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Activation of autophagy during cell death requires the engulfment receptor Draper

Christina K. McPhee^{1,2}, Mary A. Logan³, Marc R. Freeman^{3,4}, and Eric H. Baehrecke^{1,*}

¹Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605 USA

²Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742 USA

³Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605 USA

⁴Howard Hughes Medical Institute

Abstract

Autophagy degrades cytoplasmic components that are required for cell survival in response to starvation¹. Autophagy has also been associated with cell death, but it is unclear what may distinguish autophagy during cell survival and death. *Drosophila* salivary glands undergo programmed cell death that requires autophagy genes², and engulfment of salivary gland cells by phagocytes does not appear to occur³. Here we show that Draper (Drpr), the *Drosophila* orthologue of the *C. elegans* engulfment receptor CED-1, is required for autophagy during cell death. Null mutations in *drpr*, as well as salivary gland-specific knockdown of *drpr*, inhibits salivary gland degradation. *drpr* knockdown prevents the induction of autophagy in dying salivary glands, and Atg1 expression in *drpr* mutants suppresses the failure in salivary gland degradation. Surprisingly, *drpr* is cell-autonomously required for autophagy induction in dying salivary gland cells, while *drpr* knockdown does not prevent starvation-induced autophagy in the fatbody which is associated with survival. In addition, components of the conserved engulfment pathway are required for clearance of salivary glands. This is the first example of an engulfment factor that is autonomously required for self-clearance. Furthermore, Drpr is the first factor that distinguishes autophagy that is associated with cell death from cell survival.

Macroautophagy (autophagy) delivers cytoplasmic components to the lysosome for degradation in eukaryotic cells. Autophagy is an important cellular response to stress that is required for survival in response to starvation¹, and has also been associated with cell death

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*Corresponding author: Eric H. Baehrecke, Eric.Baehrecke@umassmed.edu, telephone: 508-856-6733; fax: 508-856-1310.

Author Contributions All the experiments were performed by C.K.M., and were designed by C.K.M and E.H.B., M.L. and M.R.F. provided critical DNA constructs and transgenic flies. All authors wrote and discussed the manuscript. The authors have no competing financial interests.

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in several *in vivo* contexts^{2,4}. It is not clear, however, how autophagy might function in cell death, and what might determine its function in cell death versus cell survival. *Drosophila* larval salivary gland cell death is induced by a rise in steroid 12 hours after puparium formation, and this tissue is completely degraded by 16 hours after puparium formation⁵. Both caspases and autophagy are induced by this rise in steroid, and function in an additive manner to kill and degrade salivary glands². By contrast, autophagy is induced in response to starvation in the larval fatbody⁶, and this induction is associated with cell survival.

To identify genes that may regulate autophagy in cell-specific contexts, we queried genome-wide DNA microarray data from dying salivary glands. Interestingly, several factors that have been implicated in the engulfment of apoptotic cells are induced in dying salivary glands⁷ (Supplementary Table 1), while there are no detectable changes in these genes after larval starvation⁸. Although many engulfment factors are pleiotropic through their regulation of the cytoskeleton and vesicular transport, the identification of the engulfment receptor *drpr*^{9,10} is intriguing, as salivary gland destruction is thought to be largely independent of phagocytes.

We analyzed whether Drpr is present in dying salivary glands. Whereas no Drpr protein is present in *drpr*⁻⁵ null mutants, Drpr-I, II and/or III isoforms are present at low levels and localize to the luminal/apical region of salivary gland cells at 6 hours after puparium formation (Fig. 1a and Supplementary Fig. 1). Following the rise in steroid that triggers cell death 12 hours after puparium formation¹¹, Drpr-I, II and/or III levels increase (Fig. 1a). Drpr-I protein levels remain high through 14 hours after puparium formation when a portion of Drpr changes from apical to cytoplasmic in localization, and this is coincident with a decrease in Drpr-II/III isoforms (Fig. 1a and Supplementary Fig. 1).

