Ral GTPase and the exocyst regulate autophagy in a tissue-specific manner

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Abstract

Autophagy traffics cellular components to the lysosome for degradation. Ral GTPase and the exocyst have been implicated in the regulation of stress-induced autophagy, but it is unclear whether they are global regulators of this process. Here, we investigate Ral function in different cellular contexts in *Drosophila* and find that it is required for autophagy during developmentally regulated cell death in salivary glands, but does not affect starvation-induced autophagy in the fat body. Furthermore, knockdown of exocyst subunits has a similar effect, preventing autophagy in dying cells but not in cells of starved animals. Notch activity is elevated in dying salivary glands, this change in Notch signaling is influenced by Ral, and decreased Notch function influences autophagy. These data indicate that Ral and the exocyst regulate autophagy in a context-dependent manner, and that in dying salivary glands, Ral mediates autophagy, at least in part, by regulation of Notch.

Keywords autophagy; cell death; *Drosophila*; exocyst; Notch; Ral GTPase

Subject Categories Autophagy & Cell Death; Development & Differentiation

DOI 10.15252/embr.201541283 | Received 31 August 2015 | Revised 15 October 2015 | Accepted 22 October 2015 | Published online 23 November 2015

EMBO Reports (2016) 17: 110–121

Introduction

Macroautophagy (autophagy) is a catabolic process during which cytoplasmic components, including organelles and long-lived proteins, are engulfed and trafficked to the lysosomal compartment for degradation [1]. Autophagy has been implicated in several diseases, including neurodegeneration and cancer [2]. Autophagy plays dual roles to determine cell fate depending on cell context [3]. During stress, such as nutrient deprivation or growth factor removal, autophagy promotes cellular homeostasis and survival by recycling cell components for energy production [4]. Alternatively, autophagy has been shown to function in developmentally regulated cell death, as in the case of degrading larval salivary glands during *Drosophila* development [5].

Autophagy is regulated by upstream protein and lipid kinase complexes, and these complexes in turn influence core ubiquitin-like conjugation pathways that control autophagosome formation

around cytoplasmic cargoes [6]. The serine threonine kinase Atg1 (Ulk1/2 in mammals) complex is under control of mTOR, and this is a regulatory complex that integrates nutritional status with the requirement for activation of autophagy [7]. The Vps34 lipid kinase (class III phosphatidylinositol 3-kinase in mammals) complex is required for the formation of phosphatidylinositol 3-phosphate (PI3P) and therefore has been implicated in multiple vesicle trafficking processes, including autophagy, endocytosis, and protein secretion [8–10].

Ral is a member of the Ras superfamily of small GTPases. Ral has a variety of downstream effectors and has been implicated in several cellular processes, including gene transcription, signal transduction, actin organization, and membrane dynamics [11]. Two well-characterized Ral effectors are the exocyst components, Sec5 and Exo84 [12–14]. Through its interactions with these effectors, Ral plays an important role in vesicle trafficking and protein secretion. Recently, autophagy genes have been implicated in both conventional and unconventional protein secretion [15,16]. Importantly, several regulators of autophagy, including *Atg6*, *Vps34*, *Atg1*, and *Vps15*, have been shown to be required for steroid-induced secretion of glue proteins from *Drosophila* salivary glands [16,17]. The requirement of autophagy genes for protein secretion suggests that there may be cross talk between the regulatory factors that control these distinct vesicle trafficking processes.

Ral has been implicated in the regulation of stress-induced autophagy [18]. Interestingly, through physical interaction studies between RalB and Ulk1/Atg1, components of the Vps34 complex, and the Exo84 exocyst subcomplex, a model has been proposed for this large protein complex in the regulation of autophagy [18]. This model makes multiple predictions, including that the Exo84 subcomplex of the exocyst functions as a positive regulator of autophagy and that the Sec5 subcomplex of the exocyst functions as a suppressor of autophagy. In addition, Ral may function as a broad regulator of autophagy in multiple cell contexts. However, the function of Ral and exocyst components in the control of autophagy has not been investigated in animals under physiological conditions, and it remains unclear whether these factors are global regulators of autophagy.

