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Draper acts through the JNK pathway to control synchronous engulfment of dying germline cells by follicular epithelial cells

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SUMMARY

The efficient removal of dead cells is an important process in animal development and homeostasis. Cell corpses are often engulfed by professional phagocytes such as macrophages. However, in some tissues with limited accessibility to circulating cells, engulfment is carried out by neighboring non-professional phagocytes such as epithelial cells. Here, we investigate the mechanism of corpse clearance in the *Drosophila melanogaster* ovary, a tissue that is closed to circulating cells. In degenerating egg chambers, dying germline cells are engulfed by the surrounding somatic follicular epithelium by unknown mechanisms. We show that the JNK pathway is activated and required in engulfing follicle cells. We find that the receptor Draper is also required in engulfing follicle cells, and activates the JNK pathway. Overexpression of Draper or the JNK pathway in follicle cells is sufficient to induce death of the underlying germline, suggesting that there is coordination between the germline and follicular epithelium to promote germline cell death. Furthermore, activation of JNK bypasses the need for Draper in engulfment. The induction of JNK and Draper in follicle cells occurs independently of caspase activity in the germline, indicating that at least two pathways are necessary to coordinate germline cell death with engulfment by the somatic epithelium.

KEY WORDS: Engulfment, *Drosophila*, Ovary, Cell death

INTRODUCTION

Programmed cell death (PCD) is a crucial process in metazoan development and is a major factor in many diseases. In the human body, billions of cells die daily and are removed by phagocytic cells (Birge and Ucker, 2008; Elliott and Ravichandran, 2010; Erwig and Henson, 2008; Fullard et al., 2009; Kinchen, 2010; Lleo et al., 2008). A lack of phagocytosis of apoptotic cells can lead to secondary necrosis, culminating in an inflammatory response or, in severe examples, auto-immune diseases such as lupus. Thus, phagocytosis of apoptotic cells is essential for organism homeostasis.

Phagocytosis of apoptotic cells (also called efferocytosis) occurs by the actions of both professional phagocytes, such as macrophages, and non-professional phagocytes. In *C. elegans*, which lack macrophages, efferocytosis is carried out by neighboring cells (Mangahas and Zhou, 2005). In mammals, significant effort has been placed on understanding the mechanisms of engulfment by macrophages, but much less is known about engulfment in non-professional phagocytes. Other cell types known to be involved in efferocytosis include epithelial cells, mesenchymal cells, granulosa cells, glia and neuronal progenitors (Ichimura et al., 2008; Inoue et al., 2000; Jones et al., 2008; Lu et al., 2011; Monks et al., 2008; Wiegand et al., 2001). Some non-professional phagocytes clear apoptotic cells less efficiently than professional phagocytes (Parnaik et al., 2000), but it is not known if this is a general phenomenon. Whether non-professional phagocytes can be 'activated' to increase their engulfing ability is also unknown.

Genetic analysis in *C. elegans* has defined two parallel pathways required for engulfment of apoptotic cells (Hurwitz et al., 2009; Mangahas and Zhou, 2005). One pathway involves *ced-2*, *ced-5* and *ced-12*, and activates CED-10, a Rac GTPase. A second pathway uses CED-1, a putative apoptotic cell receptor. Other genes acting in this pathway are *ced-7*, *ced-6* and *dyn-1*, and this pathway may also converge on CED-10 (Kinchen et al., 2005). Extensive study of engulfment in mammals has revealed a high level of complexity (Kinchen and Ravichandran, 2007). As in worms, the CED-1, CED-6, CED-7 and CED-2, CED-5, CED-12 pathways play prominent roles in engulfment in mammals. There are many putative apoptotic cell receptors, suggesting they may function redundantly or be specific for certain cell types.

Drosophila appears to use a smaller number of engulfment components than mammals but, unlike *C. elegans*, flies use both professional and non-professional phagocytes. Engulfment has been studied in four cell types in *Drosophila*: macrophages, embryonic epidermal cells, glia and epithelial imaginal disc cells (Fullard et al., 2009; Kinchen, 2010). The *ced-1* ortholog *draper* (*drpr*), is important in several of these contexts. *drpr* plays a role in the engulfment of neurons, severed axons, bacteria and imaginal disc cells (Freeman et al., 2003; Manaka et al., 2004; Cuttell et al., 2008; Li and Baker, 2007; MacDonald et al., 2006). The *ced-5* and *ced-10* orthologs *mbc* and *Rac1*, have partial effects in imaginal discs, suggesting that this pathway plays a more minor role in engulfment than the CED-1, CED-6, CED-7 pathway in flies (Li and Baker, 2007).

Activation of the stress-activated MAP kinases Jun N-terminal kinase (JNK) and p38 is an early event following recognition of apoptotic cells by macrophages and epithelial cells in mammals,

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but a requirement for these pathways has not been demonstrated (Patel et al., 2006; Patel et al., 2010). The role of the *Drosophila* JNK pathway in development, innate immunity and apoptosis is well characterized (Igaki, 2009; Stronach, 2005). Recently, the JNK pathway was shown to be required for the removal of imaginal disc cells succumbing to cell competition (Ohsawa et al., 2011), suggesting that JNK may play a conserved role in promoting engulfment.

The *Drosophila* ovary is an established model for many aspects of cell biology, but efferocytosis is not well characterized. Cell death of developing egg chambers can be induced during mid-oogenesis (stage 7-9) by the removal of protein from the diet (Pritchett et al., 2009). Dying mid-stage egg chambers display a reproducible series of morphological events, with the germline nurse cells (NCs) showing the first signs of degeneration (Giorgi and Deri, 1976; Nezis et al., 2000; Peterson et al., 2003). Fly ovaries have few circulating cells such as macrophages (King, 1970), indicating that germline remnants are removed largely by non-professional phagocytes. Indeed, the epithelial follicle cells (FCs), which surround developing germline cysts, engulf the NC remnants (Giorgi and Deri, 1976; Mazzalupo and Cooley, 2006; Tanner et al., 2011), but the engulfment mechanisms are unknown.

Here, we show that the *Drosophila* ovary provides a powerful in vivo system for the study of engulfment in non-professional phagocytes. Unlike the redundant pathways described in other systems, we find that the Drpr pathway alone is essential for corpse clearance by FCs. We find that Drpr activates the JNK pathway, which is also required for engulfment, and that JNK activity feeds back to promote an increase in Drpr protein in engulfing cells. Remarkably, activation of JNK can restore engulfment in the absence of Drpr. Our findings indicate that the Drpr and JNK pathways are crucial activators of engulfment in non-professional phagocytes.

MATERIALS AND METHODS

Fly strains and manipulations

All strains were obtained from the Bloomington Stock Center unless otherwise indicated. *G89* (G00089) and *G71* (G00071) are GFP gene traps from FlyTrap (Morin et al., 2001). $drpr^{\Delta 5}$ (Freeman et al., 2003) was provided by Estee Kurant (Technion-Israel Institute of Technology, Haifa, Israel). *PWIZ-Drpr RNAi* #7b (MacDonald et al., 2006) and *UAS-Drpr-I* (McPhee et al., 2010) were provided by Mary Logan (OHSU, Portland, OR, USA) and Marc Freeman (University of Massachusetts Medical School, Worcester, MA, USA). The FC-specific *GR1-GAL4* line (Trudi Schüpbach, Princeton University, NJ, USA) was used to drive all *UAS* lines unless otherwise noted. The *puc-lacZ* line was $puc^{A251.1}$*,ry/TM3* (Martín-Blanco et al., 1998). *eiger* alleles (Igaki et al., 2002) were provided by M. Miura (University of Tokyo, Japan). $dcp\text{-}1^{prev1}$, *UASp-diap1*, *UASp-t-dcp-1* and *UASp-fl-dcp-1* were generated in our laboratory (Laundrie et al., 2003; Peterson et al., 2003). The dsRNA lines used were: *P{TRiP.JF01275}*, *P{TRiP.JF01794}* and *P{TRiP. HM05075}*. To generate new alleles of *dcp-1*, we used a *P*-element strain (*d11448*, Exelixis collection at Harvard) that allows for overexpression of *dcp-1*. *d11448* males were mutagenized with EMS and crossed to *GMR-GAL4* females. Most progeny died as pupae, or had greatly reduced eyes, and suppressors were isolated. The *P* element was excised from the suppressor lines, and they were tested for complementation with $dcp\text{-}1^{prev1}$. Two non-complementers were identified and both had single nucleotide changes in *dcp-1*. Fly crosses were performed at 25°C, except for crosses involving $UAS\text{-}bsk^{DN}$ or $UAS\text{-}hep^{CA}$. Because of lethality at 25°C, these crosses were performed at 18°C; for $UAS\text{-}bsk^{DN}$, the adult progeny were incubated for several days at 25°C before analysis. $UAS\text{-}hep^{CA}$ flies were crossed to $tubulin\text{-}GAL80^{ts}$, and subsequent crosses and conditioning of progeny were performed at 18°C. To inactivate *Gal80*, flies were transferred to 29°C overnight. Before dissection, flies were conditioned on yeast paste for 2 days, and then starved for 16-20 hours on apple juice agar unless otherwise noted. This period of starvation leads to egg chambers in all phases of degeneration (supplementary material Fig. S1).

