M*/+ cells in contact with +/+ cells and up to 2–3 cells diameters away from the winner cells, Figures 2B, 2C, S2A, and S2B). It is important to note that wing discs heterozygously mutant for *drpr* (*drpr*-/+) showed increased number of apoptotic cells accumulated basally (due to a defect in the engulfing capacity of hemocytes, see below), but because those cells are outside the normal plane of the epithelium, they are not computed in this experiment. Statistical analysis showed that the average number of cells positive for activated Caspase-3 at the border where winner cells contact *M*/+ cells was comparable regardless of whether or not winner cells were mutant for *drpr*, *wasp*, *psr* (Figures 2B, 2C, 2F–2K, and 3D–3L).

In addition, we studied the area of competition between +/+ winner cells and *M*/+ loser cells in the different situations by measuring the perimeter of +/+ winner clones when they were either *wt* or mutant for *drpr*, *wasp*, and *psr*. In the case of *drpr* mutant clones, no differences were found (Figure 2D). Moreover, the average of +/+ winner clone size did not show any difference when winner clones were either *wt* or mutant for *drpr* (data not shown) as it has been previously reported (Martín et al., 2009). +/+ winner clones mutant for *wasp* showed shorter perimeter, that is, a reduced surface of competition, compared to *wt* ones (Figure 2E). Accordingly, *wasp* mutant clones were bigger than control clones, both in competitive (therefore showing reduced area of competition because they occupied more space in the wing pouch) and noncompetitive backgrounds, suggesting that it does not have a specific role in winner cells throughout cell competition (Figures 3B and 3C). Finally, +/+ winner clones mutant for *psr* did not show any difference neither in the perimeter length (Figure 2E) nor in the behavior and size in both competitive and noncompetitive backgrounds (Figures 3A and 3C).

This data underlines that *wt* winner cells do not need *drpr*, *wasp*, or *psr* to induce the apoptotic pathway in *M*/+ loser cells during *Minute*-induced competition.

drpr Deficiency Leads to Basal Accumulation of Apoptotic Corpses in *lgl*-, *scrib*-, and *dlg*-Deficient Clones

It is believed that cells mutant for some tumor suppressor genes undergo apoptosis and are engulfed by surrounding wild-type tissue (Ohsawa et al., 2011). However, it remains unclear whether this elimination is genetically related to cell competition. To address this question, we generated clones homozygously mutant for the tumor suppressor gene *lethal giant larvae* (*lgl*) (Figure 4A), both in a *wt* background (Figures 4B–4D) and in a *drpr*−/− background (Figures 4H–4J). Staining against activated Caspase-3 revealed an increased number of dead cells in *lgl*−/− clones in *drpr*−/− background compared to the *wt* background (Figures 4I and 4C, respectively; images show maximal projections, thus including apical Caspase and basally accumulated apoptotic debris). Furthermore, we observed that in the absence of *drpr* most of the apoptotic corpses were again accumulated basally compared to the *wt* background (Figures 4K–4M and 4E–4G, respectively). A similar phenotype was observed for the case of two other tumor suppressor genes: *scrib* and *dlg*. Clones expressing *RNAi* against these genes activated Caspase-3 and accumulated basally in the epithelium in the absence of *drpr* (Figure S3).

These experiments show that *lgl*, *scrib*, and *dlg*-deficient cells accumulate as apoptotic corpses and are extruded from the epithelium in the absence of *drpr*.

Hemocytes Remove Apoptotic Corpses after *Minute*-Induced Competition

Because we detected an accumulation of basally extruded cells with activated Caspase-3 in wing discs when *drpr*, *wasp*, or *psr* was impaired and this effect was bigger when *drpr*, *wasp*, or *psr* were inactivated throughout the animal than when they were specifically inactivated in winner cells, we asked whether another cell type may be helping to clean up the cellular debris after cells have been killed and extruded from the epithelium.

Drosophila hemocytes have diverse roles during development and immunity. Among other important functions, they seek out the tissue and remove apoptotic cells and debris (Abrams

Figure 2. *M*/+ Loser Cells Are Still Eliminated by +/+ Cells in the Absence of *draper*

(A) FRT-FLP technique to generate *M*+ cells mutant for *drpr*, *wasp*, or *psr* in a *M*/+ background. Note that twin *M*−/− cells (marked by double GFP) die once generated.

(B and C) Average of Caspase-3-positive cells in the periphery of the loser territory (2–3 cells diameter, that is, only apical Caspase-3-positive cells) relative to the total Caspase-3 in the loser territory in both situations: when winners are *wt* or mutant for *drpr* (B) or when they are *wt* or mutant for *wasp* or *psr* (C). Around 15 wing discs were measured for each genotype. Error bars represent SEM. There are no statistical differences ($p > 0.01$) according to a Student's t test. n.s., not significant.

(D and E) Perimeter of winner clones either *wt* or mutant for *drpr* (D) or *wt* or mutant for *wasp* and *psr* (E).

(F–K) Loser *M*/+ cells (GFP, green) are killed by winner cells (black), either *wt* (F–H) or mutant for *draper* (I–K) at 72 hr ACI. Active Caspase-3 is shown in red and DAPI in blue. Arrowheads show apoptotic loser cells at the border of competition marking apical Caspase-3-positive cells only.

Error bars represent SEM. There are no statistical differences ($p > 0.01$) for *drpr* and *psr,* according to a Student's t test (n.s., not significant). Winner mutant clones for *wasp* have statistically shorter perimeter than control ones (p value = 0.0047), according to a Student's t test. See also Figure S2A.

