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Autophagy Genes Are Essential for Dauer Development and Life-Span Extension in *C. elegans*

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Both dauer formation (a stage of developmental arrest) and adult life-span in *Caenorhabditis elegans* are negatively regulated by insulin-like signaling, but little is known about cellular pathways that mediate these processes. Autophagy, through the sequestration and delivery of cargo to the lysosomes, is the major route for degrading long-lived proteins and cytoplasmic organelles in eukaryotic cells. Using nematodes with a loss-of-function mutation in the insulin-like signaling pathway, we show that *bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene *APG6/VPS30/beclin1*, is essential for normal dauer morphogenesis and life-span extension. Dauer formation is associated with increased autophagy and also requires *C. elegans* orthologs of the yeast autophagy genes *APG1*, *APG7*, *APG8*, and *AUT10*. Thus, autophagy is a cellular pathway essential for dauer development and life-span extension in *C. elegans*.

Under conditions of high population density, limited food, or increased temperature, *Caenorhabditis elegans* nematodes reversibly arrest in an alternate third larval stage, the dauer diapause, which is specialized to survive in an unfavorable environment (*1*). Entry into dauer is regulated by different signaling pathways, including transforming growth factor (TGF-β), cyclic guanosine monophosphate, and insulin-like signaling. Single-gene mutations in the insulin-like signaling pathway that promote constitutive dauer phenotypes also prolong adult life-span (*2*). Despite extensive characterization of regulatory signals, the cellular pathways involved in dauer morphogenesis and longevity are not well understood.

The regulation of dauer entry in *C. elegans* shares similarities with the induction of autophagy in other eukaryotes. The environmental cues that promote dauer morphogenesis in *C. elegans* are also potent stimulators of autophagy in yeast and mammalian cells (*3*, *4*). Through autophagy, cells generate sufficient pools of amino acids to synthesize proteins that are essential for survival when the environmental food supply is limited. Components of the insulin-like signaling pathway that positively (e.g., *daf-18*) and negatively (e.g., *age-1* and *akt1/akt2*) regulate dauer entry in *C. elegans* may also regulate autophagy in mammalian cells (*5*, *6*). Furthermore, autophagy may be involved in life-span extension induced by dietary restriction in mammals (*7*), in delaying leaf senescence (*8*, *9*), and in developmental processes that require extensive cellular and tissue remodeling (*10*). We therefore postulated that autophagy is essential in both dauer development and life-span extension in *C. elegans*.

To test this, we used RNA interference (RNAi) (*11*) to inhibit autophagy genes in *C. elegans* that carry a loss-of-function mutation in *daf-2*, the *C. elegans* insulin-like tyrosine kinase receptor (*12*). *daf-2(e1370)* animals are temperature-sensitive *daf-c* class mutants (*13*), with inappropriate constitutive dauer entry at 25°C and extended adult life-span at lower temperatures (such as 15° to 22.5°C) (*12*, *14*) that allow reproductive growth. *C. elegans* contains candidate homologs of several yeast autophagy (*APG*) genes. We focused on *bec-1*, the *C. elegans* ortholog (T19E7.3) of yeast *APG6/VPS30*, because its mammalian counterpart, *beclin 1*, is important in autophagy, embryonic development, and tumor suppression (*15*, *16*). In both yeast and mammalian cells, Apg6/Vps30/Beclin 1 is part of a Class III phosphatidylinositol 3-kinase complex that is thought to be important in mediating localization of other Apg proteins to preautophagosomal structures (*17*, *18*).