Antibody staining and microscopy

Flies were dissected in Grace's media and egg chambers were processed as described previously (Tanner et al., 2011). Samples were mounted in VectaShield with DAPI (Vector Labs). Primary antibodies were: α-ß-gal [1:400, Promega or 1:200, Developmental Studies Hybridoma Bank (DSHB)], α-Dlg (1:1000, DSHB), α-Drpr (1:500, Marc Freeman) and α-DCAD2 (1:10, DSHB). Secondary antibodies were used at 1:200: goat-α-rabbit Alexa Fluor 488 (Invitrogen), goat-α-mouse Cy3, goat-α-rabbit Cy3 and goat-α-rat Dylight 649 (Jackson ImmunoResearch). Egg chambers were imaged on an Olympus FV10i confocal microscope. Images were processed in ImageJ and Adobe Photoshop.

Engulfment quantification

To quantify engulfment, we used ImageJ to measure the intensity of GFP in the germline compared with the GFP intensity of the entire egg chamber (germline + FCs) for each phase of death (supplementary material Fig. S2). As a second method, we quantified the area of unengulfed germline compared with the area of the entire egg chamber. The mean ratio for wild-type phase 0 egg chambers was normalized to 100%. Data for wild type were combined from *G71/+* and *GR1-GAL4 G89/TM6B* control lines. At least three egg chambers were analyzed for each phase and genotype, except *dMekk1* phase 2 (n=2). P values were determined using a two-tailed t-test.

RESULTS

Drosophila follicle cells coordinately engulf dying germline cells

To visualize engulfment in egg chambers, we obtained strains expressing GFP restricted to germline cytoplasm, so that uptake by surrounding FCs could be detected. Flies were starved to induce PCD, and egg chambers were labeled with an antibody against Discs large (α-Dlg), a scaffolding protein that labels FC membranes. As previously described (Pritchett et al., 2009), the first signs of egg chamber degeneration were observed in NC nuclei that displayed condensed chromatin, progressing to nuclear fragmentation (Fig. 1). FCs initially did not show any uptake of NC cytoplasm (Fig. 1B, phase 1), but as NC chromatin condensed, germline GFP was engulfed by surrounding FCs, which synchronously enlarged (Fig. 1C,D, phases 2 and 3). The germline appeared to be taken up through macropinocytosis, consistent with previous studies using electron microscopy (Giorgi and Deri, 1976; Tanner et al., 2011). As PCD proceeded, FCs grew and the germline shrunk as it was engulfed by FCs (Fig. 1E, phase 4). At late phases, the germline region was reduced to a small sliver and then completely engulfed by enlarged FCs (Fig. 1F, phase 5). Quantification of engulfment by measuring GFP intensity or germline area showed a decrease beginning in phase 2 (Fig. 1G; supplementary material Fig. S2). The area measurement showed a more dramatic decrease, presumably because GFP fluorescence declined during engulfment.

The receptor Draper is required for engulfment by follicle cells

The engulfment receptor Drpr and related molecules are established components of the phagocytosis machinery (Kinchen and Ravichandran, 2007). To determine whether Drpr was involved in the phagocytosis of NCs, we stained ovaries of starved flies with antibodies against Drpr (Fig. 2A-C). Healthy egg chambers showed low levels of Drpr staining around FC membranes (Fig. 2A). Once NC nuclei began to condense and FCs started to enlarge, Drpr became enriched on the apical membrane of engulfing FCs,

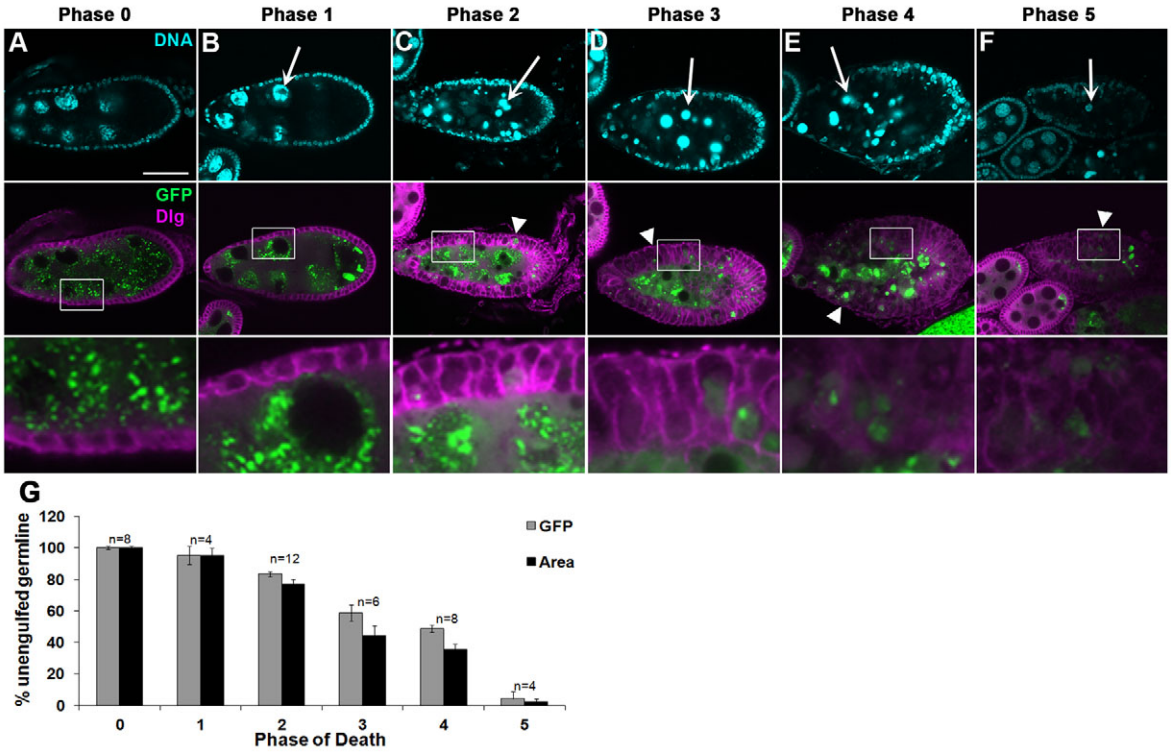

Fig. 1. Progression of cell death and engulfment. Egg chambers from starved flies were labeled with DAPI to label DNA (cyan, top panels) and α-Dlg (magenta). Egg chambers express a germline-specific GFP gene trap (G89, green). Bottom panels are enlargements of boxed regions in middle panels. The state of chromatin condensation and fragmentation is used to characterize the different 'phases' of egg chamber degeneration. Egg chambers in Figs 1-6 are from stage 8 to early stage 9 of oogenesis. (**A**) Healthy (phase 0) egg chamber shows dispersed chromatin in nurse cell (NC) nuclei, and follicle cell (FC) nuclei surround the egg chamber. (B-F) Progression of cell death. NC nuclei become highly condensed and fragmented (arrows). Middle and bottom panels show that FC membranes enlarge and engulf germline GFP (arrowheads). (**B**) Phase 1: NC chromatin is disordered. (**C**) Phase 2: NC chromatin is condensed but individual nuclear regions are still apparent. (**D**) Phase 3: NC chromatin becomes highly condensed into individual balls. (**E**) Phase 4: NC chromatin is fragmented and widely dispersed. (**F**) Phase 5: few NC nuclear fragments remain. Scale bar: 50 μm in top and middle rows. (**G**) Quantification of engulfment from control *G71/+* and *GR1-GAL4 G89/TM6B* flies shows decrease in germline GFP and area as death progresses. Data are mean±s.e.m.

bordering the germline (Fig. 2B). Drpr staining intensity peaked at phase 5 (Fig. 2C). In addition to plasma membrane staining, Drpr was detected in punctate speckles within FC cytoplasm. These data suggest that Drpr is upregulated throughout engulfment, and the puncta suggest that Drpr becomes clustered and internalized during phagosome formation.