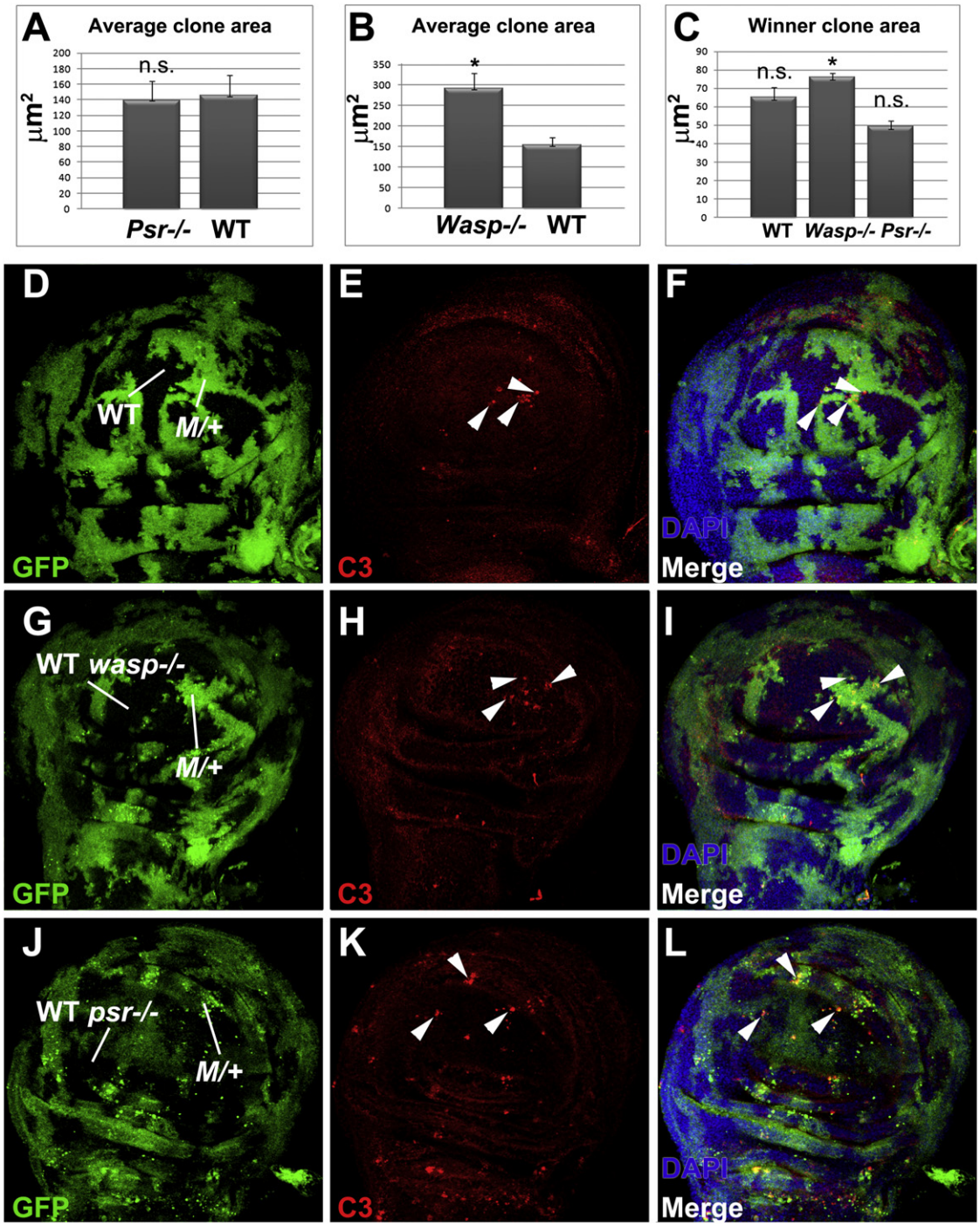

Figure 3. *M*/+ Loser Cells Are Still Eliminated and Replaced by +/+ Cells in the Absence of *psr* and *wasp*

(A and B) Average of clone area of *psr* (A) or *wasp* (B) mutant clones compared to their twin in noncompetitive backgrounds. Fifteen wing discs were measured for each genotype. Error bars represent SEM. There are no statistical differences ($p > 0.01$) according to a Student's t test for *psr* (n.s., not significant). Mutant clones for *wasp* are statistically bigger than twin controls (p value = 0.0037), according to a Student's t test.

(C) Average of winner clone area, either *wt* or mutant for *psr* or *wasp* in *Minute* competition. Fifteen wing discs were measured for each genotype. Error bars represent SEM. There are no statistical differences ($p > 0.01$) according to a Student's t test for *psr* (n.s., not significant). Winner mutant clones for *wasp* are statistically bigger than *wt* ones (p value is less than 0.0001), according to a Student's t test.

Loser *M*/+ cells (GFP, green) are killed by winner cells (black), either *wt* (D–F) or mutant for *wasp* (G–I) or *psr* (J–L) at 72 hr ACI. Active Caspase-3 is shown in red and DAPI in blue.

See also Figure S2B.