Here, we demonstrate that Ral, the Ral guanine nucleotide exchange factor (GEF) Rgl, and components of the exocyst complex are required for proper larval salivary gland degradation. We found that Ral and the exocyst function in salivary gland degradation by

regulating autophagy and that Ral may regulate autophagy by influencing Notch levels. By contrast, Ral and the exocyst are not necessary for autophagy in response to nutrient deprivation. These results indicate that Ral and the exocyst regulate autophagy in a context-dependent manner.

Results

Ral and Rgl are required for salivary gland degradation

Drosophila larval salivary gland cell death is triggered by a rise in steroid at 12 h after puparium formation, and the glands are completely degraded by 16 h after puparium formation [19]. A Ral mutant was identified in a screen of lethal *P*-element insertions for persistent larval salivary glands, suggesting that Ral could function in salivary glands during their degradation [20]. We tested whether inhibition of Ral in salivary glands would cause a salivary gland degradation defect, and found that knockdown of Ral by expression of a RNAi against *ral* (*ral^{IR}*) in salivary glands with the salivary gland-specific driver *fkh*-GAL4 resulted in a degradation defect in 100% of pupae (Figs 1A and B, and EV1). In contrast, 20% of control animals lacking the *fkh*-GAL4 driver had persistent salivary gland material at 24 h after puparium formation (Fig 1A and B). Similarly, we found that 90% of pupae with *fkh*-GAL4 driving a dominant-negative Ral, *Ral^{S25N}*, had a salivary gland degradation defect compared to 15% of control pupae with no *fkh*-GAL4 driver (Fig 1C and D). Strong Ral loss-of-function mutants are lethal early during development. Therefore, we sought to confirm whether Ral function is necessary for proper salivary gland degradation using weak *ral^{35d}* hypomorphic mutants [21]. We found that 61% of *ral^{35d}* mutant pupae failed to complete salivary gland degradation, whereas most heterozygous control pupae lack salivary gland material at 24 h after puparium formation (Fig 1E and F). Since Ral is a GTPase, we tested whether activation of Ral by its GEF, Rgl, was required for salivary gland degradation. We found that salivary gland-specific knockdown of Rgl resulted in a similar salivary gland degradation defect phenotype to both Ral knockdown and dominant-negative Ral expression (Fig 1G and H). Combined, these data indicate that Ral and its upstream activator, Rgl, function in salivary glands during degradation.

Ral is required for autophagy in dying salivary gland cells

The requirement for Ral during salivary gland degradation led us to investigate whether Ral functions in previously defined processes that participate in the destruction of this tissue. Salivary gland cell death requires both caspases and autophagy for complete salivary gland degradation [5]. Caspases and autophagy act in an additive and parallel manner to control salivary gland cell death. Specifically, decreased function of genes in either pathway alone results in a salivary gland cell fragment phenotype, whereas inhibition of both autophagy and caspases results in an additive phenotype, with a more intact salivary gland tissue fragment phenotype 24 h after puparium formation [5].

We tested whether Ral may function in the same pathway as caspases by expressing the caspase inhibitor, p35. Expression of p35 in the *ral^{35d}*/wild-type heterozygous genetic background resulted in

persistence of salivary gland cell fragments in 57% of pupae, and gland tissue fragments in 43% of pupae (Figs 2A and B, and EV2). By contrast, expression of p35 in the hemizygous *ral^{35d}* mutant background resulted in persistence of cell fragments in 20% of pupae and gland tissue fragments in 80% of pupae (Figs 2A and B, and EV2). The enhanced gland degradation defect phenotype in the *ral^{35d}* mutants expressing p35 indicates that Ral functions in an additive manner with caspases. We further tested the relationship between Ral and caspases by knocking down Ral in salivary glands and assaying for caspase activity by staining for cleaved caspase-3. At 0 h after puparium formation, well before caspases are activated during salivary gland cell death, both control and *ral^{IR}*-expressing cells have little to no cleaved caspase-3 staining (Fig 2C and E). At 13 h after puparium formation, after caspases have been activated, both control and *ral^{IR}*-expressing cells have similarly increased staining for cleaved caspase-3 (Fig 2D and E). Taken together, these data indicate that Ral is not required for caspase activity in salivary glands and that Ral and caspases function in parallel during salivary gland cell death. In addition, the presence of caspase activity at this stage in salivary glands indicates that altered *ral* function does not broadly influence development by impairing all of the steroid-triggered responses that are associated with degradation of this tissue.