To determine whether Drpr was required for engulfment, we examined ovaries from flies carrying the $drpr^{\Delta 5}$ mutation, a null allele (Freeman et al., 2003). In the absence of starvation, $drpr^{\Delta 5}$ mutant egg chambers developed normally through mid-oogenesis (Fig. 2E). However, FCs of degenerating egg chambers from starved $drpr^{\Delta 5}$ flies showed significant defects in engulfment and failed to enlarge as NC chromatin condensed (Fig. 2E′). $drpr^{\Delta 5}$ FCs also showed little engulfment, with minimal uptake of NC nuclear fragments and germline GFP. As death progressed, $drpr^{\Delta 5}$ FCs died (supplementary material Table S1) without clearing most NC debris (Fig. 2E″,I). These results suggest that Drpr is crucial for proper engulfment of the dying germline. Quantification of egg chambers revealed that $drpr^{\Delta 5}$ mutants have more than a sixfold increase in the number of phase 5 egg chambers compared with controls, indicating a pronounced defect in corpse removal (supplementary material Fig. S1, data not shown).

As the $drpr^{\Delta 5}$ mutation affects both the NCs and FCs, we assessed whether it was required specifically in the engulfing FCs. We performed a FC-specific knockdown of *drpr* by expressing $UAS\text{-}drpr^{dsRNA}$ with the FC-specific GR1-GAL4 driver (supplementary material Fig. S3). As seen in Fig. 2F′,F″,I, FC-specific knockdown of *drpr* prevented FC enlargement and NC debris uptake, similar to $drpr^{\Delta 5}$. Additionally, we rescued the $drpr^{\Delta 5}$ defect by overexpressing $drpr^{+}$ in the FCs (Fig. 2G,J). In these egg chambers, FCs enlarged and took up germline debris, similar to wild type (Fig. 2G′,G″). Furthermore, the rescued $drpr^{\Delta 5}$ FCs did not show premature death. These results show that *drpr* is specifically required in FCs for proper engulfment of NCs during starvation-induced PCD. We also made $drpr^{\Delta 5}$ germline clones (data not shown) to see whether *drpr* was required in the germline. The majority of egg chambers in these clones had proper engulfment; however, there were some that had somewhat aberrant engulfment, likely because the protocol for creating germline clones leads to some unmarked FC clones (Laundrie et al., 2003).

To further analyze the role of *drpr* in engulfment, we overexpressed *drpr* in FCs of wild-type egg chambers (Fig. 2H). Interestingly, we found that *drpr* overexpression in the FCs was sufficient to induce germline PCD in the egg chambers of unstarved flies. NCs condensed and fragmented in stage 8 and stage 9 egg chambers, but instead of engulfing, the FCs thinned out during the early phases of PCD (Fig. 2H′). By phase 4, the FCs engulfed normally, culminating in the removal of the germline (Fig.