C. elegans bec-1 encodes a predicted 375–amino acid, coiled-coil protein that shares 28% homology with yeast Apg6/Vps30p and 31% homology with human Beclin 1. To determine whether *bec-1* is a functional homolog of yeast *APG6/VPS30*, we tested its ability to restore autophagy and vacuolar protein sorting in *APG6/VPS30*-disrupted (Δ*apg6/vps30*) yeast. Using differential interference contrast microscopy, we found that Δ*apg6/vps30* yeast transformed with *C. elegans bec-1* or human *beclin 1*, but not with empty vector, showed an increase in nitrogen starvation–induced autophagy similar to that of Δ*apg6/vps30* yeast transformed with yeast *APG6/VPS30* (Fig. 1A). In contrast, Δ*apg6/vps30* yeast transformed with either *C. elegans bec-1* or human *beclin 1* were unable to properly sort and mature the vacuolar protein carboxypeptidase Y (CPY) (Fig. 1B, top). This defect was not due to a lack of stable *C. elegans* BEC-1 or human Beclin 1 protein expression in yeast (Fig. 1B, bottom) and could not be overcome by overexpression of the Apg6/Vps30p binding partner, Vps38p, that is part of the yeast vacuolar protein sorting complex (*18*) (Fig. 1B, middle). Thus, similar to human *beclin 1* (*15*), *C. elegans bec-1* complements the autophagy but not the vacuolar protein sorting function of *APG6/VPS30* in yeast. In addition, we examined the *C. elegans bec-1* expression pattern using a reporter gene in which the *bec-1* promoter was fused to green fluorescent protein (GFP) coding sequences

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(*19*). We found that the *bec-1* promoter is transcriptionally active in several different tissues (including the pharynx, intestine, hypodermis, nervous system, and reproductive organs) that undergo morphologic changes during dauer formation (*1*) (fig. S1).

To investigate the effects of inhibiting *bec-1* expression on the constitutive dauer phenotype of *daf-2(e1370)* mutants at 25°C, we used *bec-1* RNAi treatment that decreased *bec-1* mRNA levels but did not interfere with wild-type *C. elegans* development (*19*) (Fig. 2A). At 25°C, both untreated *daf-2(e1370)* (Fig, 2A) and GFP RNAi control–treated *daf-2(e1370)* formed normal dauers. These normal dauers are characterized by hyperpigmented granules in the intestine; radial constriction and elongation of the body and pharynx; the presence of a lateral, ridged, cuticular thread known as the dauer alae; increased fat storage as detected by Sudan black staining (Fig. 2B); resistance to sodium dodecyl sulfate (SDS) treatment; long-term survival at 25°C; and the ability to resume reproductive growth upon transfer to 15°C (*19*).

In contrast, at 25°C, the majority of *daf-2(e1370)*; *bec-1* (*RNAi*) animals formed abnormal dauers (Fig. 2A). Like the *daf-2(e1370)* dauers, these animals underwent a developmental arrest in the L3 gonadal stage and had some hyperpigmented granules in the intestine, some increased fat storage, and some radial constriction and elongation of the body and pharynx. However, the hyperpigmented granules were unevenly distributed and the magnitudes of fat storage, radial constriction, and elongation of the body and pharynx were less than that observed in untreated *daf-2(e1370)* animals (Fig. 2B). Furthermore, the *daf-2(e1370)*; *bec-1* (*RNAi*) animals failed to form dauer alae, were not resistant to SDS, died within a few days at 25°C, and usually did not resume reproductive growth on transfer to 15°C. These observations demonstrate that *bec-1* is not required for dauer initiation, but is required for normal dauer morphogenesis in *daf-2(e1370)* mutant animals (*20*). The death of these animals also suggests that autophagy genes may be critical for dauer survival, a finding that is consistent with the essential role of autophagy genes in survival during nutrient deprivation states in other eukaryotic organisms (*21*, *22*).

Next, we evaluated the effects of *bec-1* RNAi treatment on the life-span extension of *daf-2(e1370)* worms during reproductive growth at 15°C (Fig. 3). Consistent with previous studies (*12*, *14*), *daf-2(e1370)* animals had a significant life-span extension as compared to wild-type animals (median survival was 48 versus 28 days; $P < 0.001$, log-rank test). This life-span extension of *daf-2(e1370)* animals was reduced by *bec-1* RNAi treatment (median survival was 28 days; $P < 0.001$, log-rank test). Although *bec-1* RNAi had a slight inhibitory effect on the survival of wild-type animals (24 days for *bec-1* RNAi-treated animals versus 28 days for untreated animals), the effect of *bec-1* RNAi on shortening survival of the *daf-2(e1370)* mutants was significantly greater than its effects on shortening survival of wild-type animals. [Cox proportional-model hazard ratios were 5.4 for *bec-1* RNAi-treated *daf-2(e1370)* animals and 1.7 for *bec-1* RNAi-treated wild-type