The presence of Drpr protein in salivary glands suggested the possibility that Drpr could function directly within salivary gland cells to mediate their degradation. We tested whether *drpr*⁻⁵ null mutants have a defect in salivary gland cell death. We found that 98% of homozygous *drpr*⁻⁵ null mutants have persistent salivary gland material (Fig. 1b, c). By contrast, 0% of control *drpr*⁻⁵/wild type heterozygous animals possess salivary gland material at 24 hours after puparium formation (Fig. 1b, c), and fatbody cells fill the void where salivary gland material has been cleared. As a known engulfment factor, Drpr could also function in phagocytic blood cells to mediate salivary gland clearance, and this could explain the defect in clearance observed in whole animal *drpr*⁻⁵ null mutants. We tested this possibility by driving an upstream activating sequence (UAS)-regulated double-stranded inverse repeat construct designed to target *drpr* (*drpr*^{IR}) with the blood cell-specific *hml*-GAL4 driver and assaying for the persistence of salivary glands. We found that whereas *hml*-GAL4 is clearly expressed in blood cells but not salivary glands, *drpr* knockdown in blood cells does not lead to a defect in salivary gland clearance (Supplementary Fig. 2). By contrast, expression of *drpr*^{IR} in salivary glands with the salivary gland-specific *fkh*-GAL4 driver resulted in the persistence of salivary gland fragments in 89% of pupae compared to 0% of control animals lacking the *fkh*-GAL4 driver 24 hours after puparium formation (Fig. 1c, d). In addition, reduced function of any one of the engulfment pathway genes *simu*, *crq*, *ced-6*, *src42a*, *ced-12*, *crk*, and *mbc* also inhibited clearance of dying salivary glands (Supplementary Fig. 3). These data indicate that *drpr*, as well as other engulfment genes,

function autonomously in salivary gland cells during degradation. Similar to animals with reduced function of either caspases or autophagy genes², decreased function of *drpr* and other engulfment genes appears to inhibit clearance of dead fragmented salivary gland cells.

Drpr-I protein isoform levels remain elevated in extracts of dying salivary glands 14 hours after puparium formation (Fig. 1a). Therefore, we explored the role of Drpr-I in salivary gland cell death. Knockdown of *drpr-I* in salivary glands prevents tissue clearance in 80% of these animals compared to 0% of control animals lacking the *fkh*-GAL4 driver (Fig. 1e, f), suggesting that the defect in salivary gland cell degradation is due to decreased *drpr-I* function. In addition, salivary gland-specific expression of Drpr-I in *drpr*⁵ null mutant animals is sufficient to rescue the salivary gland persistence phenotype; 78% of Drpr-I *drpr*⁵ pupae that lack the GAL4 driver have persistent salivary gland material, while only 10% of *drpr*⁵ pupae expressing Drpr-I have persistent gland material (Fig. 1g, h). Taken together, these results indicate that *drpr-I* is required for salivary gland cell clearance.

The expression of Drpr in dying cells prompted us to investigate whether Drpr might play a role in regulating an intracellular cell death process. Caspases and autophagy have been shown to function in an additive manner in the degradation of salivary glands, and inhibition of genes in either pathway leads to partial persistence of salivary glands². Expression of the caspase inhibitor p35 alone leads to the persistence of condensed salivary gland cell fragments in 94% of pupae, and salivary gland fragments in 6% of pupae (Fig. 2a, b). Control homozygous *drpr*⁵ mutant pupae that contain the p35 transgene but lack the salivary gland GAL4 driver have persistent vacuolated salivary gland cell fragments in 80% of animals (Fig. 2a, b). However 100% of experimental *drpr*⁵ pupae that also express p35 in salivary glands contain larger amounts of persistent salivary gland material, including multi-cell gland fragments (Fig. 2a, b). Therefore, the salivary gland persistence phenotype in *drpr*⁵ is enhanced by the expression of p35, indicating that Drpr functions in an additive manner with caspases. To further test the relationship between Drpr and caspases, we tested whether *drpr* knockdown in salivary glands affects degradation of nuclear Lamin, a known caspase substrate in this tissue³. Clones of salivary gland cells that express *drpr*^{IR} and GFP possess strong Lamin staining 6 hours after puparium formation, and both control and *drpr*^{IR}-expressing cells contain decreased Lamin staining 14 hours after puparium formation (Fig. 2c), indicating that caspases are active in both control and *drpr*^{IR}-expressing cells 14 hours after puparium formation. Taken together, these results suggest that Drpr does not influence caspase activity, and functions downstream or parallel to caspases in dying salivary gland cells.