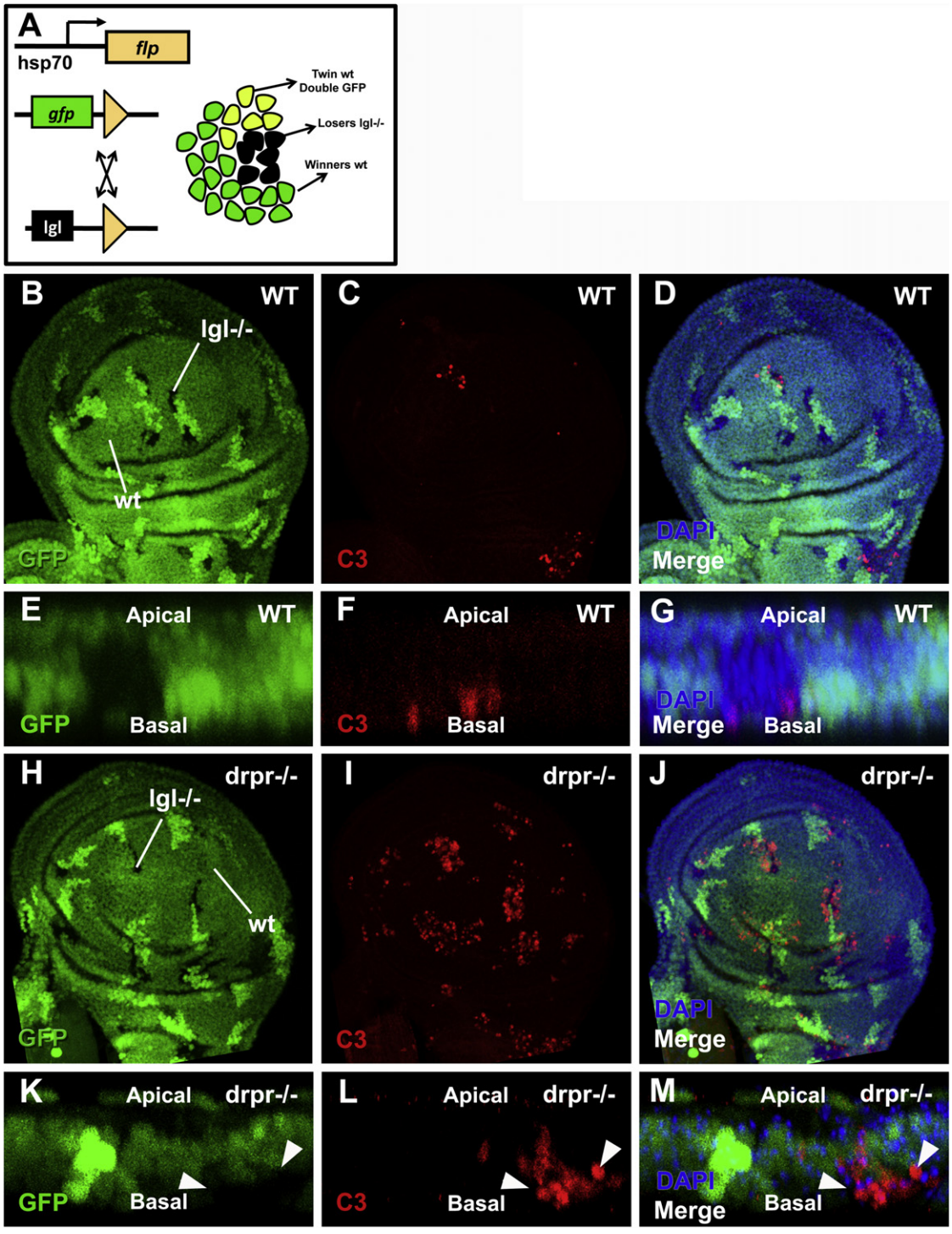

Figure 4. Preventing *draper* Function Induces Basal Apoptotic Corpses Accumulation of *lgl* Mutant Cells

(A) FRT-FLP technique used to generate *lgl*−/− cells in a *wt* background.

(B–J) Clones of *lgl* (black) are killed in a *wt* background (GFP, green) (B–D) as in a *drpr*−/− background (H–J). Active Caspase-3 is shown in red and DAPI in blue. Images show maximal projections of wing imaginal discs, thus including apical Caspase and basally accumulated apoptotic debris.

(K–M) Transversal cut of a wing imaginal disc. Arrowheads mark apoptotic corpses of *lgl*−/− cells accumulated basally in *drpr*−/− background compared to *wt* background (E–G).

See also Figure S3.