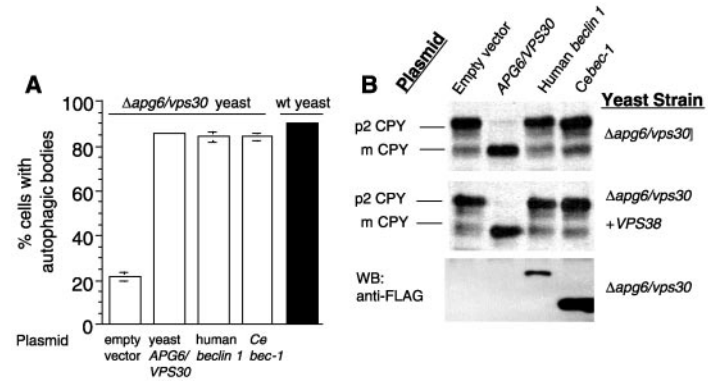

Fig. 1. *C. elegans* *bec-1* complements the autophagy but not the vacuolar protein sorting function of yeast *APG6/VPS30*. (**A**) Quantitation of autophagy in nitrogen-starved wild-type (wt) yeast and in Δ*apg6/vps30* yeast transformed with empty vector or with plasmids that encode either yeast Apg6/Vps30p, *C. elegans* (Ce) FLAG epitope–tagged BEC-1, or human FLAG epitope–tagged Beclin 1 (*19*). Results represent the mean (±SEM) percentage of cells with autophagic bodies within the vacuole for triplicate samples. Similar results were observed in three independent experiments. (**B**) Sorting of vacuolar protein CPY in (top) Δ*apg6/vps30* yeast or (middle) Δ*apg6/vps30* yeast with *VPS38* overexpression, transformed with empty vector or with plasmids that encode either yeast Apg6/Vps30p, *C. elegans* FLAG epitope–tagged BEC-1, or human FLAG epitope–tagged Beclin 1 (*19*). p2, precursor form; m, sorted mature form. Bottom: A Western blot (WB) analysis of yeast cell lysates with a polyclonal antibody to FLAG (*19*).

