

EXTRA VIEW

## Control of non-apoptotic nurse cell death by engulfment genes in *Drosophila*

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### ABSTRACT

Programmed cell death occurs as a normal part of oocyte development in *Drosophila*. For each egg that is formed, 15 germline-derived nurse cells transfer their cytoplasmic contents into the oocyte and die. Disruption of apoptosis or autophagy only partially inhibits the death of the nurse cells, indicating that other mechanisms significantly contribute to nurse cell death. Recently, we demonstrated that the surrounding stretch follicle cells non-autonomously promote nurse cell death during late oogenesis and that phagocytosis genes including *draper*, *ced-12*, and the JNK pathway are crucial for this process. When phagocytosis genes are inhibited in the follicle cells, events specifically associated with death of the nurse cells are impaired. Death of the nurse cells is not completely blocked in *draper* mutants, suggesting that other engulfment receptors are involved. Indeed, we found that the integrin subunit,  $\alpha$ PS3, is enriched on stretch follicle cells during late oogenesis and is required for elimination of the nurse cells. Moreover, double mutant analysis revealed that integrins act in parallel to *draper*. Death of nurse cells in the *Drosophila* ovary is a unique example of programmed cell death that is both non-apoptotic and non-cell autonomously controlled.

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### Introduction

Cell death occurs throughout development, helps to maintain homeostasis, and is a major contributing factor to numerous diseases in humans. Over 40 y ago, Kerr and colleagues coined the term apoptosis, describing a common morphological form of cell death observed in development and disease.<sup>1</sup> In the 1990s, several genes controlling apoptosis in mammals and programmed cell death in *C. elegans* were found to be homologous, demonstrating evolutionary conservation of this fundamental cellular process.<sup>2–4</sup> Although there are differences among upstream regulators of apoptosis in different cell types and organisms, a common event in apoptosis is the activation of caspases, a family of cysteine-aspartyl proteases that cleave cellular targets to destroy the cell.<sup>5</sup>

In addition to apoptosis, many other forms of cell death have been described. However, a molecular understanding of these forms of cell death has lagged significantly compared to apoptosis. Other forms of cell death, including necrosis, pyroptosis, and undoubtedly others, are likely to contribute significantly to

certain human diseases, and identifying treatments hinges on a molecular understanding of these “alternative” cell death pathways.<sup>6</sup> For example, necroptosis has recently been associated with multiple sclerosis,<sup>7</sup> and pyroptosis occurs in response to bacterial or viral infection.<sup>5,7</sup> Distinct biochemical requirements for different forms of cell death have been identified (see<sup>6,8,9</sup> for reviews).

While apoptosis is typically considered a form of cell suicide, apoptosis and other forms of cell death can be controlled non-autonomously, via either “assisted suicide” or “murder.”<sup>10</sup> TNF $\alpha$ -induced necroptosis and apoptosis are well-characterized examples of assisted suicide, where signaling downstream of death receptors determines the form of cell death that occurs.<sup>7</sup> Natural killer T cells can also induce apoptosis non-autonomously by releasing granzyme B, which cleaves caspase substrates and triggers the caspase cascade.<sup>11</sup> Entosis is an intriguing form of cell death where one cell commits suicide by invading another cell, and requires the lysosomal machinery of the surrounding cell for its degradation.<sup>12</sup>

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When cells die, they are typically phagocytosed and degraded by another cell. However, in some instances phagocytic cells actively participate in the death of the target cells.<sup>13</sup> This was first demonstrated in *C. elegans* when engulfment mutants showed increased survival of cells that had compromised caspase activity.<sup>14,15</sup> More recently, it has been shown that a surrounding phagocytic cell affects caspase levels and activity in the precursor of a dying cell.<sup>16</sup> In another recent example in *C. elegans*, the engulfment machinery was found to be completely required for the death of a specific cell in the male tail.<sup>17</sup> However, these cases are examples of assisted suicide, as the cell deaths are ultimately dependent on the apoptotic machinery in the dying cell.

In mammals, cell death dependent on the phagocytic machinery has been coined “phagoptosis.”<sup>13</sup> For example, LPS-stimulated microglia can kill otherwise healthy neurons and the large scale turnover of erythrocytes in mammals has been attributed to phagoptosis. Recently it has been shown that activated microglia produce nitric oxide, which promotes transient caspase activity and phosphatidylserine exposure in neurons, prior to phagocytosis.<sup>18</sup> However, it is not clear how widely this mechanism is used, or whether other mechanisms are used by phagocytes to promote the death of cells.