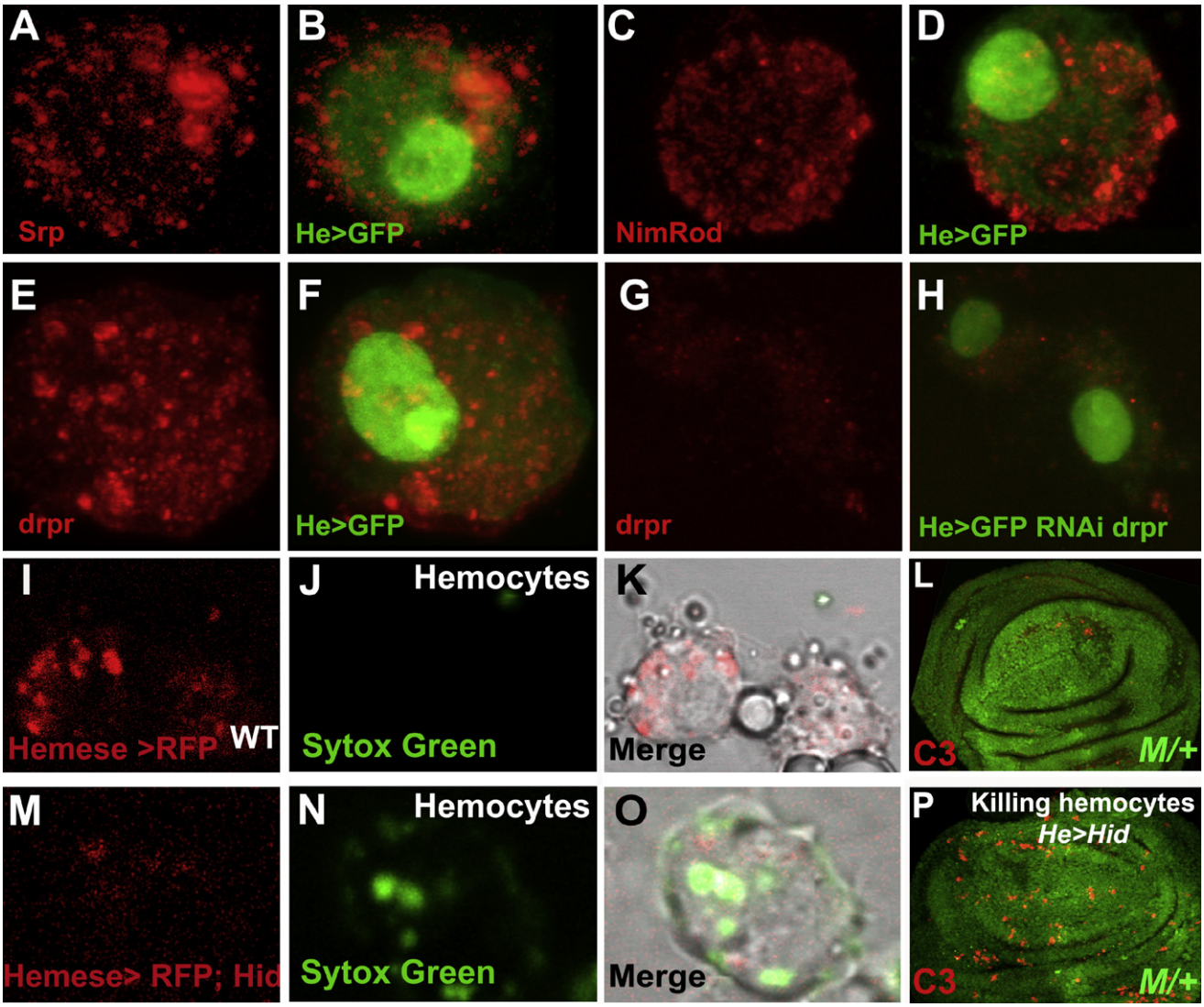

Figure 5. Hemocytes Remove Apoptotic Corpses

(A–D) Srp and Nimrod (hemocyte-specific markers, red) show the specificity of the *Hemese* driver in hemocytes (*HeGal4 UAS-GFP*).
(E–H) Drpr staining in hemocytes (marked by *HeGal4 UAS-GFP*). A *UAS-drpr RNAi* specifically expressed in hemocytes show Drpr antibody specificity (G–H).
(I–K and M–O) Hemolymph-hemocyte preparation from *wt* larvae (I–K) and from *HeGal4 RFP UAS-Hid* larvae (M–O). Sytox green is used to mark dead cells.
(L and P) Killing hemocytes (by *HeGal4 UAS-Hid*) increases Caspase-3-positive cells in a *M*/+ disc (green, P) compared to a *wt* situation (L). Active Caspase-3 is shown in red. Images show maximal projections of wing imaginal discs, thus including apical Caspase and basally accumulated apoptotic debris.
See also Figure S4.

et al., 1993; Franc et al., 1996, 1999a, 1999b; Tepass et al., 1994). Ninety-five percent of circulating hemocytes consist of phagocytic plasmatocytes, which are responsible for cell ingestion (Evans et al., 2003). Because in the wing pouch, as well as in other epithelia (Rosenblatt et al., 2001), apoptotic bodies are engulfed to preserve tissue integrity, we sought to evaluate the role of hemocytes in the elimination of outcompeted *Minute* cells.

To this end, we abolished hemocytes by overexpression of the proapoptotic gene *hid* under the control of the hemocyte-specific driver *Hemese* (Kurucz et al., 2003). Hemocyte-specific expression by *Hemese* was verified in combination with immunostaining for hemocyte-specific markers, such as the GATA transcription factor, Serpent (Srp) (Lebestky et al., 2000), and Nimrod (Kurucz et al., 2007) (Figures 5A–5D). We also stained hemocytes with a Drpr-specific antibody to corroborate that Drpr is expressed in these cells. The signal detected by anti-Drpr antibody greatly diminished when a *UAS-drpr RNAi* was specifically activated in the hemocytes, which shows the specificity of the antibody (Figures 5E–5H). Apoptotic Hid-overexpressing hemocytes, marked by RFP, stained positive for Sytox Green (used as a marker of apoptosis), in contrast to *wt* hemocytes (Figures 5M–5O and 5I–5K, respectively).

Applying this strategy to kill hemocytes, we dissected wing discs from homogeneous *M*/+ larvae (therefore without