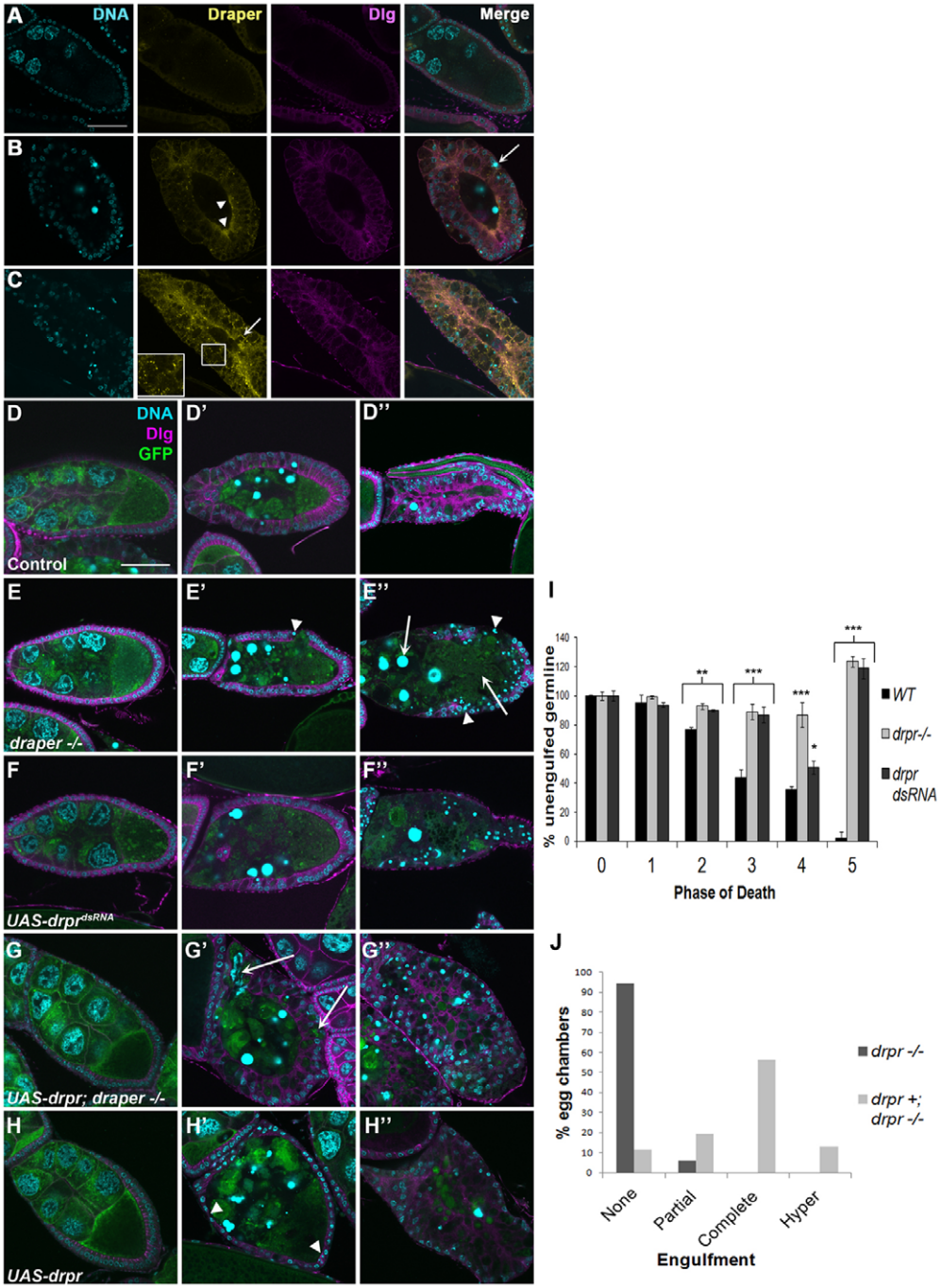

Fig. 2. Draper is required in follicle cells for proper engulfment of nurse cells. (**A-C**) Wild-type (w^{1118}) egg chambers labeled with DAPI (cyan), α-Drpr (yellow) and α-Dlg (magenta) (from starved flies). (A) Healthy egg chamber. (B) Phase 3 dying egg chamber. (C) Phase 5 egg chamber. Drpr staining intensity increases in the follicle cells (FCs) (arrowheads in B) as engulfment proceeds in dying egg chambers. Arrow in B merge shows internalization of a nurse cell (NC) nuclear fragment; arrow and inset in C indicate Drpr puncta within FCs. (D-H″) Egg chambers from starved flies expressing germline specific GFP (G71, green) stained with DAPI (DNA, cyan) and α-Dlg (magenta). Egg chambers are phases 0, 3 and 5 (left to right). (**D-D″**) Control *G71/+* egg chambers show normal death and engulfment. (**E-E″**) $drpr^{\Delta 5}$ flies show normal healthy egg chambers (E) but are defective in engulfment (E′) and show premature FC death (arrowheads) and lingering germline debris (arrows) (E″). (**F-F″**) Expression of *drpr* dsRNA in the FCs with GRI-GAL4 shows the same phenotype. (**G-G″**) Expression of $drpr^{+}$ in the FCs of $drpr^{\Delta 5}$ egg chambers rescues engulfment defects. FCs enlarge and take up NC debris (arrows). (**H-H″**) Overexpression of *drpr* in the FCs of otherwise wild-type egg chambers induces NC death (flies not starved). FCs first thin out (arrowheads) (H′) but engulfment eventually begins and proceeds normally (H″). Scale bars: 50 μm. (**I**) Quantification of unengulfed germline (wild type data from Fig. 1G). Degree and pattern of chromatin condensation were used as the primary criteria for assigning phases of death in mutant egg chambers. For phase 5 egg chambers, a reduced number of NC nuclear fragments and/or over 50% pyknotic FC nuclei were additional criteria. Phase 5 *drpr* egg chambers show over 100% unengulfed germline because of FC death. *P<0.05, **P<0.005, ***P<0.001. Data are mean±s.e.m. (**J**) Percentages of phase 3-5 egg chambers that show no engulfment, partial engulfment (less than wild type), complete engulfment (similar to wild type) and hyper-engulfment (engulfment before NC chromatin condensation) for $drpr^{\Delta 5}$ (n=34) and *UAS-drpr*; $drpr^{\Delta 5}$ *GR1-GAL4* (n=192).

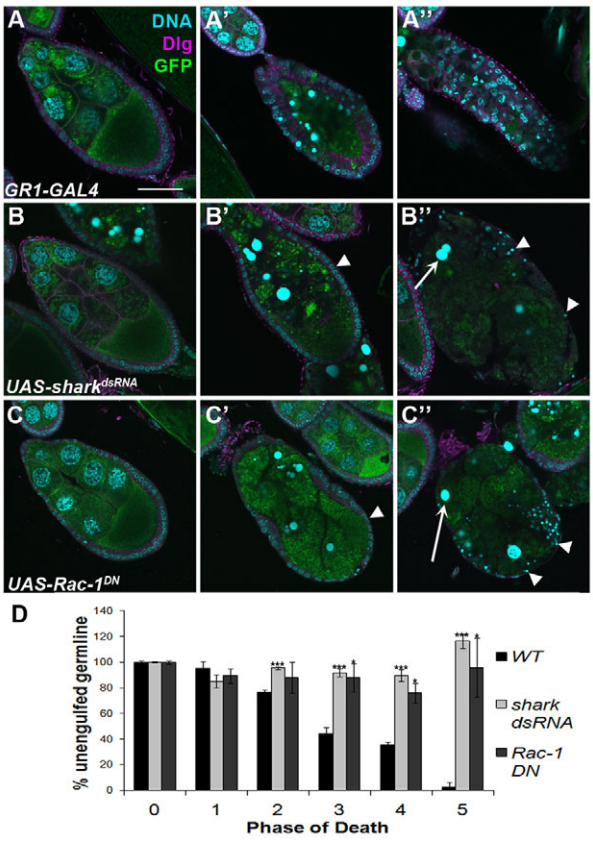

Fig. 3. Shark and Rac1 are required in follicle cells for proper engulfment. Egg chambers from starved flies express G71 GFP in the germline, stained with α-Dlg (magenta) and DAPI (cyan). (**A-A″**) *GRI-GAL4* alone shows normal progression of mid-oogenesis PCD. (**B-B″**) *UAS-shark*^{dsRNA}*/+; GRI-GAL4/+* shows defective clearance of nurse cells (arrow). Follicle cell (FC) membranes do not enlarge and GFP is largely absent in the FCs (arrowhead in B′). FCs are pyknotic (arrowheads in B″), as in *drpr*$^{\Delta 5}$ mutants. (**C-C″**) *UAS-Rac1*^{DN}*/+; GRI-GAL4/+* shows same phenotype. Scale bar: 50 μm. (**D**) Quantification of the unengulfed area. Data are mean±s.e.m. **P*<0.05, ****P*<0.001.

2H″). These findings indicate that overexpression of *drpr* is sufficient to induce NC death, but that there is a delay between the onset of death and engulfment compared with wild type. The delay could be because egg chambers from well-fed flies lack 'eat me' signals or starvation-induced signals for engulfment, or that *drpr*-induced death has delayed exposure of 'eat me' signals.

Follicle cells require a subset of known engulfment genes

We next determined whether other engulfment genes were required in FCs. The kinase Shark is known to act downstream of Drpr in glia (Ziegenfuss et al., 2008) and Rac1 is a conserved GTPase required for cytoskeletal rearrangements that occur during engulfment (Kinchen et al., 2005; Li and Baker, 2007). As seen in Fig. 3, FCs failed to enlarge in both *shark*^{dsRNA} and *Rac1*^{DN} (dominant negative) degenerating egg chambers. By phase 4-5 of cell death, NC and FC debris lingered in both *shark*^{dsRNA} and *Rac1*^{DN} egg chambers (Fig. 3B″,C″), and FCs died prematurely (supplementary material Table S1), similar to *drpr* mutants. Thus, *shark* and *Rac1* are required in FCs for proper engulfment during starvation-induced PCD. Whereas *drpr*- and *shark*-deficient FCs resembled wild type when flies were well fed, egg chambers of *Rac1*^{DN} flies displayed FC death even without starvation, indicating that *Rac1* is required for another aspect of FC function. However, *Rac1*^{DN} FCs from starved flies usually did not die until later phases, suggesting that FC death is not responsible for the engulfment defects. We also investigated mutants of *simu* (*NimC4* – FlyBase) (Kurant et al., 2008) and *prêt-a-porter* (*CG1837* – FlyBase) (Kuraishi et al., 2009), but neither displayed defects in engulfment, suggesting that only a subset of known engulfment genes are required in FCs.

JNK signaling is required for engulfment by the follicle cells

The increase in Drpr levels in engulfing FCs suggested that signaling pathways are activated in FCs to increase their competence for engulfment. The JNK pathway has been shown to be activated in engulfing mammalian macrophages and non-professional phagocytes (Patel et al., 2010; Patel et al., 2006), making it an excellent candidate pathway to be involved in germline engulfment. To visualize JNK activity, egg chambers carrying a *lacZ* enhancer trap in the JNK target gene *puckered* (Martín-Blanco et al., 1998) were analyzed with α-β-gal. Egg chambers were co-labeled with α-Drpr to visualize the timing of JNK activity relative to Drpr induction. Healthy egg chambers had no *puc-lacZ* staining and minimal Drpr staining in mid-stage egg chambers (Fig. 4A). Phase 1 dying egg chambers displayed activation of *puc-lacZ* in a few FCs and had increased Drpr staining, particularly in posterior FCs (Fig. 4B). Antibody staining indicated that the increase in Drpr levels preceded *puc-lacZ* activation. As the egg chambers progressed through death, *puc-lacZ* and Drpr staining became widespread and robust in FCs (Fig. 4C,D).

The increase in JNK activity in engulfing FCs suggested that JNK might be required for engulfment. To investigate a role for JNK (called *basket* or *bsk* in flies), egg chambers overexpressing dominant-negative *bsk* (Adachi-Yamada et al., 1999) in the FCs and germline-specific GFP were examined. Healthy egg chambers appeared wild type (Fig. 4E) and phase 1-2 dying egg chambers began the process of engulfment, including the enlargement of the FCs and uptake of germline GFP (Fig. 4E′). However, there were engulfment defects seen in phase 3-5 dying egg chambers. FCs failed to enlarge, no longer stained for Dlg, and the FC nuclei became pyknotic, indicating that the FCs were dying (supplementary material Table S1). Furthermore, NC nuclei and germline GFP were not taken up by the FCs (Fig. 4E″,E‴). Phase 3-5 egg chambers in wild type had less than 50% of unengulfed germline remaining, whereas *bsk*^{DN} mutants had 100% germline remaining (Fig. 4I). Interestingly, the NC nuclei failed to fragment properly, making it difficult to assess the specific phases of degeneration (supplementary material Fig. S4). Thus, JNK signaling is required in the FCs for fragmentation of NC nuclei and engulfment of dying NCs. Similar engulfment defects were seen by knocking down *bsk* in the FCs with dsRNA, including the lack of FC enlargement, pyknotic FCs and lingering germline material (Fig. 4F-F‴,4I; supplementary material Table S1), resembling *drpr* (Fig. 2). In unstarved flies expressing *bsk*^{DN} or *bsk*^{dsRNA} in the FCs, there were few degenerating egg chambers, indicating that the death of the FCs was due to engulfment defects and not a general requirement for *bsk*.