We next tested whether the salivary gland phenotype caused by expression of *drpr*^{IR} is enhanced by knockdown of *Atg12*, a gene required for autophagy. We found that 81% of pupae expressing *Atg12*^{IR} in salivary glands have persistence of salivary gland cell fragments, whereas 100% of those expressing *Atg12*^{IR} *drpr*^{IR} have gland cell fragments (Fig 3a, b). These phenotypes are very similar to each other, and unlike expression of p35 in *drpr*⁵ (Fig. 2a), co-expression of *Atg12*^{IR} and *drpr*^{IR} does not lead to an increase in the amount of salivary gland material that persists, and multi-cell gland fragments were absent. This led us to hypothesize that Drpr could function in the same pathway as autophagy genes. To test this possibility, we knocked down *drpr* in salivary glands expressing the autophagy

reporter GFP-LC3. Autophagy induction leads to the association of GFP-LC3 with autophagosomal membranes, which are visible as GFP puncta. We found that whereas autophagy is highly induced in control salivary glands 14 hours after puparium formation, those expressing *drpr^{IR}* contain few GFP-LC3 puncta at the same stage (Fig 3c, d). Autophagosomes are trafficked to and fuse with lysosomes, where autophagic content is degraded by lysosomal hydrolases. To test whether *drpr* knockdown affects lysosome numbers, we expressed *drpr^{IR}* in salivary glands and assayed for the number of Lysosome Associated Membrane Protein 1 (LAMP1)-GFP puncta. We found that salivary glands expressing *drpr^{IR}* contained more lysosomes than control salivary glands (Fig. 3e, f). Therefore, *drpr* is required for the induction of autophagy in salivary glands. The increase in lysosome numbers in *drpr* knockdown salivary gland cells is consistent with the failure in autophagy induction, given that lysosome numbers decrease when autophagy and autolysosome formation are abundant¹².

Expression of Atg1 is sufficient to induce ectopic autophagy and cell death in several *Drosophila* tissues, including the fatbody and salivary glands^{2,13}. Therefore, we tested whether expression of Atg1 is sufficient to suppress the *drpr⁵* null mutant defect in degradation of salivary glands. Whereas 100% of control Atg1^{6A} *drpr⁵* animals that lack the GAL4 driver have persistent salivary gland material 24 hours after puparium formation, this phenotype is completely suppressed by the expression of Atg1 in *drpr⁵* null mutant salivary glands (Fig. 3g, h). This suggests that *Atg1* functions downstream of *drpr*.

The question of whether autophagy plays dual roles in cell survival versus cell death has been controversial, and raises the possibility that different factors may regulate autophagy in different cell contexts. We analyzed whether Drpr protein is present in the larval fatbody, since *drpr* RNA was not detected in microarray analyses of starving larvae⁸. We found that Drpr is expressed in larval fatbody, but the levels and localization of Drpr do not change in response to starvation (Fig. 4a, b). Since *drpr* is required for autophagy during salivary gland cell death, we wondered whether *drpr* is required for starvation-induced autophagy in the *Drosophila* larval fatbody⁶. We starved third instar larvae expressing GFP-Atg8a (fly homologue of LC3 in mammals) in all cells and *drpr^{IR}* specifically in dsRed-marked cell clones, and assayed for the induction of autophagy. Surprisingly, we found that GFP-Atg8a puncta form in both *drpr^{IR}*-expressing cells, as well as neighboring control cells (Fig. 4c). In addition, loss of *drpr⁵* function in mutant clones of cells failed to suppress autophagy in starved fatbody (Supplementary Fig. 4). Therefore, unlike in dying salivary glands, *drpr* is not required for starvation-induced autophagy in the *Drosophila* fatbody. One possibility is that *drpr* mediates a tissue-wide autophagic response, as we had not tested whether *drpr* functions in a cell autonomous manner in dying salivary glands. To test the cell-autonomous requirement of *drpr* for autophagy, salivary glands of animals expressing GFP-Atg8a in all cells, and *drpr^{IR}* specifically in dsRed-marked clone cells, were assayed for GFP-Atg8a puncta. Strikingly, we found that salivary gland cells expressing *drpr^{IR}* contain diffuse GFP-Atg8a, whereas neighboring control cells contain numerous GFP-atg8a puncta (Fig. 4d). Thus, *drpr* functions in a cell-autonomous manner upstream of autophagy in dying salivary glands, but not in the larval fatbody in response to starvation.