We next investigated whether Ral is required for autophagy. We first asked whether the salivary gland degradation phenotype of *ral^{35d}* mutants is enhanced by knocking down the autophagy gene, *Atg6*. We found that 45% of animals expressing *Atg6^{IR}* had a persistent cell fragment phenotype. Similarly, 46% of *ral^{35d}* mutants expressing *Atg6^{IR}* had a cell fragment phenotype; neither group had any persistence of gland tissue fragments (Fig 3A and B). The lack of phenotypic enhancement when *Atg6^{IR}* is expressed in the *ral^{35d}* background is different from the increased persistence of gland material that we observed in *ral^{35d}* mutants expressing p35 (Fig 2A and B), and suggests that Ral and *Atg6* may function in the same pathway. Consistent with our histological data suggesting that Ral functions in the autophagic pathway, clonal knockdown of *ral* function by expression of *ral^{IR}* in salivary gland cells 14 h after puparium formation lead to a significant decrease in autophagy reporter mCherry-Atg8a puncta formation when compared to neighboring control cells (Fig 3C and D). Similarly, loss-of-function *ral^{PG89}* mutant cells possess fewer autophagy reporter GFP-Atg8a puncta (Fig 3E and F), and possess more autophagy cargo receptor Ref(2)p/p62 puncta than control cells (Fig 3G and H). Combined, these data indicate that *ral* is required for autophagy in dying salivary gland cells.

Several factors have been identified that regulate autophagy in a dying cell context-specific manner [22–24]. Since RalB was shown to be required for resource-deprivation-triggered autophagy in mammalian cell lines [18], this prompted us to ask whether Ral is required for starvation-triggered autophagy *in vivo*. We tested this by assaying for GFP-Atg8a puncta formation in fat bodies of starved animals with loss-of-function *ral^{PG89}* mutant cells. In contrast to what we observed in dying salivary gland cells, fat bodies of starved larvae had similar levels of GFP-Atg8a puncta in both cells lacking *ral^{PG89}* function and their neighboring control cells (Fig 3I and J). This indicates that Ral functions in a context-specific manner to regulate autophagy, as it is required for autophagy in dying salivary gland cells, but it appears to be expendable for starvation-induced autophagy in the fat body.

Figure 1. *Ral* and *Rgl* are required for salivary gland degradation.

A, B Control animals (+/*w*; UAS-*ral*^{IR}/+, *n* = 20) and those with salivary gland-specific knockdown of *ral* (*fkh*-GAL4/*w*; UAS-*ral*^{IR}/+, *n* = 20) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation (A). Quantification of the data is shown in (B).
 C, D Control animals (+/*w*; UAS-*ral*^{S25N}/+, *n* = 20) and those with salivary gland-specific expression of dominant-negative Ral (*fkh*-GAL4/*w*; UAS-*ral*^{S25N}/+, *n* = 20) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (D).
 E, F Control animals (*ral*^{35D}/+, *n* = 25) and *ral* hypomorph mutants (*ral*^{35D}/*Y*, *n* = 23) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (F).
 G, H Control animals (+/*w*; UAS-*rgl*^{IR}/+, *n* = 19) and those with salivary gland-specific knockdown of *rgl* (*fkh*-GAL4/*w*; UAS-*rgl*^{IR}/+, *n* = 20) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (H).

Data information: Data in bar graphs are represented as means. Statistical significance was determined using a chi-square test.

Figure 2. Loss of *ral* does not affect caspase activity during salivary gland degradation.