### Developmental nurse cell death

One mysterious type of cell death is the developmental cell death of nurse cells in the *Drosophila melanogaster* ovary.<sup>19-21</sup> Each egg is derived from a germline cyst of 16 cells, where one cell differentiates as the oocyte and the other 15 cells become polyploid nurse cells. At the end of oogenesis during stages 10-11, the 15 nurse cells transfer their cytoplasm to the oocyte and then during stages 12-14, the nurse cell nuclei are degraded. Genetic studies have ruled out a major role for apoptosis in developmental nurse cell death.<sup>20-23</sup> Mutants of caspase genes or the apoptosis initiators, *reaper*, *hid* and *grim*, fail to show any strong disruption to nurse cell death.<sup>21,23</sup> Similar mild phenotypes are observed with over-expression of caspase inhibitors.<sup>20-22</sup> There is some evidence that autophagy is induced during nurse cell death, but mutants of autophagy genes also fail to significantly disrupt nurse cell death suggesting that nurse cells do not die by autophagic cell death.<sup>24,25</sup> Even combined inhibition of apoptosis and

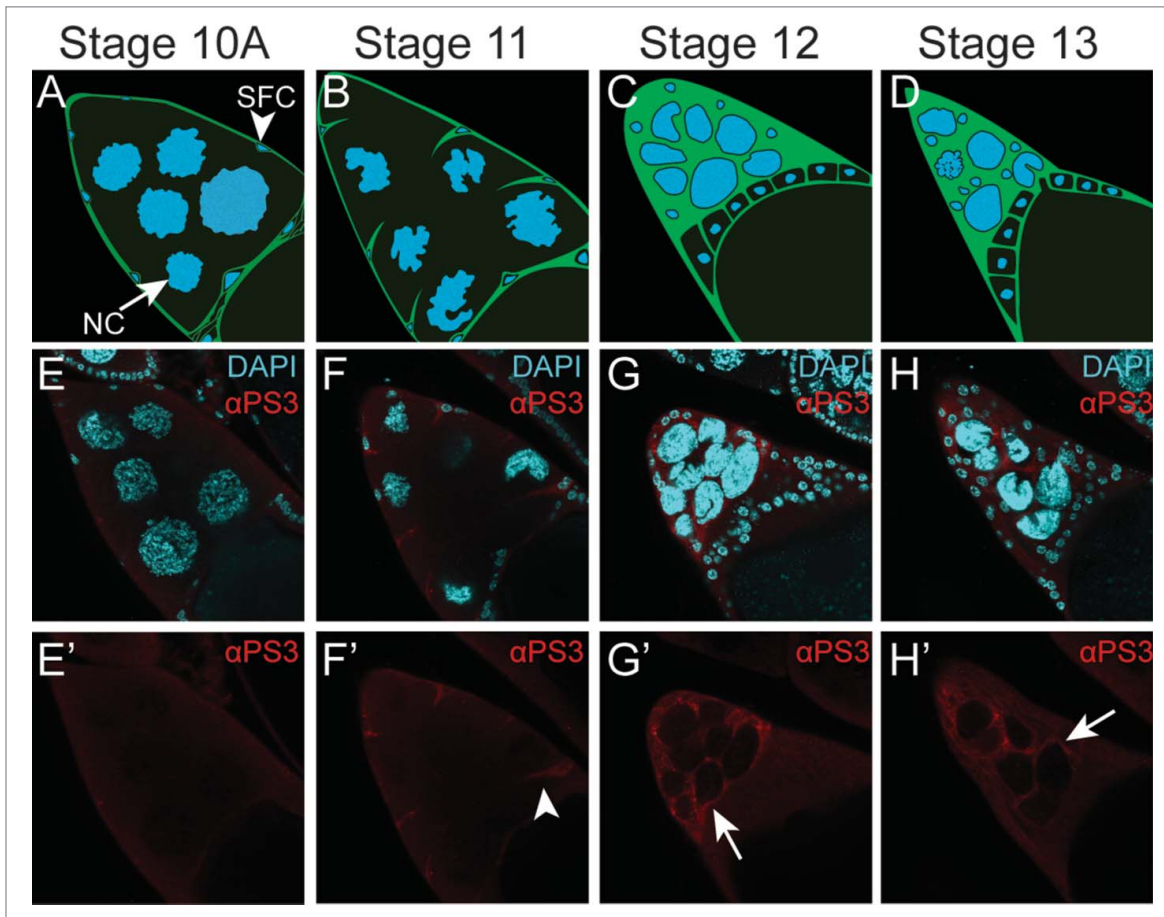
autophagy does not cause a disruption to nurse cell death.<sup>26</sup> So how do nurse cells die?