Our findings indicate that Drpr is autonomously required for the clearance of cells that are not removed by phagocytosis. Although autophagy has been associated with engulfment in studies of TLR4- and CD46-mediated clearance of pathogens¹⁴, this is the first example of an “engulfment factor” regulating cell-autonomous clearance. Moreover, our work suggests that Drpr is required for the induction of autophagy in a cell death-specific context. Little is known about how autophagy induction leads to different outcomes in different contexts. It is possible that autophagy functions to deplete specific survival factors during cell death¹⁵. Alternatively, different levels of autophagy could also be induced during different contexts, and extensive autophagy and depletion of cell resources could kill a cell. How Drpr functions to regulate autophagy specifically in a cell death context remains to be determined. Given recent interest in manipulation of autophagy for therapy¹⁶, it is possible that factors such as Drpr could be used as biomarkers to distinguish autophagy leading to cell death versus cell survival.

METHODS SUMMARY

Genetic, histological, protein expression and localization, and reporter assays were performed as previously described^{2,3,17}. Detailed genotypes of control and experimental individuals, as well as the detailed methods for each experiment, are described in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Appendix

Methods

Drosophila strains

The Canton-S strain was used as the wild-type control. For loss of function studies, *drpr*⁵, *simu*² 18, *ced-6*^{J26} 19, and *Df(2R)*^{w73-1} mutants were used. For RNAi studies, the following fly strains were used: *pWiz-drpr*^{IR}10, *pWiz-drpr*^{IR}, *UAS-Atg12-RNA*^{IR}20. The following Vienna *Drosophila* RNAi Center (VDRC) RNAi stocks were used: *UAS-crq*^{IR} VDRC Transformant ID (TID) 45884, *UAS-src42a*^{IR} VDRC TID 46019, *UAS-ced-12*^{IR} TID 10455, *UAS-crk*^{IR} TID 19081, *UAS-mbc*^{IR} TID 16044. The sequences used for VDRC knock-down strains are available for each TID at <http://stockcenter.vdrc.at/control/main>. For ectopic expression studies, *UAS-Drpr-I*, *UAS-p3521*, and *UAS-Atg1*^{6A} 13 were used. *UAS-GFP-LC322*, *hs-GFP-Atg8a6* and *tub-LAMP1-GFP22* were used as markers of autophagy and lysosomes.

Transgenic strains

To generate the UAS-Drpr-I strain, full length *drpr-I* cDNA (GH24127) was inserted into the XhoI site of the pUAST transformation vector²³. The *pWiz-drpr-I^{IR}* RNAi construct was made by amplifying a 1041 nucleotide sequence unique to Drpr-I (nucleotides 1331-2371 from GH 24127) and inserting this fragment into the NheI and XbaI sites of the pWIZ transformation vector²⁴. The same sequence was then inserted in the XhoI and EcoRI sites in the 3' to 5' direction to generate the final *pWiz-drpr-I^{IR}* RNAi construct. Constructs were sequenced and used to generate transgenic *Drosophila* (BestGene, Inc.).

Protein Extracts and Western Blotting

Protein extraction and Western Blotting were performed as described previously¹⁷. Primary antibodies used were rabbit anti-Drpr9 (1:1000), and mouse anti-Beta-Tubulin (1:50) (Developmental Studies Hybridoma Bank).

Histology

Histology was performed as described previously²⁵.

Immunolabeling and Microscopy

Dissection, fixation, and antibody labeling of *Drosophila* tissues was performed as described previously³. Imaging was performed on a Zeiss Axiophot II microscope. Primary antibodies were used at the following concentrations: rabbit anti-Drpr9(1:500), mouse anti-Lamin DMO (1:10) (Developmental Studies Hybridoma Bank), mouse anti-Crumbs (1:10) (Developmental Studies Hybridoma Bank), and mouse anti-GFP (1:100)(Invitrogen Monoclonal). For LC3 and LAMP1 quantification, salivary glands were imaged without fixation on a Zeiss Axiophot II microscope and GFP puncta were quantified using Zeiss Automeasure software.

Induction of cell clones

To induce RNAi-expressing cell clones in *Drosophila* tissues, an overnight egg lay was obtained at 25°C, and following the egg lay, embryos were heat shocked at 37°C for 15 min. To induce expression of hsGFPAtg8, larvae were heat shocked at 37°C for 30 minutes, and then recovered at 25°C until the time of dissection. To induce *drpr*⁵ null mutant cell clones, *y w* hsFLP; UAS-pCherry-Atg8; UbiGFPnls, FRT2A virgins were crossed to *CG-Gal4*; UbiGFPnls, FRT2A, *drpr*⁵ males. An overnight egg lay was obtained at 25°C, and following the egg lay, embryos were heat shocked at 37°C for 1 hour.