Upstream components of the JNK signaling pathway are required for engulfment

In *Drosophila*, the JNK pathway is simpler compared with that in mammals, with only one JNK (*bsk*) and two JNK kinases (JNKKs)

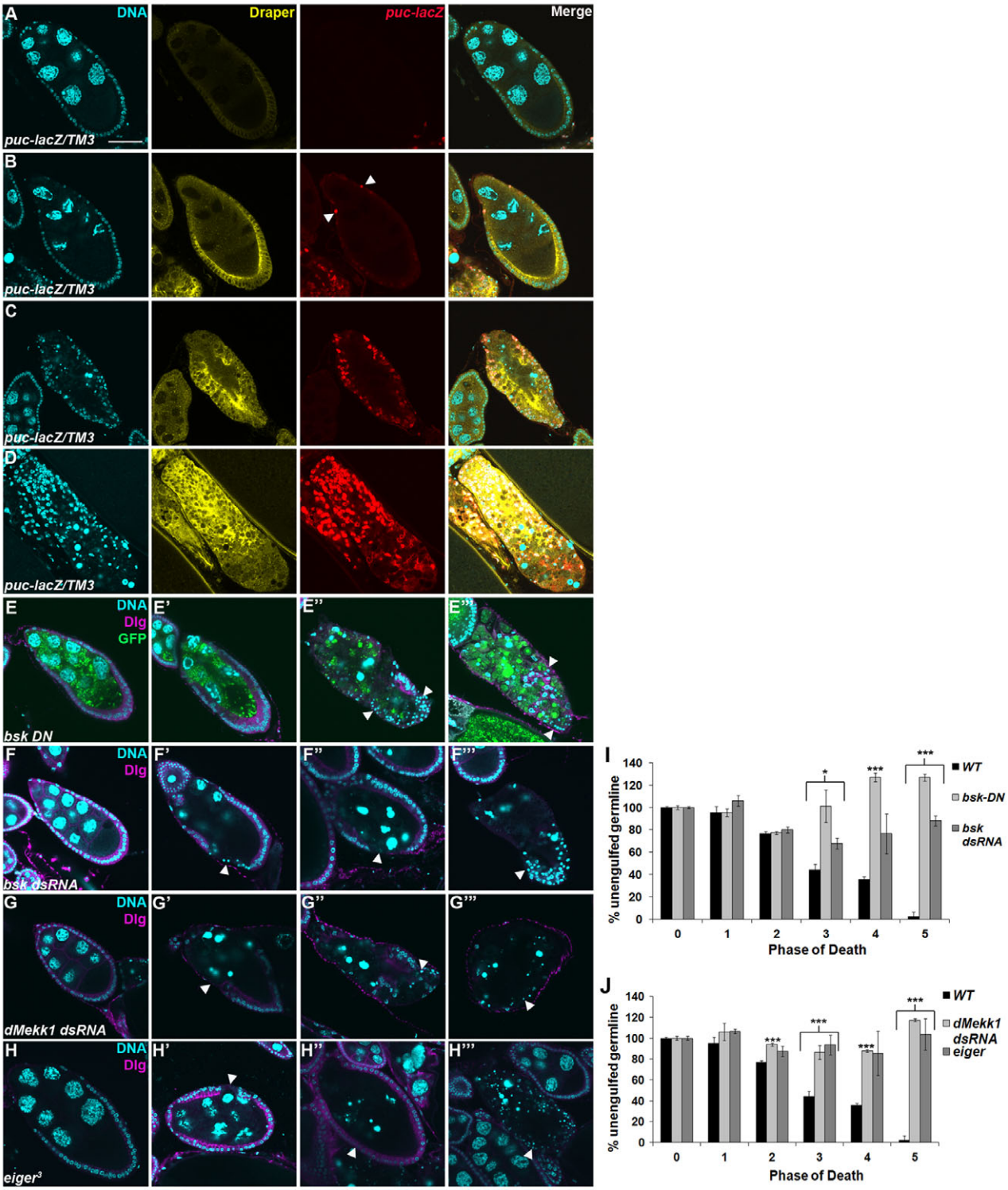

Fig. 4. The JNK pathway is activated and required in follicle cells during engulfment. (A-D) Healthy and progressively dying egg chambers from starved flies are stained with DAPI (cyan), α-Drpr (yellow) and α-β-gal (red). Egg chambers carry a *lacZ* enhancer trap in *puc*. (**A**) Healthy egg chambers express minimal Drpr and no *puc-lacZ*. (**B**) Phase 1 dying egg chambers begin to express Drpr and *puc-lacZ* in the follicle cells (FCs) (arrowheads). (**C**,**D**) Drpr and *puc-lacZ* are expressed robustly in actively engulfing FCs in phase 4-5 dying egg chambers. (E-G‴) Healthy and progressively dying egg chambers from starved JNK pathway mutants stained with DAPI (cyan) and α-Dlg (magenta). Arrowheads indicate the pyknotic nuclei of dying FCs or FCs that have failed to enlarge. (**E-E‴**) Egg chambers from *UAS-bsk*^{DN}*/+; GR1-GAL4 G89/+* flies express *bsk*^{DN} in the FCs and germline GFP (green). Healthy (E, phase 0) and dying (E′, phase 1) egg chambers look normal but later phase egg chambers (E″,E‴, phase 4,5) display defects in engulfment. (**F-G‴**) Egg chambers expressing *bsk*^{dsRNA} or *Mekk1*^{dsRNA} in FCs show a similar phenotype. Egg chambers in F-F‴ are phases 0, 1, 3 and 5, and egg chambers in G-G‴ are phases 0, 3, 4 and 5. (**H-H‴**) Phase 0, 1, 2 and 5 *eiger*^{3} egg chambers show little FC enlargement and premature death of the FCs. Scale bar: 50 μm. (**I**,**J**) Quantification of the unengulfed area. Data are mean±s.e.m. **P*<0.05, ****P*<0.001.