In our recent paper,<sup>27</sup> we investigated whether nurse cell death was controlled non-autonomously. In late oogenesis, the cluster of 15 nurse cells is surrounded by a thin layer of approximately 50 “stretch” follicle cells (Fig. 1A-D). As nurse cell death proceeds, the stretch follicle cells extend processes between the nurse cell nuclei, gradually surrounding each nurse cell nucleus individually. This intimate association with the nurse cells implicated the stretch follicle cells as candidates for controlling nurse cell death. Indeed, we found that the stretch follicle cells are required for nurse cell death, and that many of the nurse cell death events are mediated by the phagocytic machinery of the stretch follicle cells.

### Role of engulfment genes in nurse cell death

The *Drosophila* engulfment receptor Draper has been shown to play an important role in the clearance of apoptotic cells by hemocytes, glia, and epidermal cells.<sup>28-31</sup> Previously, we found that Draper was required earlier in oogenesis for the clearance of nurse cells undergoing apoptosis in response to starvation.<sup>32</sup> Interestingly, we also found that overexpression of Draper in follicle cells was sufficient to kill the underlying germline cells. Thus, our previous work suggested that follicle cells were capable of phagoptosis.

We examined homozygous *draper* mutant ovaries and found that there was a profound effect on developmental nurse cell death.<sup>27</sup> Whereas wild-type egg chambers showed an average of 0.2 nurse cell nuclei remaining by stage 14, *draper* mutants had an average of 8 persisting nurse cell nuclei. A similar phenotype was also observed when *draper* was knocked down by RNAi specifically in stretch follicle cells, demonstrating that *draper* was non-autonomously required for nurse cell removal. Knockdown of another engulfment gene, *Ced-12*, showed a comparable phenotype. In *C. elegans*, *Ced-12* and the *draper* ortholog *Ced-1* have been shown to function in parallel.<sup>33</sup> To determine whether this held true in the stretch follicle cells, we generated double *Ced-12 draper* knockdowns. Indeed, double mutants showed a stronger phenotype with over 10 persisting nuclei, suggesting that *Ced-12* and *draper* function in parallel during nurse cell death. We screened through a number of other engulfment genes using RNAi and identified several other genes



**Figure 1.** Integrin  $\alpha$ PS3 (Scab, FBgn0003328) is expressed in stretch follicle cells in late oogenesis. (A-D) Illustration of stretch follicle cell interactions with nurse cells at the end of oogenesis during stages 10-13. Stretch follicle cells (green with small blue nuclei, arrowhead) extend processes that surround each nurse cell (large blue nuclei, arrow). SFC = stretch follicle cell, NC = nurse cell. (E-H') Egg chambers stained with antibodies against  $\alpha$ PS3<sup>37,42</sup> and DAPI to visualize nuclei.  $\alpha$ PS3 is first detectable during stage 11 (arrows, F'), and increases during stages 12-13 (G'-H').

important for nurse cell removal (e.g. *shark*, *Gprk2*, *Src42A*, *mbc*, *Rac1* and the JNK pathway).

The persistence of nurse cell nuclei could indicate a defect in clearance, or a defect in cell death.<sup>27</sup> To distinguish between these possibilities, we stained ovaries for several cell death markers. In wild-type ovaries, signs of nurse cell death first become apparent in stage 10B, when nurse cell nuclei become permeable, releasing nuclear proteins such as the reporter nuclear  $\beta$ -galactosidase.<sup>34</sup> Cytoplasmic actin bundles also form during stage 10B, prior to the rapid transfer of nurse cell cytoplasm to the oocyte.<sup>34</sup> After the transfer of cytoplasm in stage 11, nurse cell nuclei become more compact, and eventually become acidified and stain positively for TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)<sup>23,24,35,36</sup> which labels the 3'OH groups of fragmented DNA. We found that TUNEL and acidification were almost

completely abolished in *draper* mutants.<sup>27</sup> While nurse cell dumping was not affected, the leakage of nuclear proteins was impaired. Together these findings indicated that the nuclei remained intact in *draper* mutants, suggesting that nurse cell death as well as clearance was inhibited.