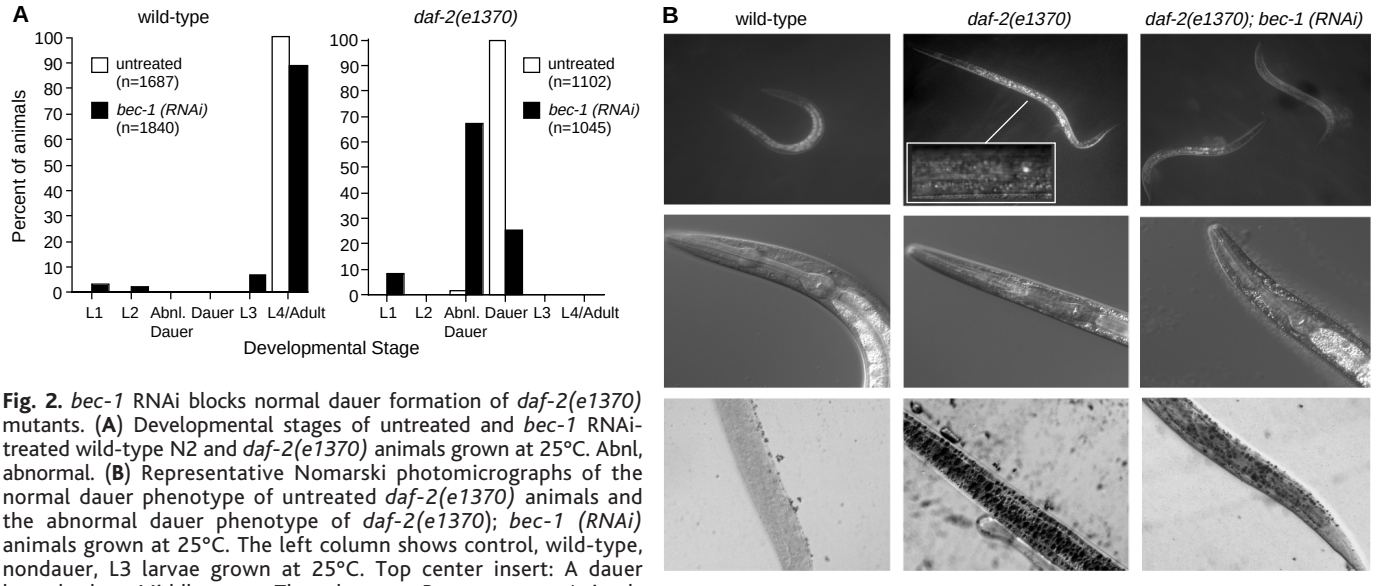

Fig. 2. *bec-1* RNAi blocks normal dauer formation of *daf-2(e1370)* mutants. (**A**) Developmental stages of untreated and *bec-1* RNAi-treated wild-type N2 and *daf-2(e1370)* animals grown at 25°C. Abnl, abnormal. (**B**) Representative Nomarski photomicrographs of the normal dauer phenotype of untreated *daf-2(e1370)* animals and the abnormal dauer phenotype of *daf-2(e1370)*; *bec-1 (RNAi)* animals grown at 25°C. The left column shows control, wild-type, nondauer, L3 larvae grown at 25°C. Top center insert: A dauer lateral alae. Middle row: The pharynx. Bottom row: Animals stained with Sudan black.

animals; $P < 0.001$]. These data indicate that *bec-1* is essential for the adult life-span extension of *daf-2(e1370)* nematodes.

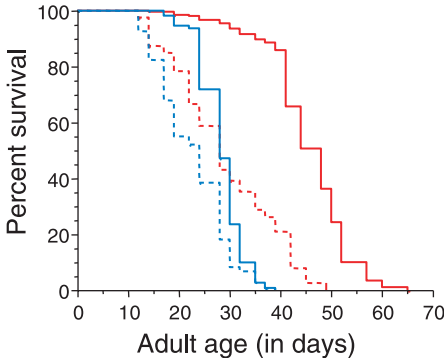

Fig. 3. *bec-1* is required for life-span extension in *daf-2(e1370)* mutants. A Kaplan-Meier survival curve shows the life-span of adult offspring of wild-type N2 (blue) and mutant *daf-2(e1370)* (red) untreated (solid line) or *bec-1* RNAi-injected (dotted line) worms maintained at 15°C (n = 81 to 273 animals per experimental group) (*19*).

The requirement for *bec-1* in *daf-2(e1370)* dauer formation and life-span extension suggested that autophagy may be involved in these phenotypes. To visualize autophagy in intact nematodes, we generated transgenic wild-type N2 and *daf-2(e1370)* animals that expressed a GFP-tagged version of the *C. elegans* gene product LGG-1 (*19*), which is an ortholog of yeast Apg8/Aut7p and mammalian MAP-LC3. During autophagy, Apg8/Aut7p and MAP-LC3 localize to preautophagosomal and autophagosomal membranes (*23–25*), and their punctate staining pattern (versus a diffuse pattern in the absence of autophagy) provides a useful marker of autophagy (*25–27*). Wild-type N2 and *daf-2(e1370)* animals carrying an extrachromosomal array that expressed GFP::LGG-1 had diffuse cytoplasmic GFP expression in multiple tissues in different stages of development, including the nervous system, pharynx, intestine, hypodermis, somatic gonad, and vulva (*28*). The tissue distribution of GFP::LGG-1 expression was similar to that observed for GFP regulated by the endogenous *bec-1* promoter.