A, B *ral* hypomorph mutants (*ral*^{35d}/Y; UAS-p35/+; *n* = 20), animals with salivary gland-specific expression of p35 (*ral*^{35d}/+; UAS-p35/*fkh*-GAL4; *n* = 21), and *ral* hypomorph mutants with salivary gland-specific expression of p35 (*ral*^{35d}/Y; UAS-p35/*fkh*-GAL4; *n* = 20) were analyzed by histology for the presence of salivary gland material (red dotted circles) 24 h after puparium formation. Quantification of the data is shown in (B). Data are represented as means. Statistical significance was determined using a chi-square test comparing the percentages of gland fragments.

C–E Salivary glands dissected from 0 (C) or 13 h (D) after puparium formation control animals (+/w; UAS-*ral*^{IR}/+) and those with salivary gland-specific knockdown of *ral* (*fkh*-GAL4/w; UAS-*ral*^{IR}/+) and stained with cleaved caspase-3 antibody (green) and DAPI (blue). Scale bars represent 20 μ m. Quantification of the data is shown in (E). Data are represented as means $\pm$ SEM; *n* $\geq$ 10. Statistical significance was determined using a Student's *t*-test.

The exocyst is required for autophagy associated with cell death, not starvation-induced autophagy

Ral has several effector proteins, the best characterized are RALBP (or Rlip) which is involved in endocytosis, and the exocyst subunits, Sec5 and Exo84 [25]. The exocyst is an evolutionarily conserved octameric complex involved in the tethering of secretory vesicles to the plasma membrane [26]. Previous studies in mammalian cell lines suggest that Ral regulates autophagy through its interactions with Sec5 and Exo84 [18,27]. Since our results indicate that Ral regulates autophagy in a context-dependent manner, we wondered whether the exocyst could be regulating autophagy in a similar way. We tested whether knockdown of exocyst subunits could affect glue peptide exocytosis from salivary glands, and identified RNAi strains against *sec5*, *sec3*, *sec8*, and *exo84* that inhibited secretion (Fig EV3), indicating that these RNAis are functional. In addition, knockdown of *ral* in the salivary gland led to a similar secretion defect (Fig EV3).

We next asked whether the exocyst could regulate starvation-induced autophagy in the fly. We tested this by clonally knocking down several exocyst subunits in fat body cells, starving these animals, and then assaying for mCherry-Atg8a puncta formation. Similar to our results with *ral* loss of function (Fig 3), we did not observe a difference in mCherry-Atg8a puncta formation between control cells and the various exocyst subunit knockdown cells (Fig 4). Furthermore, when we clonally knocked down the exocyst subunits and checked for mCherry-Atg8a puncta formation in the fat bodies of feeding animals, we observed no difference (Fig EV4). Taken together, these results suggest that similarly to Ral, the exocyst is not required for starvation-induced autophagy in the fly.

Since the exocyst, like Ral, is not required for starvation-induced autophagy, we next asked whether it is required for cell death-associated autophagy. We tested this by knocking down several exocyst subunits in salivary glands and assaying for mCherry-Atg8a puncta formation during salivary gland degradation. We found

Figure 3.

that in dying salivary glands from animals with *fkh*-GAL4-driven expression of either *sec5^{IR}*, *sec15^{IR}*, *sec3^{IR}*, *sec8^{IR}*, or *exo84^{IR}*, there were significantly fewer mCherry-Atg8a punctae when compared to salivary

glands from control animals lacking RNAi to any of the tested exocyst components (Fig 5). These data indicate that the exocyst is required for autophagy in dying salivary gland cells. In addition,

Figure 3. *ral* is required for autophagy in dying salivary glands, but not for starvation-induced autophagy in fat body.