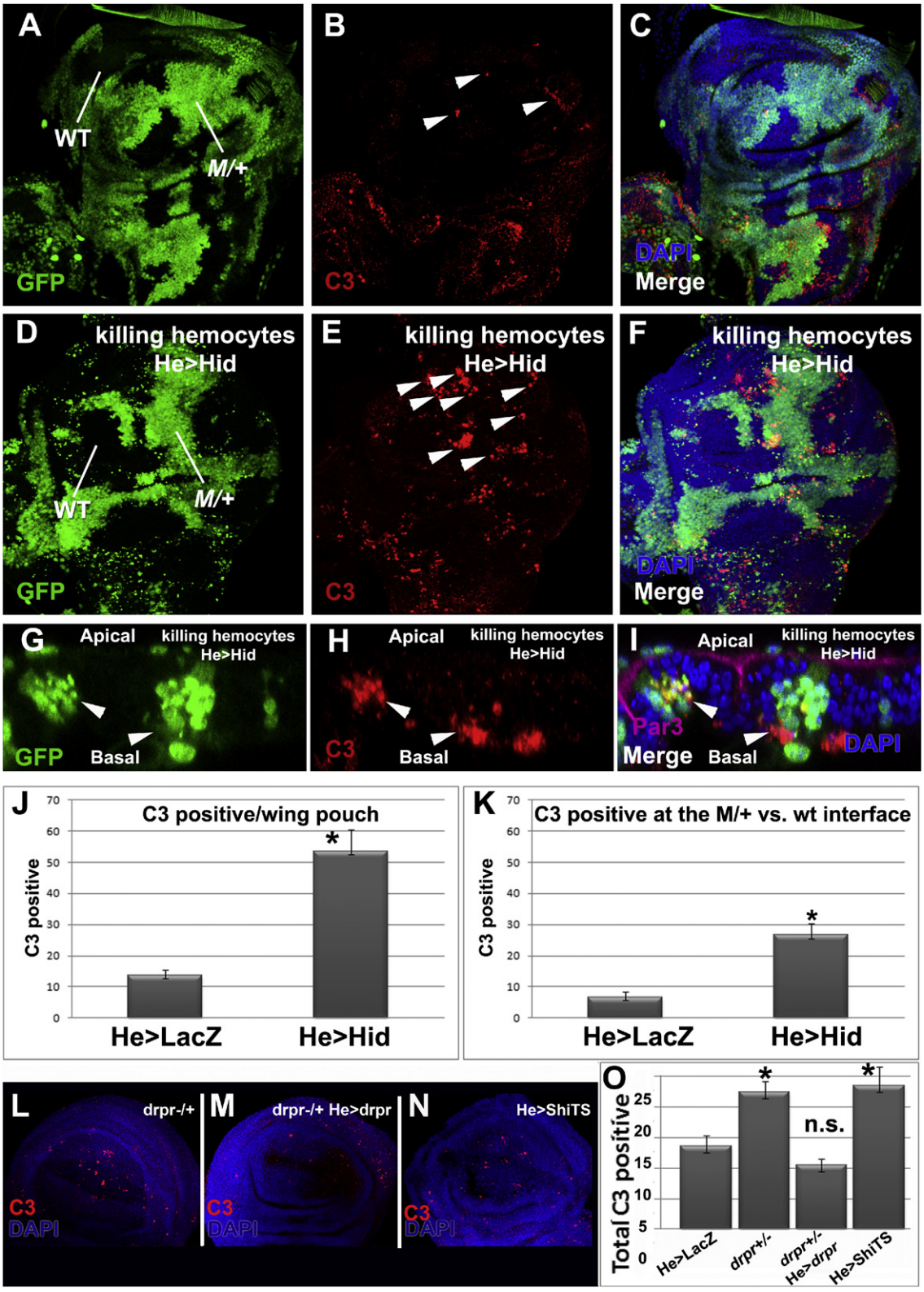

Figure 6. Hemocytes Clean Up the Apoptotic Corpses during *Minute* Competition

(A–F) Killing hemocytes increases the accumulation of apoptotic corpses (arrowheads) during *Minute* competition (D–F) compared to a *wt* situation (A–C). Active Caspase-3 is shown in red and DAPI in blue. Arrowheads show apoptotic *M*/+ loser cells at the border of competition.

competition) either with intact or ablated hemocytes. When staining against activated Caspase-3, we observed a high number of apoptotic corpses in discs from larvae lacking phagocytic hemocytes compared to *wt* larvae (Figures 5P and 5L, respectively, images show maximal projections, thus including apical Caspase and basally accumulated apoptotic debris). We also confirmed that these apoptotic corpses were not dead hemocytes sticking to the epithelium both in competitive and noncompetitive backgrounds (Figures S4A–S4L) and that we were able to eliminate almost 80% of the hemocytes by *hid* overexpression (Figure S4M).

Next, we generated *Minute*-induced cell competition, opposing *M*/+ cells with *wt* cells and observed a clear accumulation of apoptotic corpses when hemocytes were killed in contrast to discs from larvae with intact hemocytes (Figures 6A–6F; images show maximal projections, thus including apical Caspase and basally accumulated apoptotic debris). This increase in active Caspase-3-positive cells was statistically significant, both when total number of apoptotic cells in the wing pouch were compared (including apical Caspase and basally accumulated apoptotic debris) and when only the active Caspase-3-positive loser cells at cell boundaries facing winner cells were compared (apical Caspase signal found 2–3 cell diameters apart from +/+ cells, Figures 6J and 6K). Furthermore, we noted that ablation of hemocytes coincided with persistence of active Caspase-3-positive cellular debris that was extruded basally but failed to be removed (Figures 6G–6I).

To determine if indeed hemocytes are the main engulfing cells, we quantified the number of apoptotic corpses in wing discs that lacked Drpr (*drpr* −/−) and observed the same increase in apoptotic bodies compared to discs where hemocytes were ablated (*hemese-gal4; UAS-hid*) (Figure S4N, considering both apical Caspase and basally accumulated apoptotic debris). This result indicates that the Drpr-mediated ingestion of apoptotic debris in the wing disc is mainly performed by hemocytes. To verify this observation, we tried to rescue the accumulation of dead cells in *drpr* mutants by restoring Drpr function only in hemocytes. Wing discs heterozygously mutant for *drpr* (*drpr*−/+) showed an increased number of apoptotic cells accumulated basally (i.e., outside the normal plane of the epithelium). This phenotype could be rescued if *drpr* expression was provided exclusively in hemocytes using the hemocyte-specific driver *hemese-gal4* (Figures 6L, 6M, 6O, and S5; Movies S1 and S2).

Finally, in an attempt to test whether genes related to endocytic pathways, such as *dynamin/shibire* (Awasaki and Ito, 2004), were implicated in the elimination of apoptotic debris by hemocytes, we used a thermosensitive allele and performed the experiments at the restrictive temperature. We measured the number of apoptotic cells, considering both apical Caspase and basally accumulated apoptotic debris, and observed a significant increase in the number of apoptotic cells, especially those basally located (Figures 6N and 6O), suggesting that this gene is involved in the process of eliminating apoptotic corpses from the wing imaginal disc.

We have shown that lack of hemocytes leads to a piling up of apoptotic corpses in the wing pouch during development and cell competition. Interestingly, the association of hemocytes to mutant cells has been previously described (Cordero et al., 2010; Pastor-Pareja et al., 2008); therefore, circulating hemocytes must reach the places where competition is taking place in order to efficiently clear dead cells.

To study the recruitment process, we stained against Serpent (Lebestky et al., 2000) in different scenarios of cell competition. Serpent staining revealed the presence of hemocytes close to *wt* loser cells in a *tub* > *dmyc* background (Figures 7A–7C). Hemocytes were also associated with *M*/+ loser cells during *Minute* competition (Figures 7D–7F) and *lgl*−/− clones in mutant *lgl*-triggered cell elimination (Figures 7G–7I). In all cases the number of hemocytes was higher than in a control situation (Figures 7J and 7K).

Taken together, these results demonstrate that hemocytes are not required to trigger apoptosis in loser cells during *Minute*-competition. However, after apoptotic cell death and basal extrusion are finished, hemocytes are needed to remove apoptotic corpses and active Caspase-3-positive cellular debris.

DISCUSSION

Previous work described that the genes *drpr* and *wasp*, implicated in the engulfment process, and *psr*, unrelated to the engulfment process, appeared to be required in winner cells in different scenarios of cell competition (Li and Baker, 2007).

Here, we have decided to reassess the significance of these genes in a supercompetitor assay where dMyc-overexpressing supercompetitors (*tub* > *dmyc*) outcompete surrounding wild-type cells (*tub* > *gal4*). A time-course experiment revealed that *drpr*, the gene that showed the strongest phenotype in the

(G–I) Transversal cut of a wing imaginal disc. Arrowheads mark dying *M*/+ loser cells (GFP, green) that remained basally extruded. The apical marker Par3 is shown in magenta and DAPI in blue.