During dauer formation, we observed a marked change in the subcellular localization pattern of GFP::LGG-1 in hypodermal seam cells, a cell type known to be important for certain dauer-associated morphological changes, including the formation of the dauer alae and the radial constriction of the body (*29–31*) (Fig. 4A). Therefore, to quantitate differences in autophagic activity, we counted the number of GFP-positive punctate areas per hypodermal seam cell in N2 and *daf-2(e1370)* L3 larvae grown at 15°C (during reproductive growth) and at 25°C [during dauer diapause for *daf-2(e1370)* but not N2 animals] (Fig. 4B). In N2 animals, similar numbers of GFP-positive punctate areas were observed during growth at 15°C and at 25°C. In contrast, there was a marked increase in the number of seam cell, GFP-positive, punctate areas in *daf-2(e1370)* animals during dauer formation at 25°C as compared to during reproductive growth at 15°C ($P < 0.001$, t test). This increase demonstrates that dauer morphogenesis is associated with autoph-

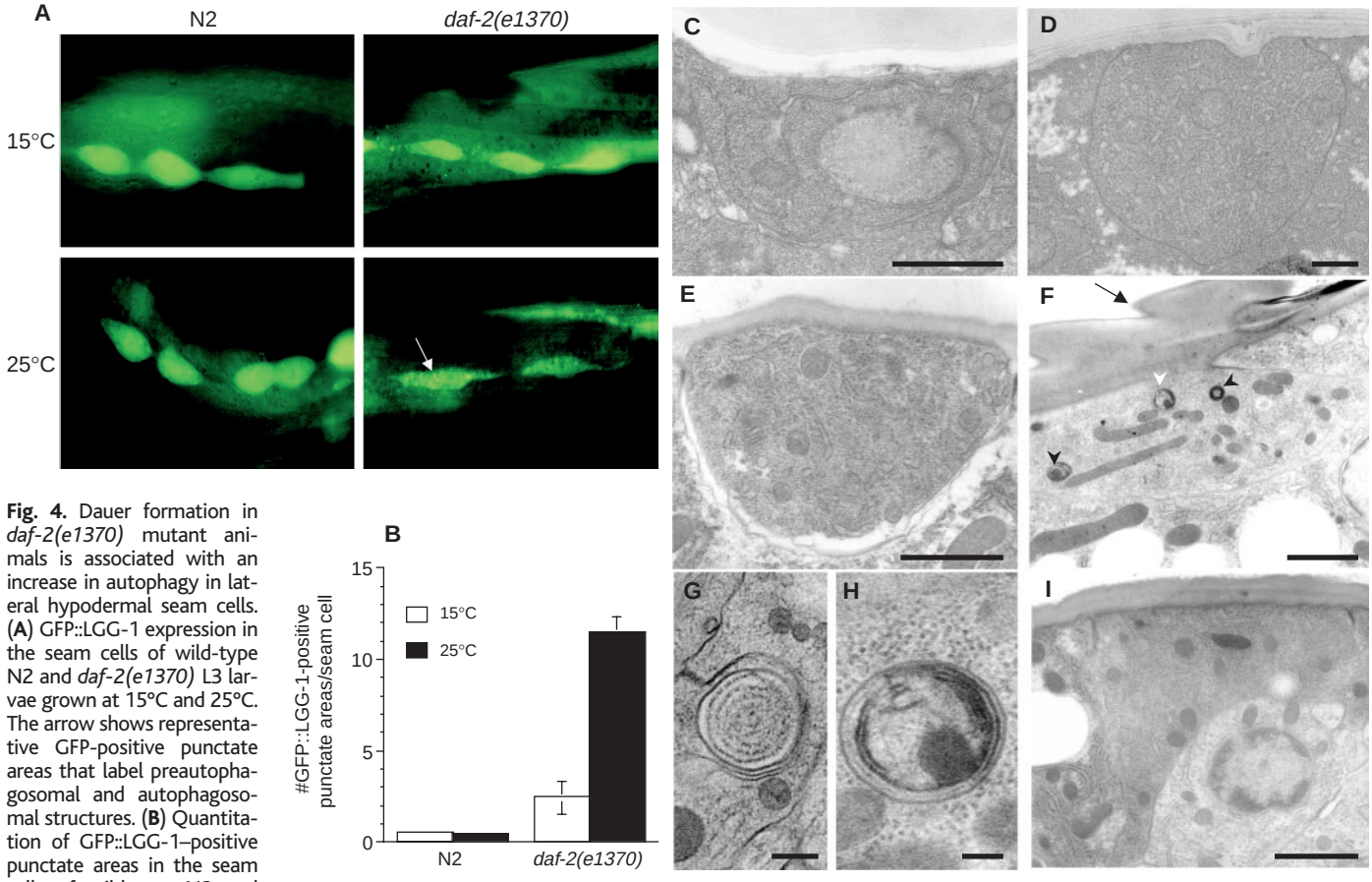

Fig. 4. Dauer formation in *daf-2(e1370)* mutant animals is associated with an increase in autophagy in lateral hypodermal seam cells. (**A**) GFP::LGG-1 expression in the seam cells of wild-type N2 and *daf-2(e1370)* L3 larvae grown at 15°C and 25°C. The arrow shows representative GFP-positive punctate areas that label preautophagosomal and autophagosomal structures. (**B**) Quantitation of GFP::LGG-1–positive punctate areas in the seam cells of wild-type N2 and *daf-2(e1370)* animals grown at 15°C and 25°C (mean + SEM for at