#### **Stretch follicle cells control nurse cell death**

To directly address the requirement of stretch follicle cells in nurse cell death, we eliminated them by expressing RNAi against the caspase inhibitor *Diap1* specifically in the stretch follicle cells.<sup>27</sup> We found that these egg chambers not only had persisting nuclei, but they were also “dumpleless,” indicating that there was a failure in the transport of nurse cell cytoplasm to the oocyte. Actin bundles failed to form in the cytoplasm, and the nurse cell

nuclei failed to become acidified or TUNEL-positive. These findings demonstrate that the stretch follicle cells control all aspects of nurse cell death. Moreover, it shows that the stretch follicle cells activate other pathways in addition to the phagocytic machinery, as actin bundles and nurse cell cytoplasm dumping occurred normally in phagocytosis mutants.

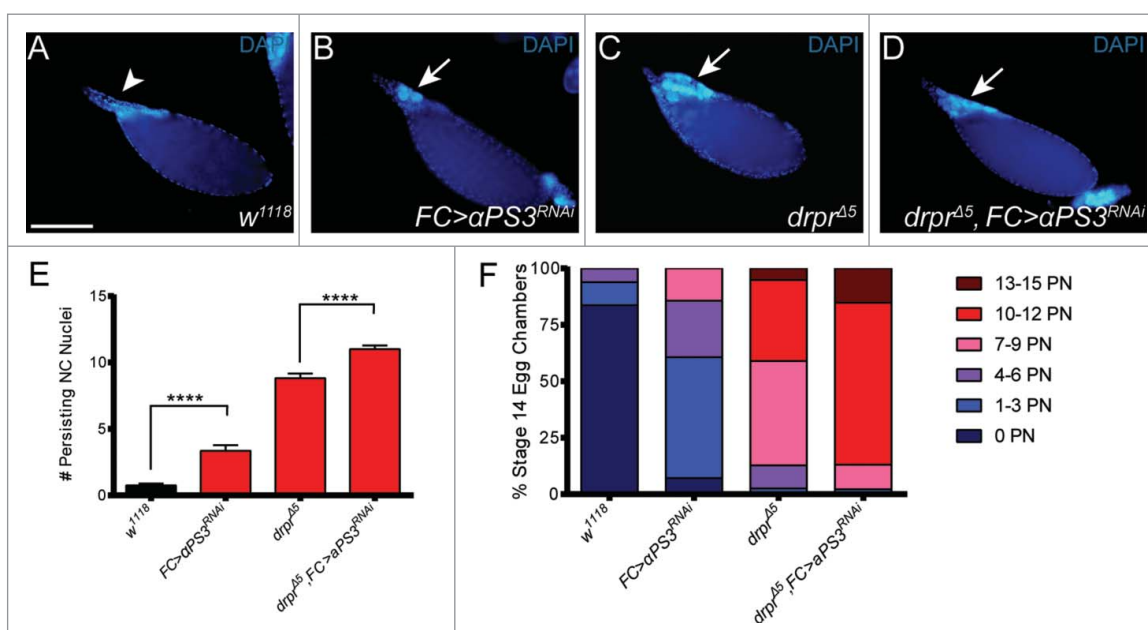
### Engulfment receptors integrins and Draper function in parallel

One puzzling aspect of our analysis was that nurse cell removal was not completely blocked in *draper* mutants. One possibility is that another receptor acts in parallel to *draper* in late oogenesis. A strong candidate is integrins, which have been shown to function during engulfment in *C. elegans*, mammals, and recently flies.<sup>37</sup> In flies, there are 7 different integrin subunits that can form different combinations of  $\alpha/\beta$  heterodimers. Knockdown of most of the integrin subunits did not affect programmed cell death in late oogenesis.<sup>38</sup> However, knockdown of either the  $\alpha$ PS3 or  $\beta$ PS subunit in follicle cells led to moderate to strong persisting nurse cell nuclei phenotypes in late oogenesis.<sup>27</sup> The

knockdown of  $\beta$ PS had additional defects in egg chamber development,<sup>38</sup> so we focused on  $\alpha$ PS3, which seemed to have a specific defect in nurse cell removal.