- A, B *ral* hypomorph mutants (*ral*^{35d}/Y; UAS-Atg6^{IR}/+, *n* = 27), animals with salivary gland-specific expression of Atg6^{IR} (*ral*^{35d}/+; UAS-Atg6^{IR}/fkh-GAL4, *n* = 20), and *ral* hypomorph mutants with salivary gland-specific expression of Atg6^{IR} (*ral*^{35d}/Y; UAS-Atg6^{IR}/fkh-GAL4, *n* = 24) were analyzed by histology for the presence of salivary gland material (red dotted circles) 24 h after puparium formation. Quantification of the data is shown in (B).
- C, D Salivary glands dissected from 14 h after puparium formation animals expressing mCherry-Atg8a in all cells, and *ral*^{IR} specifically in GFP-marked clone cells (*yw*, *hsflp/w*; pmCherry-Atg8a/UAS-*ral*^{IR}; *act* < FRT, *cd2*, FRT > GAL4, UAS-GFP/+), analyzed for mCherry-Atg8a puncta. GFP-marked clone *ral*^{IR}-expressing cells contain a yellow asterisk. Quantification of the data is shown in (D).
- E, F Salivary glands dissected from 14 h after puparium formation animals that have *ral* loss of function (*ral*^{PC89}) specifically in non-RFP cells, and that express GFP-Atg8a in all cells (*yw*, *hs-Flp*, FRT19A, *Ubi-RFP/ral*^{PC89}, FRT19A; pGFP-Atg8a) analyzed for GFP-Atg8a puncta. *ral*^{PC89} mutant cells are marked with a yellow asterisk. Quantification of the data is shown in (F).
- G, H Salivary glands dissected from 14 h after puparium formation animals that have *ral* loss of function (*ral*^{PC89}) specifically in non-RFP cells (*yw*, *hs-Flp*, FRT19A, *Ubi-RFP/ral*^{PC89}, FRT19A) and are stained for Ref(2)p (green) and DAPI (blue). *ral*^{PC89} mutant cells are marked with a yellow asterisk. Quantification of the data is shown in (H).
- I, J Fat bodies dissected from 4 h starved animals that have *ral* loss of function (*ral*^{PC89}) specifically in non-RFP cells, and that express GFP-Atg8a in all cells (*yw*, *hs-Flp*, FRT19A, *Ubi-RFP/ral*^{PC89}, FRT19A; pGFP-Atg8a) analyzed for GFP-Atg8a puncta. *ral*^{PC89} mutant cells are marked with a yellow asterisk. Quantification of the data is shown in (J).

Data information: Scale bars represent 20 μ m. Data are represented as means $\pm$ SEM; *n* $\geq$ 10 (D, F, H, J). Statistical significance was determined using a chi-square test (B) or a Student's *t*-test (D, F, H, J).

we tested whether inhibition of the exocyst in salivary glands would cause a salivary gland degradation defect. Expression of either *sec5*^{IR} (Fig 6A and B), *sec15*^{IR} (Fig 6C and D), *sec3*^{IR} (Fig 6E and F), *sec8*^{IR} (Fig 6G and H), or *exo84*^{IR} (Fig 6I and J) resulted in a significantly higher percentage of animals with a salivary gland degradation defect when compared to their control animals. These results suggest that the exocyst is necessary for proper salivary gland degradation by autophagy during *Drosophila* development.

Notch is regulated by Ral and required for autophagy in salivary glands

Previous work in *Drosophila* identified both the Janus Kinase/STAT and Notch pathways as regulatory targets of Ral [28,29]. Therefore, we tested whether reporters of STAT signaling [30] and Notch signaling [31] are induced in dying salivary glands. Although reporters of both of these pathways are active in salivary glands, the reporter of Notch exhibited a significant induction prior to salivary gland destruction (Fig 7A and B). Moreover, decreased function of *Ral* by expression of an RNAi specifically in salivary glands significantly reduced Notch reporter activity (Fig 7A and B). These data prompted us to investigate whether Notch influences autophagy in salivary glands. Significantly, clonal knockdown of *notch* function by expression of *notch*^{IR} in salivary gland cells 14 h after puparium formation resulted in a decrease in autophagy reporter mCherry-Atg8a puncta formation compared to neighboring control cells (Fig 7C and D). These data indicate that Ral may regulate autophagy via Notch.

Discussion

In this study, we investigated the role of Ral GTPase in the regulation of autophagy *in vivo* and demonstrated that Ral regulates autophagy in the context of developmentally programmed cell death. Our data indicate that the exocyst, a downstream Ral effector, is also required for developmentally programmed cell death.