Starvation of larvae

Third instar larvae were either allowed to remain in the food (fed) or removed from food and placed on moist petri dishes for 4 hours (starved).

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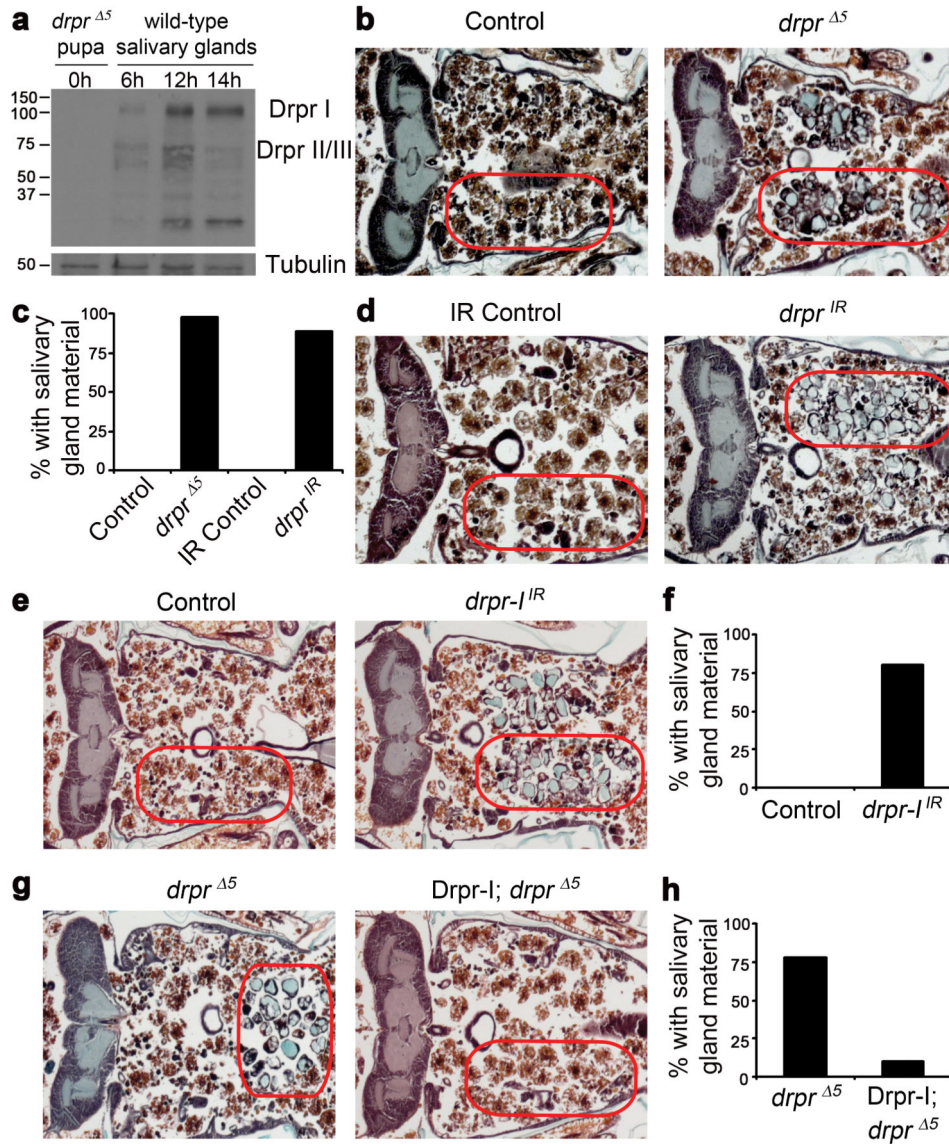


Figure 1. Draper is required for salivary gland cell degradation

a, Protein extracts from *drpr* null (*w*; *drpr*⁵/*drpr*⁵) pupae at puparium formation (0h) and wild type (Canton-S) salivary glands 6h, 12h, and 14h after puparium formation, were analyzed by Western Blotting with anti-Drpr antibody. **b**, Control animals (*+w*; *+drpr*⁵), *n*=12, and *drpr* null mutants (*w*; *drpr*⁵/*drpr*⁵), *n*=47, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **c**, quantification of data from **b** and **d**. **d**, Control animals (*+w*; *+UAS-drpr*^{IR}), *n*=11, and those with salivary gland-specific knockdown of *drpr* (*fkh-GAL4/w*; *UAS-drpr*^{IR/+}), *n*=19, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **e**, Control animals (*+w*; *+UAS-drpr-I*^{IR}), *n*=9, and those with salivary gland-specific knockdown of *drpr-I* (*fkh-GAL4/w*; *UAS-drpr-I*^{IR/+}), *n*=20, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **f**, quantification of data from **e**. **g**, *drpr* null animals (*+w*; *+UAS-Drpr-I*; *drpr*⁵/*drpr*⁵), *n*=11, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **h**, quantification of data from **g**.