To determine where  $\alpha$ PS3 was expressed in late oogenesis, we stained ovaries with antibodies against  $\alpha$ PS3. In early stages of oogenesis,  $\alpha$ PS3 was not detected (Fig. 1E) but by stage 11 (Fig. 1F),  $\alpha$ PS3 was specifically detected on stretch follicle cell membranes. Staining became more intense during stage 12 and 13 (Fig. 1G-H), resembling the expression and staining pattern of Draper.<sup>27</sup>  $\beta$ PS, while expressed at low levels throughout oogenesis, also became enriched on stretch follicle cells during stages 10-13.<sup>38</sup>

To determine if integrins and Draper functioned in parallel in late oogenesis, we generated double mutants and counted persisting nuclei in stage 14 egg chambers (Fig. 2). As we reported previously,<sup>27</sup> the control *w*<sup>1118</sup> had on average less than one nurse cell nucleus remaining at stage 14 (Fig. 2A, E, F). Knockdown of  $\alpha$ PS3 in follicle cells led to an average of 3 persisting nurse cell nuclei (Fig. 2B, E, F). The double mutants, *draper* <sup>$\Delta$ 5</sup> (null) combined with the  $\alpha$ PS3 RNAi knockdown in follicle cells, showed a stronger phenotype than *draper* alone, with an average of 11 persisting nuclei (Fig. 2C-F). In over 15% of egg chambers, the



**Figure 2.**  $\alpha$ PS3 and *draper* act in parallel to remove nurse cells in late oogenesis. (A-D) Stage 14 egg chambers of the indicated genotypes stained with DAPI to label nuclei. (A) The control *w*<sup>1118</sup> does not have persisting nurse cell nuclei. (B-D) Loss of engulfment receptors leads to persisting nurse cell nuclei (arrows). The follicle cell (FC)  $\alpha$ PS3 RNAi knockdown was generated using GR1-GAL4<sup>43</sup> and TRiP strain JF02696. *draper* <sup>$\Delta$ 5</sup> (FBgn0027594) was generated by Marc Freeman.<sup>29</sup> The genotype of the double mutant in (D) is *draper* <sup>$\Delta$ 5</sup> *UAS- $\alpha$ PS3* (*dsRNA JF02696*)/*draper* <sup>$\Delta$ 5</sup>, *GR1-GAL4*. (E) Persisting nuclei were quantified in stage 14 egg chambers. (F) Data from (E) sorted into bins. PN = Persisting Nuclei. Number of egg chambers was >22 for all genotypes.



disruption to nurse cell removal was nearly complete with 13-15 nuclei remaining. These data indicate that  $\alpha$ PS3 acts in parallel to *draper* for the removal of nurse cell nuclei in late oogenesis. The strength of the  $\alpha$ PS3 *draper* double mutant phenotype was comparable to the phenotype of the *Ced-12 draper* double mutant,<sup>27</sup> suggesting that integrins could act upstream of Ced-12, as has been shown in *C. elegans*.<sup>39</sup>