least 50 seam cells from 10 different animals). (**C** to **I**) Representative electron micrographs of lateral hypodermal seam cells in a wild-type N2 control animal in (C) the L2 larval stage and (D) the L3 larval stage; a *daf-2(e1370)* animal grown for (E) 1 day at 25°C and [(F) to (H)] 2 days at 25°C; and (I) a *daf-2(e1370); bec-1 (RNAi)* animal grown for 2 days at 25°C. In (F), arrowheads denote representative vacuolar structures in the late stages of the autophagolysosomal pathway; the white arrowhead denotes structure that is shown at higher magnification in (H); the arrow denotes the dauer cuticle. (G) A representative early autophagosome and (H) a multilamellar body/autolysosome are shown in a seam cell of a *daf-2(e1370)* dauer animal. Scale bars, 1 μM [(C) to (F) and (I)]; 0.1 μM [(G) and (H)].

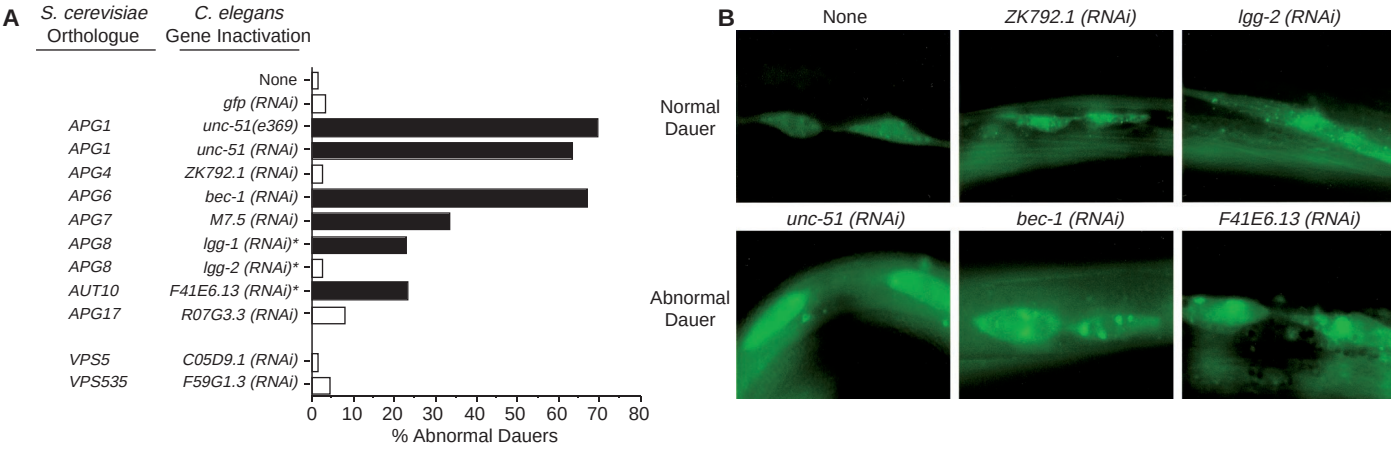

Fig. 5. The effect of *apg* and *vps* gene inactivation on dauer formation and autophagy in *daf-2(e1370)* mutant animals at 25°C. (**A**) The percentage of abnormal dauers observed with different *C. elegans* gene inactivations. Black bars correspond to gene inactivations that resulted in ≥20% abnormal dauers, and white bars correspond to gene inactivations that resulted in <20% abnormal dauers (n = 296 to 1840 animals per experimental group). Asterisks denote that undiluted RNAi is lethal in the N2 background and that data are shown for a 1:1 dilution of RNAi that is not lethal in the N2 background [see (*19*)]. (**B**) Representative photomicrographs of GFP::LGG-1 staining in seam cells from *daf-2(e1370)* animals treated with RNAi as indicated.