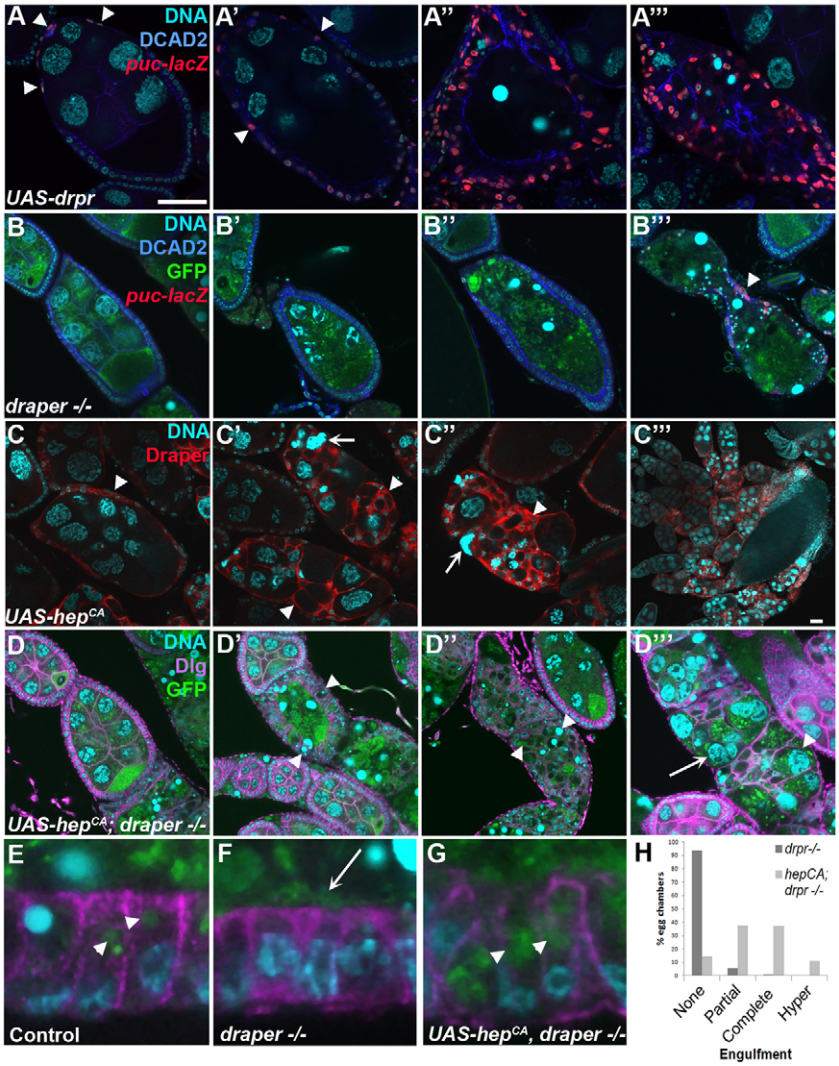

Fig. 5. Draper and JNK regulate each other during engulfment. (**A-A‴**) Egg chambers overexpressing *drpr* in follicle cells (FCs) show activation of *puc-lacZ* (red, arrowheads; flies not starved). Egg chambers stained with DAPI (cyan) and α-DCAD2 to label FC membranes (blue). (**B-B‴**) $drpr^{\Delta 5}$ egg chambers from starved flies do not express *puc-lacZ* (red, arrowhead) until late phases of death. Egg chambers are stained as in A and express G71 GFP (green). (**C-C‴**) FCs that overexpress hep^{CA} (*tubulin-GAL80^{ts}/+; UAS-hep^{CA}/GR1 G89*) induce Drpr expression (red) in the absence of starvation. Arrowheads indicate FCs and arrows indicate dying nurse cells (NCs). (C‴) Lower magnification image shows widespread induction of Drpr and few late stage egg chambers. (**D-D‴**) Overexpression of hep^{CA} in $drpr^{\Delta 5}$ background (*tubulin-GAL80^{ts}/+; UAS-hep^{CA}/G71; drpr$^{\Delta 5}$ GR1-GAL4/drpr$^{\Delta 5}$* flies incubated at 29°C, starved) suppresses the *drpr* phenotype. (D) Healthy egg chamber appears normal. (D′,D″). FCs enlarge (arrowheads) and engulf germline GFP. (D‴) Some egg chambers show a hyper-engulfment phenotype, where FCs (arrowhead) engulf intact NCs (arrow). Scale bar: 50 μm. (**E-G**) Enlargements of FCs from Fig. 2D′ (E), Fig. 2E′ (F) and Fig. 5D′ (G) show that control and *UAS-hep^{CA}; drpr$^{\Delta 5}$* FCs engulf GFP (arrowheads) whereas $drpr^{\Delta 5}$ FCs do not (F, arrow). Scale bars: 50 μm. (**H**) Quantification of engulfment as in Fig. 2J for $drpr^{\Delta 5}$ (n=128) and *UAS-hep^{CA}; drpr$^{\Delta 5}$* (n=745). Egg chambers from flies incubated at 29°C.

(Stronach, 2005). However, upstream of the JNKKs, there is increased complexity with six putative JNKK kinases, activated by multiple upstream pathways. One upstream regulator is Rac1, which we have demonstrated to be required for engulfment by FCs. To determine whether other upstream activators of the JNK signaling pathway were required, we expressed dsRNA against several components of the JNK pathway specifically in the FCs of starved flies, including the JNKK *hemipterous* (*hep*), the JNKKKs *slipper* (*slpr*) and *Mekk1*, and the JNKKKK *misshapen* (*msn*). We found that FCs in $Mekk1^{dsRNA}$ egg chambers failed to enlarge and died prematurely, similar to *bsk* (Fig. 4E-G,I,J; supplementary material Table S1). In the absence of starvation, mid-stage egg chambers appeared normal, indicating that the FC phenotype was due to defective engulfment and not to a general requirement for *Mekk1*. The death of the NCs and subsequent engulfment by the FCs occurred normally with expression of *hep*, *slpr* or *msn* dsRNA (data not shown). However, these knockdowns may not be strong enough to produce a phenotype. We additionally analyzed mutants of *eiger*, which encodes the fly TNF ortholog and is a ligand known to activate the JNK pathway (Igaki et al., 2002; Moreno et al., 2002). Like *bsk* and *Mekk1*, *eiger* mutant FCs failed to enlarge and take up NC debris, and died prematurely (Fig. 4H,J; supplementary material Table S1). Importantly, excessive FC death was observed only in late phases of degeneration (supplementary material Table S1) after engulfment defects were apparent in the mutants (Fig. 4I,J), indicating that engulfment defects were not a consequence of FC death. Furthermore, many mid-stage egg chambers remained healthy (phase 0) under starvation conditions, and these did not show FC death (supplementary material Table S1). Our findings demonstrate the requirement for JNK signaling in the FCs and the involvement of the upstream signaling components *eiger*, *Rac1* and *Mekk1*.

JNK acts downstream of Draper to promote engulfment

Because both *drpr* and JNK pathway mutants displayed defects in engulfment by FCs, we wished to determine whether they acted in the same pathway, and, if so, which gene acted upstream. To determine whether overexpression of *drpr* was sufficient to activate JNK, we examined expression of *puc-lacZ* in ovaries from well-fed flies overexpressing *drpr* in FCs (Fig. 5A). *puc-lacZ* was induced in these egg chambers before they showed any signs of degeneration (Fig. 5A′). *puc-lacZ* was first detected in anterior FCs, but then gradually spread to all FCs, and increased as the germline began to die (Fig. 5A″). Interestingly, *puc-lacZ* was not detected until stage 8 of oogenesis, although *drpr* overexpression was observed by stage 6 with the *GR1-GAL4* driver (data not shown, see supplementary material Fig. S3). These findings