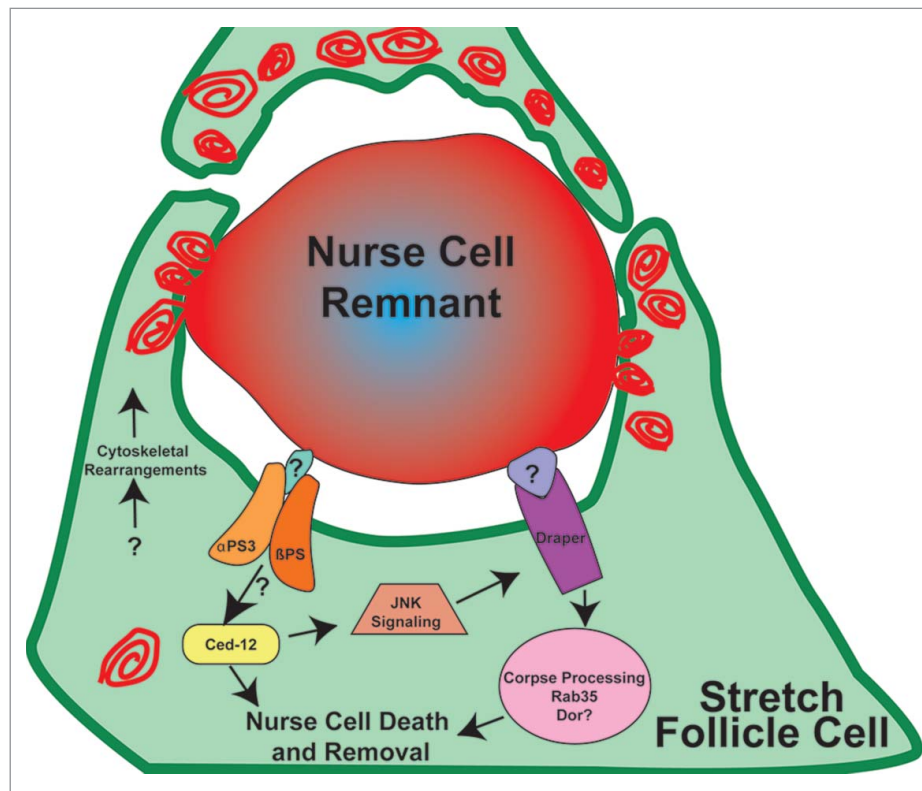
It was intriguing, however, that the double mutants still did not show a complete block to nurse cell removal. We also examined a triple mutant including the CD36 family receptor Croquemort, and found that nurse cell death was still not completely inhibited.<sup>38</sup> These findings suggest that there may be redundant or compensatory mechanisms, perhaps involving another engulfment receptor, or some of the nurse cells may be removed by other mechanisms that do not involve phagocytosis genes.

### Future perspectives

One major open question is: how does the phagocytosis machinery mediate nurse cell death and removal?

When nurse cells die in mid-oogenesis in response to starvation, they are engulfed by the surrounding follicle cells in a typical fashion, with the formation of phagosomes containing apoptotic debris.<sup>32,37</sup> The phagosomes are then processed through the canonical phagosome maturation pathway.<sup>40</sup> Phagosome formation and processing during starvation-induced cell death in mid-oogenesis are dependent on the phagocytosis genes *draper*, *Ced-12*, integrins, and the JNK pathway. However, during developmental nurse cell death in late oogenesis, we have not seen any evidence of vesicle uptake into the stretch follicle cells.<sup>27</sup> We do see LysoTracker-positive puncta in follicle cells,<sup>27</sup> but these may be trafficking toward the nurse cell nuclei, rather than away from them, which would be expected if the phagocytic material was being processed.

Another possibility is that the stretch follicle cells form a very large phagocytic cup to surround each nurse cell nucleus, which then fuses with multiple lysosomes until each nurse cell remnant is acidified (Fig. 3). Our observations suggest that multiple stretch follicle cells may collaborate to remove each nucleus,<sup>27</sup> so a simple phagocytic cup model is not likely. It is



**Figure 3.** Model of nurse cell death and removal in late oogenesis. In the drawing, one nurse cell nucleus is surrounded by stretch follicle cells (green). Red swirls represent lysosomes and/or other acidified organelles that surround the nurse cell remnant, eventually leading to its acidification, indicated by the gradient of red staining.

clear however, that nurse cell removal involves lysosomal activity because we identified many lysosomal trafficking genes required for nurse cell removal.<sup>27</sup> It is possible that the stretch follicle cells utilize this lysosomal machinery to acidify and degrade the nurse cells in a mechanism distinct from phagosome formation. For example, osteoclasts utilize lysosomal machinery to secrete hydrogen ions to acidify and resorb bone, specifically by targeting V-ATPases to the plasma membrane.<sup>41</sup>

In addition to the acidification step, it is possible that the phagocytic machinery signals to the nurse cells to promote their death. We showed previously that overexpression of *draper* in follicle cells can induce the death of the germline, prior to any phagocytic activity.<sup>32</sup> This implicates pro-death signaling between follicle cells and nurse cells. The molecules that mediate nurse cell death in response to *draper* overexpression remain to be determined. One clue is that *draper* and *Ced-12* can both activate the JNK pathway, and the transcription factor Kayak is required for nurse cell removal.<sup>27</sup> Thus there may be a transcriptional response activated by the phagocytic machinery that mediates nurse cell death independent of phagocytic activity *per se*. Further investigation into exactly how phagocytic cells can contribute to the death of cells by phagoptosis remains to be determined.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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