drpr⁻⁵), n=9, and those with salivary gland-specific expression of Drpr-I (*fkh-GAL4/w; UAS-Drpr-I/+; drpr⁻⁵/drpr⁻⁵*), n=20, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **h**, quantification of data from **g**.

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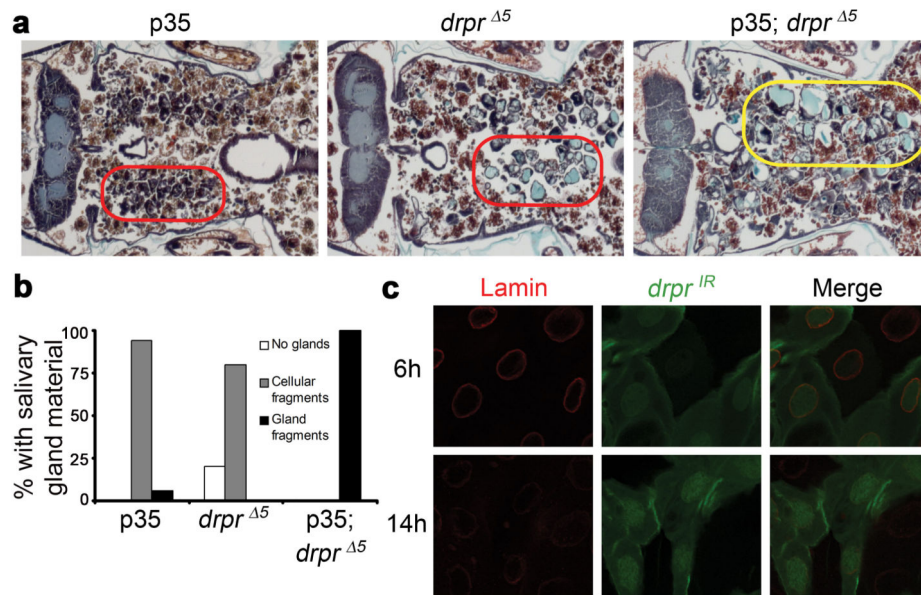


Figure 2. Draper functions downstream or in parallel to caspases during salivary gland cell death

a, Animals with salivary gland-specific expression of p35 (*fkh-GAL4/+; UAS-p35/+*), $n=18$, *drpr* null animals ($+/w; +/UAS-p35; drpr^{5/drpr^{5}}$), $n=10$, and *drpr* null animals with salivary gland-specific expression of p35 (*fkh-GAL4/w; UAS-p35/+; drpr^{5/drpr^{5}}*), $n=16$, were analyzed by histology for the presence of salivary gland material 24h after puparium formation. Cell fragments are in red circles, and gland fragments are in the yellow circle. **b**, quantification of data from **a**. **c**, Salivary glands were dissected from animals expressing *drpr*^{IR} specifically in GFP-marked clone cells (*hsflp/w; UAS-drpr*^{IR} $+/+$; *act<FRT,cd2, FRT>Gal4, UAS-GFP/+*) 6h and 14h after puparium formation. Salivary glands were stained with GFP antibody (green) to label cells expressing *drpr*^{IR}, and Lamin antibody (red).