(J and K) Quantification of total number of Caspase-3-positive cells of wing pouch (J, including both apical Caspase and basally accumulated apoptotic debris), and (K) total number of Caspase-3-positive cells in the periphery of the loser territory. Between 15 and 20 wing discs were measured for each genotype. Error bars represent SEM. The number of Caspase-3-positive cells appeared to be statistically higher when hemocytes were eliminated (He > Hid) compared to a *wt* situation (He > LacZ), according to a Student's t test (p value = 0.0005 of J and p value = 0.0003 of K).

(L and M) Wing imaginal disc showing Caspase-3-positive cells in a *drpr*-/+ background (L), compared with the same mutant background when hemocytes overexpressed *drpr* (M). Images show maximal projections of wing imaginal discs, thus including apical Caspase and basally accumulated apoptotic debris.

(N) Overexpression of the thermosensitive form of the protein Shibire in hemocytes significantly increased the number of Caspase-3-positive cells (p value equals 0.0182) compared to a control situation (*He* > *LacZ*), according to a Student's t test. Between 15 and 20 wing discs were measured for each genotype.

(O) The number of Caspase-3-positive cells is significantly reduced (to basal levels, *He* > *LacZ*) when hemocytes overexpressed *drpr* in a *drpr*+/− background compared with *drpr*+/− background (p value = 0.0034), according to a Student's t test.

Error bars represent SEM. See also Figure S5 and Movies S1 and S2.

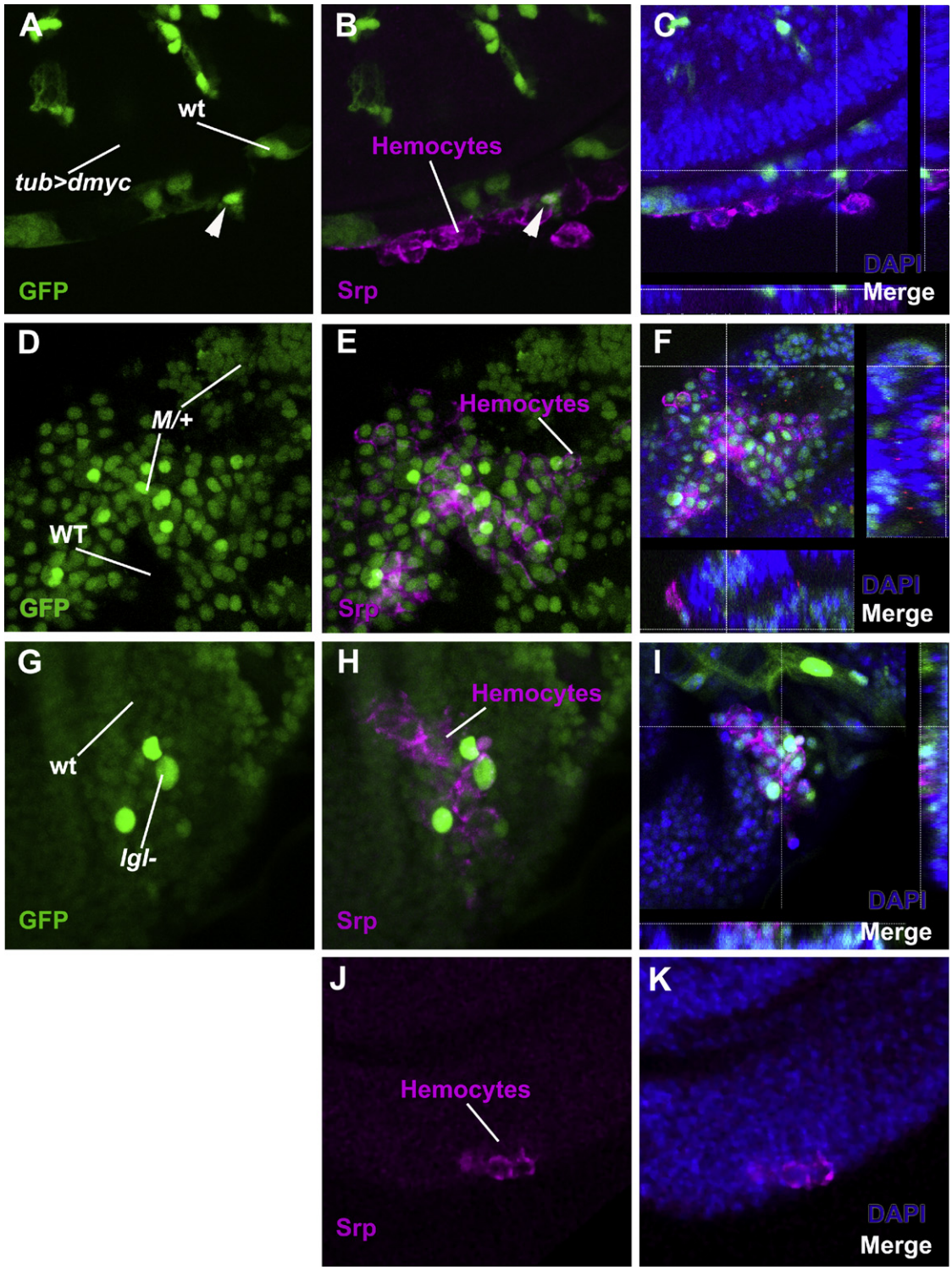

Figure 7. Hemocytes Are Recruited during Cell Competition

Hemocytes marked by Serpent expression (magenta) migrate and appear in large numbers in different scenarios of cell competition surrounding cells to be eliminated compared with a control situation (without cell competition, J and K): in a supercompetitor assay (A–C), arrowhead indicates a loser cell inside of a hemocyte, in *Minute* competition (D–F) and in *lgl* cell elimination (G–I).

previous report (Li and Baker, 2007), was dispensable for the elimination of *wt* loser cells.