agy induction in hypodermal seam cells. In addition, *daf-2(e1370)* mutant animals grown at 15°C had significantly more seam cell GFP-positive punctate areas than wild-type N2 animals grown at 15°C ($P < 0.001$, t test). This latter observation demonstrates that the wild-type *daf-2* gene product also functions to suppress autophagy during reproductive growth and that the insulin-like signaling pathway negatively regulates autophagy in *C. elegans*.

We performed electron microscopy to further study the process of seam cell autophagy during dauer development (Fig. 4, C to I). In wild-type L2 and L3 animals, we observed rare early autophagosomes, but no evidence of later structures in the autophagy pathway (Fig. 4, C and D). In *daf-2(e1370)* "pre-dauer" animals grown at 25°C for 1 day, we saw an increase in the number of multivesicular bodies (a structure of endosomal origin), but very few autophagosomes or autolysosomes (Fig. 4E). In contrast, the seam cells from *daf-2(e1370)* animals that entered dauer diapause after 2 days of growth at 25°C displayed marked ultrastructural differences (Fig. 4F) from those in the pre-dauer *daf-2(e1370)* or wild-type L2 or L3 animals. Although some early autophagosomes were detected (Fig. 4G), the most notable finding was the accumulation of vacuolar structures that appear similar to those described in the late stages of autophagy in mammalian cells (Fig. 4, F and H). Furthermore, seam cells from the *daf-2(e1370); bec-1 (RNAi)* abnormal dauer animals completely lacked any early or late autophagic structures (Fig. 4I). Together with the immunofluorescent studies of GFP::LGG-1 localization in *daf-2(e1370)* animals, these data indicate that there is active autophagy in hypodermal seam cells during dauer development that is blocked by inactivation of the *bec-1* autophagy gene.

To further evaluate whether autophagy is required for *daf-2(e1370)* dauer formation, we tested the effects of RNAi of other *C. elegans* orthologs of yeast autophagy genes on *daf-2(e1370)* dauer formation (Fig. 5). In addition, because the yeast ortholog, *APG6/VPS30*, of *bec-1* is involved in both vacuolar protein sorting and autophagy, we examined the RNAi phenotypes of two VPS genes, *VPS5* and *VPS35*, that have no documented role in autophagy. RNAi-mediated interference of the *C. elegans* orthologs of yeast *APG4*, *APG17*, and the *C. elegans lgg-2* ortholog of yeast *APG8*, as well as of the *C. elegans* orthologs of yeast *VPS5* and *VPS35*, had minimal effects on dauer morphogenesis in *daf-2(e1370)* mutant animals (Fig. 5A) and did not alter the autophagosomal punctate staining pattern in the seam cells of GFP::LGG-1 dauer animals (Fig. 5B). In contrast, RNAi inactivation of the *C. elegans* orthologs of yeast *APG1* (*unc-51*), yeast *APG7*, yeast *AUT10*, and the *C. elegans lgg-1* ortholog of yeast *APG8/AUT7*, as well as a loss-of-function mutation in *unc-51* [*unc-51(e369)*] (*32*), resulted in the formation of abnormal *daf-2(e1370)* dauers (Fig. 5A). These abnormal dauers displayed characteristics similar to those described for *daf-2(e1370); bec-1 (RNAi)* animals and also had aberrant localization of GFP::LGG-1 (Fig. 5B) (*33*). Thus, several *C. elegans* orthologs of yeast and mammalian *APG* genes, including *APG1*, *APG6/VPS30/beclin1*, *APG7*, *APG8*, and *AUT10*, are required for normal dauer morphogenesis in *C. elegans*.