Previous studies have implicated the Ral/exocyst effector complex in the regulation of autophagy [18,27]. In mammalian cells, RalB is proposed to regulate starvation-induced autophagy through binding of an Exo84 subcomplex of the exocyst. In contrast, we found that neither Ral nor the exocyst has an observable role in autophagy triggered by nutrient deprivation *in vivo*. This could be due to the apparent cell type specificity in which Ral regulates autophagy. Additionally, our data contradicts the idea of Ral regulating autophagy through distinct exocyst subcomplexes. When we knocked down the different exocyst subunits in salivary glands, we observed a similar inhibition of pmCherry-Atg8a puncta formation across all subunits, suggesting that the exocyst may function as a whole complex during autophagic cell death. In addition, knockdown of Sec5, a key component of the so-called autophagy suppression complex [18], failed to cause premature autophagy in the fly fat body (Fig EV4). These discrepancies could be due to either organism differences or differences in the regulation of autophagy depending on context; exocyst subcomplexes could regulate starvation-induced autophagy, while the octameric exocyst complex regulates autophagic cell death.

It remains unclear why Ral regulates autophagy in a context-dependent manner. Ral can be activated by Ras or Rap via its Ral-GEF or by Ca²⁺/calmodulin binding [21,32–36]. Our data indicate that the Ral-GEF, Rgl, is necessary for salivary gland cell death; however, this does not preclude Ca²⁺/calmodulin binding from being involved. Several studies have linked calcium signaling to autophagy and recent results indicate that calmodulin functions downstream of IP3 signaling during salivary gland cell death, but not during starvation-induced autophagy in the fat body [24,37]. It could be possible that in the context of cell death, Ral is activated by both Ral-GEF and Ca²⁺/calmodulin binding and potentiates these signals to promote autophagy.

Signaling downstream of Ral could also affect how Ral regulates autophagy in different cell and tissue contexts. In mammalian cells, it has been proposed that Ral regulates autophagy through mTOR [18,27]. However, mTOR is not the only factor that has been implicated in both Ral signaling and autophagy. Notch signaling has been shown to both regulate autophagy [38,39], and to be regulated by

Figure 4. The exocyst is not required for starvation-induced autophagy in fat body.

A–J Fat bodies dissected from 4-h starved animals expressing mCherry-Atg8a in all cells, and (A) *sec5^{IR}*, (C) *sec15^{IR}*, (E) *sec3^{IR}*, (G) *sec8^{IR}*, (I) *exo84^{IR}* specifically in GFP-marked clone cells (*yw*, *hsflp/w*; *pmCherry-Atg8a/+*; *act < FRT*, *cd2*, *FRT > GAL4*, *UAS-GFP/+*) analyzed for mCherry-Atg8a puncta. Quantification of the data from (A, C, E, G, I) is shown in (B, D, F, H, J), respectively.

Data information: Scale bars represent 20 μ m. Data are represented as means $\pm$ SEM; $n \geq 10$. Statistical significance was determined using a Student's *t*-test.

autophagy [40,41]. Interestingly, there is evidence that Ral regulates Notch in *Drosophila* [29]. Here, we have shown that Notch is required for autophagy in degrading salivary glands with Notch activity increasing prior to salivary gland degradation (Fig 7). Additionally, knockdown of *ral* reduces Notch reporter activity in

salivary glands (Fig 7). These results raise the possibility that Ral regulates autophagy through the modulation of Notch levels in degrading salivary glands. Further studies are required to determine the mechanistic link between Ral, Notch, and autophagy during salivary gland cell death.

Figure 5. The exocyst is required for autophagy in dying salivary gland cells.