Based on these findings, the role of Draper was re-evaluated in a classic *Minute-induced* competition, where *M*/+ cells (heterozygously mutant for a ribosomal protein gene) are opposed to winner +/+ cells, either *wt* or mutant for *drpr*. Quantification of the cell competition process and the resulting apoptotic corpses showed that *drpr* was neither necessary for the induction of apoptosis in loser cells nor necessary for the basal extrusion of the ensuing apoptotic debris. Regarding the average size of +/+ winner clones mutant for *drpr*, we also did not detect any significant difference when winner clones were either *wt* or mutant for *draper* (Martín et al., 2009).

The same experiments were repeated with mutants for *wasp* and *psr*, and these genes were also dispensable for cell competition-induced cell death and did not impair clonal growth.

Similar results were achieved when *lgl*-, *scrib*-, *or dlg*-deficient clones coexisted with *wt* tissue: *wt* cells still induced death of deficient cells in the absence of *draper* function.

Altogether, these experiments show that winner cells do not need the genes *draper*, *wasp*, or *psr* to kill the loser cells, neither during *dmyc*-induced supercompetition nor during *Minute*-induced competition. This is further supported by the observation that $fwe^{Lose\text{-}A}$-overexpressing cells also die in the absence of *draper* (Rhiner et al., 2010).

However, upon removal of those genes in the whole organism, we observed an accumulation of basally located cellular debris and apoptotic corpses. Interestingly, this effect was less prominent when the same genes were inactivated specifically in the winner cells. This raised the possibility that another cell type may be helping in the removal of most of the cellular debris after cells have been killed and the resulting apoptotic corpses extruded from the epithelium.

Our experiments provide evidence that clearance of most of the apoptotic corpses is executed by circulating hemocytes. Ablation of hemocytes by overexpression of the proapoptotic gene *hid* under a specific hemocyte driver, leads to the accumulation of apoptotic corpses and Caspase-3-positive cellular debris. However, those remnants of cellular competition rest outside the plane of the epithelium, where they have been effectively extruded.

These findings seem to contradict previous studies regarding the role of *psr*, *wasp*, and *draper* in the induction of apoptosis in loser cells, corpse removal, and clone size of the winner cells (Li and Baker, 2007).

One possibility is that basally extruded apoptotic corpses and Caspase-3-positive cellular debris may have been erroneously scored as living loser cells. In addition, the basal extrusion of apoptotic corpses from the epithelium may not have been taken into account. To avoid this, we have distinguished between apical Caspase activation, a marker of apoptotic induction, from basal Caspase accumulation, a consequence of deficient engulfment by hemocytes. This distinction between early apoptotic induction (apical) and extruded apoptotic debris (basal and outside the plane of the epithelium) helps to follow the time line of cell competition. Previous reports may have not made this distinction. In addition, if no hemocyte-specific markers are used, then this circulating cell type could be mistaken for an epithelial cell. This may significantly affect the interpretation of a result because we show that hemocytes are not only recruited to epithelia with ongoing cell competition but also are intimately associated with loser cells. Finally, we also carefully estimate the area of competition between winners and losers.

Along the same lines, we would like to note that some of the genes that were tested to assess if engulfment is necessary for competition, are actually not involved in the process (i.e., *psr*) and that some of the markers detected do not necessarily imply engulfment. Lysotracker, for example, also marks processes, such as autophagy. This may have caused confusion between autophagy and engulfment. We think this is possibly the case. First, other proteins related to autophagy are also expressed by loser cells, such as Lysosome Associated Membrane Protein (LAMP) (Rhiner et al., 2010), and second, if engulfment by winner cells is crucial, lysosome markers should be expressed in winner cells in order to degrade the engulfed material; but, this is not the case (Ohsawa et al., 2011; Rhiner et al., 2010). Expression of lysosome markers has been observed in the loser cells (Ohsawa et al., 2011; Rhiner et al., 2010), further supporting the view that autophagy within the loser cells is a more likely explanation than is engulfment by the winner cells.

The results described here strongly support the view that cell competition is a mechanism that identifies and kills viable cells from a growing population in the absence of *psr*, *wasp*, and *draper*. *draper* is required after the discrimination between winners and losers is completed and the losers have been killed and extruded from the epithelium. After dead loser cells are extruded basally, the cellular debris is cleared by circulating hemocytes. Our results imply that the relevant molecules that allow winner cells to recognize and eventually impose cell death on the loser cells during cell competition still remain to be elucidated.

EXPERIMENTAL PROCEDURES

wt Clones in *tub > dmyc* and *tub > dmyc drpr−/−*, wasp*−/−*, and *psr−/−* Background, Mitotic Recombination Clones, and Hemocyte Experiments

The fly stocks used were obtained from the Bloomington Stock Center, except where indicated. To generate *wt* clones in *tub > dmyc* background, crosses were performed as previously described (Moreno and Basler, 2004). In the case of *drpr−/−*, *wasp−/−*, and *psr−/−* backgrounds, females of genotype *ywhs-FLP; tub > dmyc > gal4/Cyo;* $drpr^{\Delta 5}$*FRT80/TM6B*, *FRT82B wasp[1]/TM6B* or *FRT82B psr[null]/TM6B* (a gift of N. Baker) were crossed to *ywhs-FLP; UAS-GFP/Cyo;* $drpr^{\Delta 5}$*FRT80/TM6B, FRT82B wasp(1)/TM6B* or *FRT82B psr(null)/TM6B* males. The larvae were subjected to a 15 min heat shock at 37°C and harvested 24, 48, and 72 hr ACI.