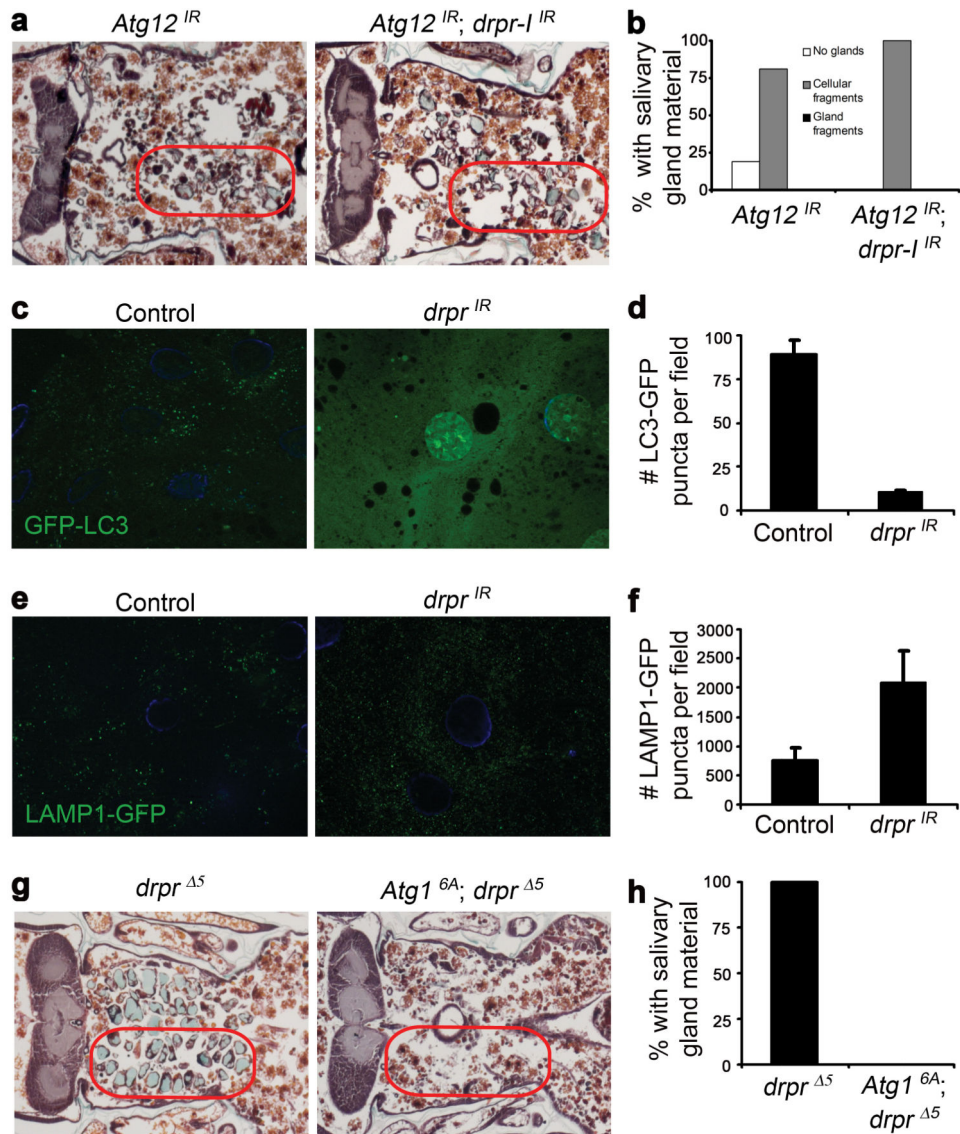


Figure 3. Draper is required for the induction of autophagy in dying salivary gland cells
a, Animals with salivary gland-specific knockdown of *Atg12* (*fkh-GAL4/w*; *UAS-Atg12^{IR/+}*), *n*=21, and those with salivary gland-specific knockdown of both *Atg12* and *drpr-I* (*fkh-GAL4/w*; *UAS-Atg12^{IR/+}*; *UAS-drpr-I^{IR/+}*), *n*=19, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **b**, quantification of data from **a**. **c**, GFP-LC3 was expressed in salivary glands of control animals (*+/w*; *UAS-GFP-LC3/+*; *fkh-GAL4/+*) and those with salivary gland-specific knockdown of *drpr* (*w*; *UAS-drpr^{IR}/UAS-GFP-LC3*; *fkh-GAL4/+*). Salivary glands were dissected 6h and 14h after puparium formation, imaged for GFP-LC3, and LC3 puncta were quantified using Zeiss Automeasure software. **d**, quantification of data from **c**. Error bars represent s.e.m.; *n* = 10; *p* < 0.0000001. **e**, LAMP-GFP was expressed in control animals (*tub-LAMP-GFP/w*; *+fkh-GAL4*) and those with salivary gland-specific knockdown of *drpr* (*tub-LAMP-GFP/w*; *UAS-drpr^{IR/+}*; *fkh-GAL4/+*). Salivary glands were dissected 14h after puparium formation, imaged for LAMP-GFP, and LAMP puncta were quantified using

Zeiss Automeasure software. **f**, quantification of data from **e**. Error bars represent s.e.m.; n 10; $p < 0.05$. **g**, *drpr* mutant animals (+/w; +/UAS-*Atg1^{6A}*; *drpr⁵/drpr⁵*), n=10, and those with salivary gland-specific expression of *Atg1^{6A}* (*fkh-GAL4/w*; UAS-*Atg1^{6A}/+*; *drpr⁵/drpr⁵*), n=16, were analyzed by histology for the presence of salivary gland material (red circles) 24h after puparium formation. **h**, quantification of data from **g**.