A, B Salivary glands dissected from 14 h after puparium formation (top left) control (*fkh-GAL4/+; pmCherry-Atg8a/+*), and those with salivary gland-specific knockdown of (middle left) *sec5* (*fkh-GAL4/w; pmCherry-Atg8a/+; UAS-sec5^{IR}/+*), (bottom left) *sec15* (*fkh-GAL4/w; pmCherry-Atg8a/+; UAS-sec15^{IR}/+*), (top right) *sec3* (*fkh-GAL4/w; pmCherry-Atg8a/UAS-sec3^{IR}*), (middle right) *sec8* (*fkh-GAL4/w; pmCherry-Atg8a/UAS-sec8^{IR}*), (bottom right) *exo84* (*fkh-GAL4/w; pmCherry-Atg8a/UAS-exo84^{IR}*) analyzed for mCherry-Atg8a puncta. Scale bars represent 20 μ m. Quantification of the data is shown in (B). Data are represented as means $\pm$ SEM; $n \geq 10$. Statistical significance was determined using a Student's *t*-test (** $P < 0.001$, *** $P < 0.0001$).

The Ral/exocyst effector complex is an important spatiotemporal regulator of several membrane trafficking processes. Our findings indicate a previously unknown role for the Ral/exocyst effector complex in the regulation of autophagy that is involved in cell death. Importantly, we have shown that Ral and the exocyst

regulate autophagy in a context-dependent manner, and question a global role for these factors in their regulation of autophagy in animals. Given recent interest in the targeting of Ral [42] and autophagy [43] for cancer therapy, it will be important to understand how Ral regulates autophagy in a variety of cell contexts.

Figure 6. The exocyst is required for salivary gland degradation.

A, B Control animals (+/+; UAS-*sec5^{IR}*/+, $n = 19$) and those with salivary gland-specific knockdown of *sec5* (*fkh-GAL4/w*; UAS-*sec5^{IR}*/+, $n = 20$) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (B).

C, D Control animals (+/+; UAS-*sec15^{IR}*/+, $n = 20$) and those with salivary gland-specific expression *sec15* (*fkh-GAL4/w*; UAS-*sec15^{IR}*/+, $n = 20$) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (D).

E, F Control animals (+/+; UAS-*sec3^{IR}*/+, $n = 20$) and those with salivary gland-specific expression *sec3* (*fkh-GAL4/w*; UAS-*sec3^{IR}*/+, $n = 20$) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (F).

G, H Control animals (+/+; UAS-*sec8^{IR}*/+, $n = 20$) and those with salivary gland-specific knockdown of *sec8* (*fkh-GAL4/w*; UAS-*sec8^{IR}*/+, $n = 19$) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (H).

I, J Control animals (+/+; UAS-*exo84^{IR}*/+, $n = 19$) and those with salivary gland-specific knockdown of *exo84* (*fkh-GAL4/w*; UAS-*exo84^{IR}*/+, $n = 20$) were analyzed by histology for the presence of salivary gland material (red dotted circle) 24 h after puparium formation. Quantification of the data is shown in (J).

Data information: Data are represented as means. Statistical significance was determined using a chi-square test.

Figure 7. Notch activity is regulated by *ral* and is required for autophagy in dying salivary gland cells.

A, B Salivary glands dissected from late third-instar larvae (*Gbe Su(H)-lac-Z/w⁻;+;Fkh-GAL4/+*) show less *notch* activity (left) compared to glands from 14 h after puparium formation (APF) animals (middle). Downregulation of *ral* in salivary glands from 14 h APF animals (*Gbe Su(H)-lac-Z/w⁻; UAS-*ral*^{IR}/+; Fkh-GAL4/+*) reduces the levels of *notch* activity (right). Scale bar represents 20 μ m. Quantification of the data is shown in (B).
C, D Salivary glands dissected from 14 h after puparium formation animals expressing mCherry-Atg8a in all cells, and *notch*^{IR} specifically in GFP-marked clone cells (*yw, hsf1p/w; UAS-*notch*^{IR}; pmCherry-Atg8a/+; act < FRT, cd2, FRT > GAL4, UAS-GFP/+*) analyzed for mCherry-Atg8a puncta. Scale bar represents 20 μ m. Quantification of the data is shown in (D).

Data information: Data are represented as means $\pm$ SEM; $n \geq 4$ (B); $n > 10$ (D). Statistical significance was determined using a Student's *t*-test.