For *wt drpr−/−* clones in *M*/+ *discs,* females of genotype *ywhs-FLP;* $drpr^{\Delta 5}$*FRT80* were crossed to *ywhs-FLP; ubi-GFP Rps17 [4] FRT80B/TM6b* males. As a control, females of genotype *ywhsFLP; mwh FRT80B /TM6b* were crossed to *ywhsFLP; ubi-GFP Rps17[4] FRT80B /TM6b* males. For *wt wasp−/−* and *psr−/−* clones in *M*/+ *discs,* females of genotype *ywhs-FLP; FRT82B wasp[1]* and *ywhs-FLP; FRT82B psr[null]* were crossed to *ywhs-FLP; FRT82B Ubi-GFP RpS3[Plac92]/TM6B* males. As a control, females of genotype *UAS-flp/Cyo; FRT82B/TM6b* were crossed to *ywhs-FLP; FRT82B ubi-GFP RpS3[Plac92)/TM6b* males. Heat shock was performed as previously described (Li and Baker, 2007). For *wt drpr−/−* clones in noncompetitive backgrounds, females of genotype *ywhs-FLP;* $drpr^{\Delta 5}$*FRT80* were crossed to *ywhs-FLP; ubi-GFP FRT80B/TM6b* males. As a control females of genotype *ywhsFLP; mwh FRT80B /TM6b* were crossed to *ywhsFLP; ubi-GFP*

FRT80B /TM6b males. For *wt wasp−/−* and *psr−/−* clones in noncompetitive backgrounds, females of genotype *ywhs-FLP; FRT82B wasp[1]* and *ywhs-FLP; FRT82B psr[null]* were crossed to *ywhs-FLP; FRT82B Ubi-GFP/TM6B* males. As a control, females of genotype *UAS-flp/Cyo; FRT82B/TM6b* were crossed to *ywhs-FLP; FRT82B ubi-GFP/TM6b* males. Heat shock was performed as previously described (Li and Baker, 2007).

For *lgl−/−* clones in *wt* or *drpr−/−* backgrounds, females of genotype *ywhs-FLP; lgl^4 FRT40A/Cyo* or *ywhs-FLP; lgl^4 FRT40A/Cyo; drpr$^{\Delta5}$FRT80/TM6B*, respectively, were crossed to *ywhs-FLP; UbiGFP FRT40A/Cyo* or *ywhs-FLP; UbiGFP FRT40A/Cyo; drpr$^{\Delta5}$FRT80/TM6B* males. The larvae were subjected to a 10 min heat shock at 37°C and harvested 48 ACI.

For hemocyte elimination experiments, the specific driver *Hegal4 UAS-GFP/TM6B* (a gift of P. Pareja) was crossed to *UAS-Hid* or *UAS-LacZ* in *M/+* backgrounds. For *wt* clones in *M/+* discs, females of genotype *ywhs-FLP; arm-LacZ FRT40A/Cyo; Hegal4 UAS-GFP/TM6B* were crossed to *UAS-Hid; UbiGFP M FRT40A/; TM6B/+* males to kill hemocytes or *UAS-LacZ; UbiGFP RpL27A[1] FRT40A/+; TM6B/+* males as a control. Larvae were subjected to 10 min heat shock at 37°C and harvested at 72 hr ACI.

To study the relation between hemocytes and *draper$^{\Delta5}$*, females of the genotype *ywhs-FLP; Cyo/if; Hegal4 UAS-GFP/TM6B* were crossed to *drpr$^{\Delta5}$FRT80/''* or *ywhs-FLP; UAS-Drprl; drpr$^{\Delta5}$FRT80/''* males. Larvae were maintained at 25°C until third larval instar appeared. As another way to block engulfment *UAS-shibire^{ts}* flies were used: females of the genotype *ywhs-FLP; Cyo/if; Hegal4 UAS-GFP/TM6B* were crossed to *UAS-shibire^{ts}/UAS-shibire^{ts}* males. Larvae were maintained at 17°C until second larval instard appeared, then put at 29°C and harvested at 72 hr.

Hemocyte Extraction and Preparation

For hemocyte images larvae of genotype *Hegal4 UAS-myr-RFP UAS-Hid* or *Hegal4 UAS-myr-RFP UAS-LacZ* were dried and opened with forceps. Hemolymph was loaded onto coverslips in a 24-well culture plate with 250 μl of Schneider medium. After 30 min, to allow hemocytes to sediment, they were fixed with FA 4% for 20 min, immunostained as usual, and mounted. The following primary antibodies were used: rabbit anti-draper (1/125) (Marc Freeman), mouse anti-Serpent (1/125) (Pastor Pareja), mouse anti-Nimrod (1/125) (István Andó). The following secondary antibodies were also used: Alexa Fluor donkey anti-rabbit 555 (1:250) (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor donkey anti-mouse 555 (1:250) (Invitrogen). Sytox Green was used as a marker for cell death. Images were obtained with a LEICA TCS-SP2-AOBS.

Immunohistochemistry

The following primary antibodies were used: rabbit anti-cleaved Caspase-3 (1/100) (Cell Signaling Technology, Danvers, MA, USA), mouse anti-Serpent (1/1,000) (Pastor Pareja), rabbit anti-draper (1/500) (Marc Freeman), mouse anti-Nimrod (1/500) (István Andó), and mouse anti-Par3 (1/10) (Alberto Ferrus). The following secondary antibodies were used: Alexa Fluor donkey anti-rabbit 555 (1:250) (Invitrogen), Alexa Fluor donkey anti-mouse 555 (1:250) (Invitrogen), and Alexa Fluor donkey anti-mouse 647 (1:250) (Invitrogen).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick end labeling (TUNEL; Roche, Indianapolis, IN, USA) assay was performed as previously described (Udan et al., 2003), complementary to cleaved Caspase-3 staining. All images were obtained with a LEICA TCS-SP2-AOBS.

Quantifications

The number of GFP-positive clones was quantified in all the discs using ImageJ A 1.44a software. For the number of active Caspase-3-positive cells, wing pouch area was marked in Photoshop CS3 Extended, and Caspase-3-positive dots were counted by using the Counter tool. Quantifications of experiments shown in Figures 2B, 2C, and Figures 6K were made taking into account only apical active Caspase-3-positive cells. The rest of the quantifications (Figures 1D, 1S, 6J, and 6O) were made using maximal projections of wing imaginal discs (therefore, considering both apical and basal active Caspase-3-positive cells). Averages and SEM were calculated for the different time points (24, 48, and 72 hr). All error bars represent SEM. Statistical significance was calculated with the Student's t test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.012.

LICENSING INFORMATION

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