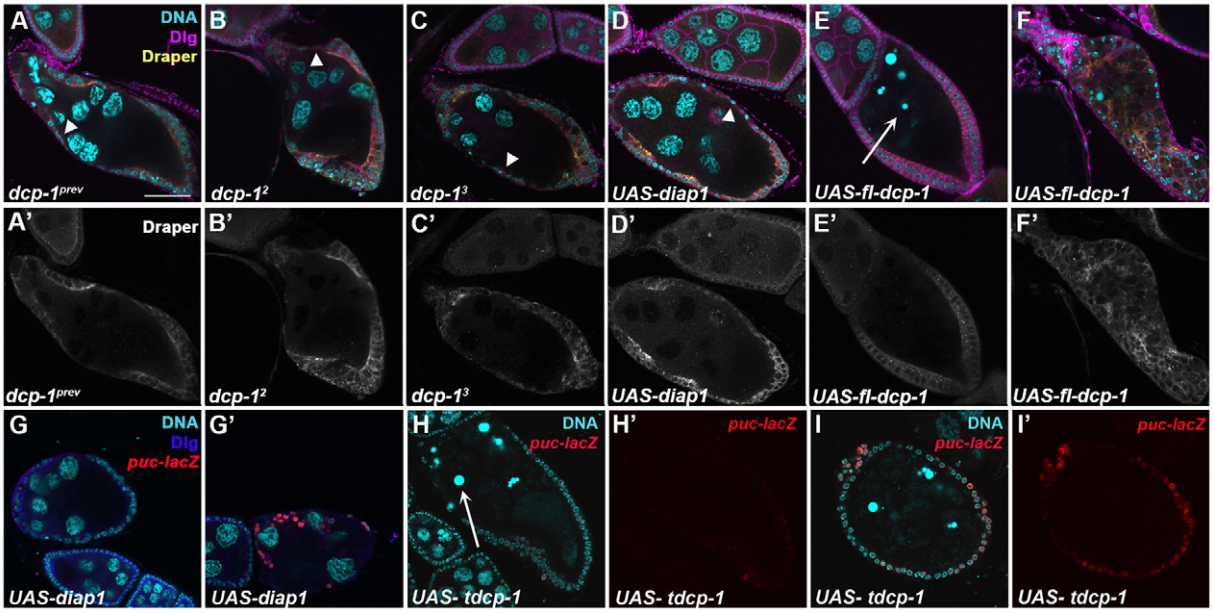

Fig. 6. Caspase activity is required for proper engulfment but is not required for upregulation of Drpr or JNK activity. (A-F) Caspase mutant egg chambers stained with DAPI (cyan), α-Drpr (yellow) and α-Dlg (magenta). (A′-F′) Drpr staining only (white). (**A-C′**) Egg chambers from three homozygous alleles of *dcp-1* (*dcp-1*^{prev1}, *dcp-1*^{2} and *dcp-1*^{3}) show Drpr upregulation in the follicle cells (FCs) when starved. However, most FCs fail to enlarge and display thinning out of their membranes (arrowheads). (**D**,**D′**) Egg chambers overexpressing *diap1* in the germline (*NGT/UASp-diap1; nanos-GAL4/+;* starved flies) show Drpr upregulation. FCs fail to enlarge and display thinning out of their membranes (arrowhead). (**E-F′**) Egg chambers from unstarved flies overexpressing full-length *dcp-1* [*nanos-Gal4-tubulin (NGT)/UASp-fl-dcp-1; nanos-GAL4/+*] in the germline show germline death (arrow) but delays in engulfment and Drpr induction (compare E′,F′ with Fig. 2B,C). (G-I′) Caspase mutant egg chambers stained with α-β-gal to detect *puc-lacZ* (red) and DAPI (cyan). (**G**,**G′**) Egg chambers from starved flies overexpressing *diap1* in the germline (*NGT/UASp-diap1; nanos-GAL4/puc-lacZ*) show an induction in *puc-lacZ*. G′ is probably a later phase egg chamber than G because of the FC loss. (**H-I′**) Germline overexpression of truncated *dcp-1* (*nanos-GAL4 UASp-tdcp-1/ puc-lacZ*) in the absence of starvation leads to death of the germline with delayed *puc-lacZ* expression. The same egg chamber is shown in H-I′ with the red channel only shown in H′,I′. I,I′ is a later phase egg chamber than H,H′ based on nuclear morphology. Arrow in H indicates condensed nurse cell nucleus. Scale bar: 50 μm.

indicate that Drpr is sufficient to activate JNK, but only at mid-oogenesis. To confirm that Drpr acts upstream of JNK, we examined *puc-lacZ* expression in starved *drpr*$^{\Delta 5}$ mutants (Fig. 5B). Consistent with the overexpression results, *puc-lacZ* was not induced in phase 1-2 (Fig. 5B′,B″), but surprisingly was detected in some FCs of late phase degenerating egg chambers (Fig. 5B‴). These observations suggest that Drpr activates JNK in engulfing FCs, but another pathway can activate JNK in the late phases of germline PCD.

To determine whether JNK activity promotes the increase in Drpr levels during engulfment, we examined egg chambers from starved flies expressing *bsk*^{DN} in the FCs. Drpr staining was observed on FC membranes in early dying egg chambers, but it declined as the FCs died (not shown). We additionally overexpressed constitutively active *hep* (Adachi-Yamada et al., 1999), which encodes a JNKK. Activation of the JNK pathway led to an increase in Drpr in the FCs of healthy egg chambers in the absence of starvation (Fig. 5C). Furthermore, the *hep*^{CA}-expressing FCs from unstarved flies displayed a hyper-engulfment phenotype in which they invaded and surrounded apparently healthy NCs (Fig. 5C-C″), culminating with FCs engulfing intact NCs and inducing their death (Fig. 5C″). These results indicate that JNK activity is sufficient for the upregulation of Drpr observed during engulfment.

To further examine the relationship between JNK and Drpr, we carried out epistasis analysis. First, we expressed *hep*^{CA} in a *drpr*$^{\Delta 5}$ mutant background (Fig. 5D). Overexpression of *hep*^{CA} in FCs led to the formation of many abnormal egg chambers, but close examination showed that *hep*^{CA} could restore engulfment in *drpr*$^{\Delta 5}$ mutant egg chambers, seen by enlargement of FCs and uptake of germline GFP (Fig. 5D′,D″,H). Furthermore, expression of *hep*^{CA} still led to hyper-engulfment of the germline in a *drpr* mutant background (Fig. 5D‴,H). These findings support our previous conclusion that JNK acts downstream of Drpr, but also reveal that, remarkably, *drpr* is not required for engulfment in the presence of activated JNK. We also conducted epistasis analysis with *bsk*^{DN} and *UAS-drpr*, but found that this combination was lethal even at low temperatures.

Caspase activity in the dying germline is necessary but not sufficient for engulfment

We next determined whether engulfment by FCs was dependent on caspase activity in the dying germline. Inhibition of caspases in the germline leads to a striking ‘undead’ egg chamber phenotype where NC nuclei fail to condense, but FCs disappear (Laundrie et al., 2003). To determine whether engulfment was initiated when caspases were inhibited in the germline, we starved flies and examined egg chambers that were starting to show morphological abnormalities, but still had most of their FCs (Fig. 6A-D). We first examined egg chambers from *dcp-1*^{prev1} homozygous mutants (where both FC and NC lack *Death caspase-1*), and found that engulfment was largely inhibited with only occasionally enlarging FCs (Fig. 6A). *dcp-1* is the only caspase mutant found to completely disrupt mid-stage NC death, and only one allele has

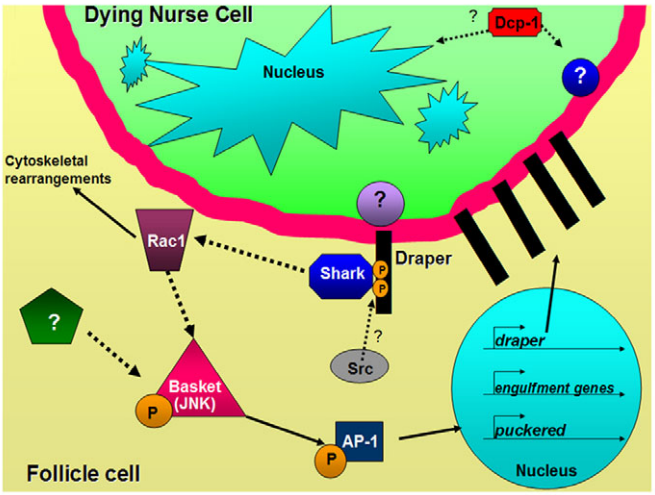

Fig. 7. Model for Draper-JNK circuit in engulfing follicle cells. Drpr (black rectangles) recognizes an unknown ligand(s) (purple circle) on germline cells, leading to its initial activation in follicle cells (FCs). Drpr becomes phosphorylated by Src kinase and physically interacts with Shark. Shark activates Rac1 and leads to cytoskeletal changes and activation of Basket (JNK). The phosphorylation of Basket leads to the activation of the transcription factor AP-1, which translocates to the nucleus and activates its downstream target, *puckered*. Additionally, AP-1 is proposed to transcriptionally activate *drpr*, leading to enrichment of Drpr on the membrane and other engulfment genes. Our data suggest that the caspase Dcp-1 activates an independent pathway that contributes to engulfment.

been described. Therefore, we generated additional alleles of *dcp-1* to confirm that the partial phenotype was not due to residual activity in the *dcp-1*^{prev1} allele. Two new EMS-induced alleles were generated: *dcp-1*^{2} (P92L) and *dcp-1*^{3} (W243Stop). The new alleles showed ovary phenotypes that were largely indistinguishable from the *dcp-1*^{prev1} allele, with very little engulfment by FCs (Fig. 6B,C). Some engulfment by FCs was seen in all *dcp-1* alleles, so we examined whether inhibition of additional caspases would show a stronger phenotype. Egg chambers overexpressing the caspase inhibitor Diap1 in the germline also showed a partial inhibition of engulfment (Fig. 6D). Thirty percent (n=138) of *dcp-1*^{2} undead egg chambers showed some FC enlargement and, similarly, 36% (n=64) of Diap1 overexpressing undead egg chambers showed some FC enlargement. These findings indicate that Dcp-1 is the major caspase mediating engulfment signals in the ovary. To determine whether Dcp-1 was sufficient to induce engulfment, we overexpressed *dcp-1* in the germline. Although engulfment by FCs was observed, it was delayed compared with wild type (Fig. 6E-F), suggesting that Dcp-1 does not induce engulfment directly, but that subsequent events in the dying NCs can induce engulfment.