Materials and Methods

Drosophila strains

We used the following fly stocks: Canton-S as wild-type control, *fkh-GAL4, w; Sgs43-GFP* (Bloomington Drosophila Stock Center), *yw, hsf1p; pmCherry-Atg8a; act > CD2 > GAL4, UAS-GFP/TM6B, fkh-GAL4; pmCherry-Atg8a, yw, hsf1p, FRT19A, Ubi-RFP* and *yw, hsf1p, FRT19A, Ubi-RFP; pGFP-Atg8a*. For loss-of-function studies, we used *ral*^{35d} provided by J. Camonis [21] and *ral*^{PG89}, FRT19A provided by J. Fischer [28]. For RNAi studies, we used the following stocks from the Vienna Drosophila RNAi Center (VDRC): UAS-*ral*^{IR} VDRC Transformant ID (TID) 43622, UAS-*rgl*^{IR} TID 23639, UAS-*sec5*^{IR} TID 28873, UAS-*sec15*^{IR} TID 35162, UAS-*sec3*^{IR} TID 35806, UAS-*sec8*^{IR} TID 45032, UAS-*exo84*^{IR} TID 108650. For ectopic expression studies, we used UAS-*ral*^{S25N} and UAS-p35 (Bloomington Drosophila Stock Center). For the *notch* analyses, we used the *Gbe Su(H)-lac-Z* line [31] provided by N. Tapon and the UAS-*notch*^{IR} [44] (Bloomington Drosophila Stock Center).

Histology

Histology was performed as described previously [45].

Immunostaining and microscopy

For immunostaining, salivary glands were dissected in PBS and fixed overnight in 4% formaldehyde in PBS at 4°C. They were blocked in PBS, 1% BSA, and 0.1% Tween (PBSBT) and incubated with primary antibody (rabbit anti-cleaved caspase-3 1:400, Cell Signaling Technology; rabbit anti-Ref(2)p 1:2,000, gift from G. Juhasz; rabbit anti-beta galactosidase 1:3,000, Cappel/MP Biomedicals) overnight at 4°C. Then, salivary glands were washed in PBSBT for 2 h at room temperature, incubated with the appropriate secondary antibody for 2 h at room temperature, and washed for 1 h in PBSBT. Salivary glands were mounted in Vectashield (Vector Laboratories). For mCherry-Atg8a and GFP-Atg8a analysis, salivary glands and fat bodies were dissected from animals staged at 25°C in PBS and mounted in 50% glycerol in PBS containing 2 μ M Hoescht stain. mCherry-Atg8a, GFP-Atg8a, and Ref(2)p puncta were quantified using ImageJ software. Imaging was performed on either a Zeiss Axiophot II microscope or on a Zeiss LSM700 confocal microscope.

Induction of cell clones

The induction of RNAi-expressing cell clones was performed as described previously [22]. For *ral* loss-of-function clones, females

ral^{PG89}/Fm7-GFP flies were crossed to *yw*, *hs-Flp*, FRT19A, *Ubi-RFP* or *yw*, *hs-Flp*, FRT19A, *Ubi-RFP*; pGFP-Atg8a male flies and were left to lay eggs for 8 h. The egg lay was heat shocked at 37°C for 1 h to induce cell clones.

Protein secretion assay

Salivary glands were dissected at 6 h after pupariation, fixed briefly with 4% formaldehyde, and stained with Hoescht.

Starvation

Feeding third-instar larvae either remained in the food (fed), or were removed from the food and placed in a moist Petri dish for 4 h (starved) at 25°C.

Expanded View for this article is available online.

Acknowledgements

We thank J. Camonis, N. Tapon, J. Fischer, the Bloomington Stock Center, the *Drosophila* Genetic Resource Center, and the VDRC for flies, and T. Fortier for technical support. This work was supported by NIH grant GM079431 to E.H.B. E.H.B. is an Ellison Medical Foundation Scholar.

Author contributions

KT, PDV, and EHB designed the experiments and wrote the manuscript. Experiments were performed by KT and PDV.

Conflict of interest

The authors declare that they have no conflict of interest.

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