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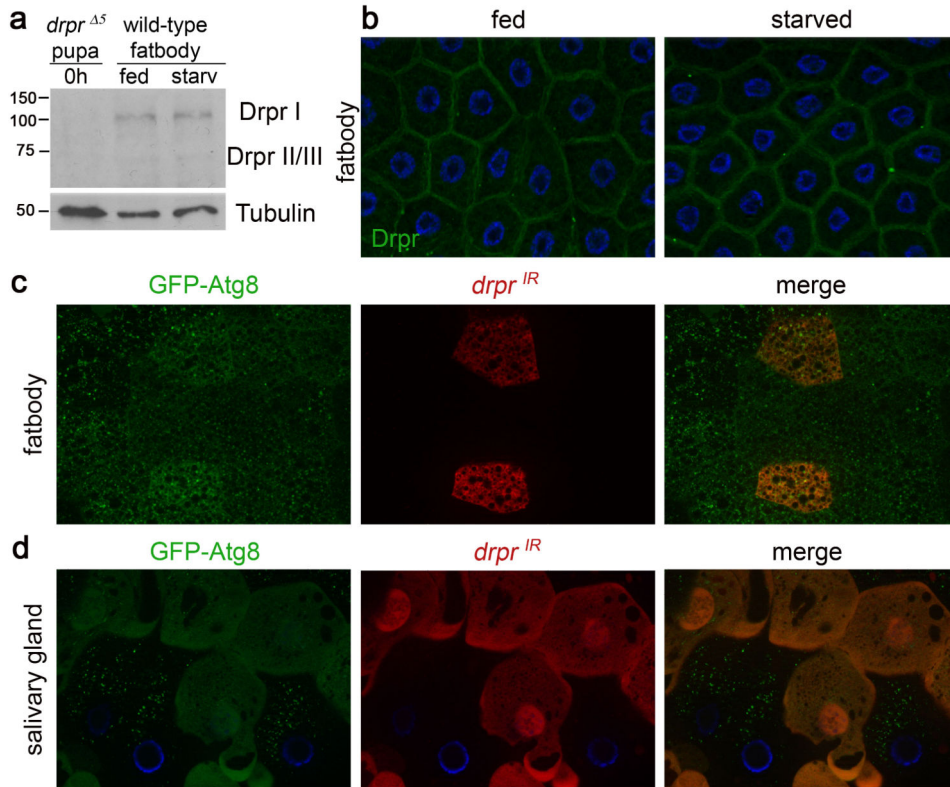


Figure 4. Drpr is cell-autonomously required for autophagy in dying salivary glands, but not in response to starvation in the fatbody

a, Protein extracts from *drpr* null (*w*; *drpr*⁵/*drpr*⁵) pupae at puparium formation (0h) and from the fatbodies of wild type (Canton-S) third instar larvae were analyzed by Western Blotting with anti-Drpr antibody. Third instar larvae were either fed or starved 4h. **b**, Wild-type (Canton-S) third instar larvae were either fed or starved 4h, and fatbodies were dissected, stained with anti-Drpr antibody, and imaged for Drpr (green). Nuclei were stained with DAPI (blue). **c**, Third instar larvae expressing GFP-Atg8 in all cells, and *drpr*^{IR} specifically in dsRed-marked clone cells (*hsflp/w*; *UAS-drpr*^{IR/+}; *hsGFPA*tg8b, *act*<FRT,cd2, FRT>Gal4, *UAS-dsRed/+*), were starved 4h. Larval fatbodies were dissected and imaged for GFPAtg8 (green) and dsRed (red). **d**, Salivary glands of animals expressing GFP-Atg8 in all cells, and *drpr*^{IR} specifically in dsRed-marked clone cells (*hsflp/w*; *UAS-drpr*^{IR/+}; *hsGFPA*tg8b, *act*<FRT,cd2, FRT>Gal4, *UAS-dsRed/+*) were dissected 14h after puparium formation. Salivary glands were imaged for GFPAtg8 (green) and dsRed (red). Nuclei were stained with Hoescht (blue).