To further investigate the role of caspases in activating engulfment in the surrounding FCs, we examined Drpr and JNK induction. Surprisingly, we saw induction of both Drpr and JNK in *dcp-1* mutants and in egg chambers overexpressing *diap-1* in the germline (Fig. 6A′-D′,G,G′, starved flies). This indicates that *dcp-1* acts in a pathway independent of JNK and Drpr induction (Fig. 7). Consistent with these findings, we found that germline overexpression of *dcp-1* in the absence of starvation led to Drpr and JNK induction (Fig. 6E′-F′,H-I′), but it was delayed compared with wild type, based on NC chromatin morphology. These findings indicate that JNK and Drpr are activated independently of caspase activity in NCs, and their activation alone is not sufficient to drive engulfment. This suggests that at least two pathways are required for engulfment: one caspase dependent and one acting through JNK and Drpr.

DISCUSSION

How non-professional phagocytes respond to dying cells and modulate their phagocytic capabilities is unclear. Here, we have used the *Drosophila melanogaster* ovary as a model to study engulfment by non-professional phagocytes. In this system, the germline can be induced to undergo PCD upon starvation. Following the initiation of PCD, a layer of epithelial FCs synchronously engulfs the dying germline. We have shown that the engulfment genes *drpr*, *shark* and *Rac1* are required for engulfment by FCs. We have also found that the JNK pathway is specifically activated during engulfment and is required for proper engulfment by FCs. Our analysis suggests that *drpr* and JNK are involved in a circuit, where the dying germline activates Drpr, which activates JNK, and JNK signaling leads to an increase in Drpr and likely other engulfment genes (Fig. 7). Surprisingly, activation of JNK is sufficient to rescue *drpr* engulfment defects, indicating that other pathways can carry out engulfment in the absence of *drpr*. A likely candidate pathway is CED-2, CED-5, CED-12, which can promote engulfment in the absence of *ced-1* in *C. elegans*.

In other systems, such as *C. elegans* and mammalian macrophages, there is redundancy among engulfment pathways (Kinchen, 2010; Kinchen and Ravichandran, 2007). In *Drosophila* embryos lacking *drpr*, unprocessed apoptotic particles are detected within glia (Kurant et al., 2008), suggesting that other pathways can facilitate engulfment of corpses. However, in FCs, *drpr* is essential for corpse removal. This may be because FCs die if they are engulfment-defective, and there may not be time to activate redundant pathways prior to FC death. It is important to note that *drpr* (and JNK pathway) mutant FCs survive in healthy egg chambers under starvation conditions; it is only during terminal phases of egg chamber degeneration that they die (supplementary material Table S1). Why do the FCs die if they are engulfment defective? Perhaps they have a metabolic requirement, and starve if they cannot obtain nutrients from the germline. Another possibility is that wild-type FCs are programmed to die after completing engulfment, and this PCD may be activated prematurely if engulfment is defective. Mammalian macrophages eliminate themselves after engulfment of specific pathogens or following efferocytosis in ABC transporter mutants (Navarre and Zychlinsky, 2000; Yvan-Charvet et al., 2010). Alternatively, FCs may die because of death ‘by confusion’, where disruption of the proper signaling network culminates in PCD. We attempted to block FC death by expression of caspase inhibitors p35 and Diap1, but FC death was still observed in control, *drpr*$^{\Delta 5}$- and *bsk*^{DN}-expressing egg chambers, indicating that FCs die via a caspase-independent pathway.

In mammals, JNK is activated in engulfing professional and non-professional phagocytes (Patel et al., 2010; Patel et al., 2006), although it remains to be determined whether JNK is required for engulfment. Recently in *Drosophila*, JNK has been found to be required for the removal of imaginal disc cells succumbing to cell competition (Ohsawa et al., 2011), and for the removal of severed axons (M. Freeman, personal communication). These findings suggest that JNK may play a conserved role in engulfment. To our knowledge, a role for JNK in engulfment has not been explored in *C. elegans* and no transcription factor has been shown to activate

engulfment genes. This is surprising as levels of CED-1 increase in engulfing cells (Zhou et al., 2001).

How does JNK become activated during engulfment? It may occur via Shark, a kinase that has been shown to interact with both Drpr and JNK in *Drosophila* (Fernandez et al., 2000; Tran and Berg, 2003; Ziegenfuss et al., 2008). Another candidate is Rac1, which can act upstream of JNK and may act downstream of Drpr (Kinchen et al., 2005). Interestingly, JNK activity is sufficient to restore engulfment in *drpr*-null egg chambers, suggesting that the primary role of Drpr is to activate JNK. Thus, functions attributed to Drpr such as actin reorganization, Ca^{2+} signaling, the formation of junctional complexes and autophagy (Cuttell et al., 2008; McPhee and Baehrecke, 2010), may depend on JNK activity. Indeed, JNK has been shown to induce autophagy genes in *Drosophila* (Wu et al., 2009).

Remarkably, *drpr* or hep^{CA} overexpression in FCs promoted death of egg chambers even when flies were not starved. To our knowledge, this is the first time that overexpression of an engulfment gene has been shown to induce non-autonomous cell death. In other systems, engulfment can promote the death of cells that are weakened, perhaps on the brink of death. For example, mutations in engulfment genes can lead to the survival of cells fated to die in *C. elegans ced-3* hypomorphs (Reddien et al., 2001; Hoeppner et al., 2001), and to the survival of 'loser' cells in *Drosophila* imaginal discs (Li and Baker, 2007). In mammals, neuronal exposure to amyloid Aβ peptide or LPS leads to cell death, which can be inhibited by blocking phagocytosis (Neher et al., 2011; Neniskyte et al., 2011). Interestingly, treated neurons transiently expose phosphatidylserine, perhaps to announce their vulnerability. Our findings differ from these scenarios in that the egg chambers are healthy. However, mid-stage egg chambers are more susceptible to death stimuli than egg chambers at other stages of oogenesis (McCall, 2004). Overexpression of Drpr in early oogenesis did not lead to egg chamber death, but death was observed later in mid-oogenesis. Thus, it may be that Drpr is not sufficient to kill the germline until mid-oogenesis, when it is more vulnerable. The factors that contribute to this vulnerability are unknown.

Overexpression of *drpr* in FCs led to death of the underlying NCs before there was any engulfment by the FCs, suggesting that *drpr* produces a death signal that is sent to the germline. Overexpression of the JNKK hep^{CA} led to destruction of egg chambers earlier in oogenesis than overexpression of *drpr*, suggesting that JNK did not require the vulnerability at mid-oogenesis. Furthermore, hep^{CA}-expressing FCs engulfed intact NCs ('hyper-engulfment'), rather than inducing death first. This phenotype resembles the process of entosis, where living cells are engulfed by their neighbors (Overholtzer et al., 2007).

Germline PCD in mid-oogenesis requires caspases, and our results indicate that caspase activity is required to stimulate FCs to engulf the germline. Surprisingly, germline caspase activity was not necessary or sufficient to activate JNK or induce Drpr in the FCs. This suggests that a caspase-dependent pathway, distinct from the pathway(s) that activate Drpr-JNK, is required for engulfment in mid-oogenesis. The caspase-dependent signal and the responding pathway in the FCs remain to be elucidated. Another open issue is how Drpr, and thereby JNK, becomes activated in response to the dying germline. The complexity of cell surface modifications that occur during apoptosis will make this a challenge to determine. Draper and JNK may become activated directly in the FCs in response to starvation; however, this scenario seems less likely than activation by the dying germline for two reasons. First, many egg chambers do not die immediately upon starvation, and activation of Draper and JNK was observed only in egg chambers that had begun to die. Second, germline death triggered by overexpression of *dcp-1* could lead to Draper and JNK activation in FCs in the absence of starvation. The activation of JNK and Drpr illustrate ways in which non-professional phagocytes change in response to apoptotic cells. Future work will reveal the network of pathways activated in non-professional phagocytes to enhance apoptotic cell clearance.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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