Our findings demonstrate that autophagy genes are required for normal dauer morphogenesis and life-span extension in *C. elegans*. In the unicellular organism *Saccharomyces cerevisiae*, autophagy is essential for survival during starvation and for sporulation and differentiation (*21*). We propose that autophagy is also essential for the cellular and tissue remodeling that permits multicellular organisms to successfully adapt to environmental stress. Furthermore, our results suggest that autophagy functions in multicellular organisms not only in development and in stress responses but also in cellular processes that regulate life-span during nonstressed conditions. Thus, the degradation of cellular contents by autophagy may underlie, in part, the diverse metabolic, developmental, and life-span processes controlled by the insulin-like signaling pathway in nematodes and, possibly, in higher eukaryotic organisms.

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Dishevelled 2 Recruits β-Arrestin 2 to Mediate Wnt5A-Stimulated Endocytosis of Frizzled 4

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Wnt proteins, regulators of development in many organisms, bind to seven transmembrane–spanning (7TMS) receptors called frizzleds, thereby recruiting the cytoplasmic molecule dishevelled (Dvl) to the plasma membrane. Frizzled-mediated endocytosis of Wg (a *Drosophila* Wnt protein) and lysosomal degradation may regulate the formation of morphogen gradients. Endocytosis of Frizzled 4 (Fz4) in human embryonic kidney 293 cells was dependent on added Wnt5A protein and was accomplished by the multifunctional adaptor protein β-arrestin 2 (βarr2), which was recruited to Fz4 by binding to phosphorylated Dvl2. These findings provide a previously unrecognized mechanism for receptor recruitment of β-arrestin and demonstrate that Dvl plays an important role in the endocytosis of frizzled, as well as in promoting signaling.

The secreted glycoprotein signaling molecules of the Wnt family (Wingless or Wg in *Drosophila*) are conserved in evolution and play important developmental roles (*1*). Wnt activity is mediated by interaction with the frizzled 7TMS receptors, which signal by stabilizing β-catenin, thereby enhancing the activity of LEF/Tcf transcription factors. The most proximal signaling intermediate in this pathway is a cytoplasmic molecule, Dvl, which is recruited to the plasma membrane by Fz (*2*, *3*). In *Drosophila*, Wg is endocytosed in a frizzled-dependent manner, and spatially restricted differences in the rates of Wg endocytosis and lysosomal degradation correlate with the formation of Wg morphogen gradients (*4*).

β-arrestins are ubiquitous multifunctional adaptor proteins that universally regulate numerous aspects of 7TMS receptor function (*5*). When receptors are activated, they are rapidly phosphorylated by G protein–coupled receptor kinases (GRKs) (*6*), and then bind β-arrestin 1 or 2. β-arrestins desensitize second-messenger generation by sterically blocking receptor–G protein interaction (*5*); mediate endocytosis of the receptors in clathrin-coated pits by binding clathrin, AP-2, and other elements of the endocytic machinery (*7*, *8*); and serve as scaffolds that link the receptors to other signaling pathways (*9*–*11*). Here we have investigated the mechanisms responsible for Fz internalization and the possible involvement of β-arrestins in this process.

To visualize Fz4 receptors in live cells, we constructed a Fz4–green fluorescent protein (GFP) molecule and expressed it in human embryonic kidney 293 (HEK293) cells (*12*). It was present predominantly at the cell surface (Fig. 1A) with a few green puncta also observed in the cytosol, possibly due to the presence of partially processed Fz4-GFP. When cells were stimulated for 30 min with Wnt5A conditioned medium (Wnt5A), which activates signaling through Fz4 (*13*), no internalization of Fz4-GFP was observed (Fig. 1B). Similarly, activation of protein kinase C (PKC) with 1 μM phorbol myristoyl acetate (PMA) for 30 min resulted in no apparent receptor internalization (Fig. 1C). However, when cells were stimulated concurrently with both Wnt5A and an activator of PKC such as PMA (Fig. 1D) or substance P, which activates receptors coupled to the G protein G_q (Fig. 1E), Fz4-GFP was internalized and the receptor was present in intracellular vesicles.

The prototypic 7TMS receptor, the β_2-adrenergic receptor (β_2-AR), is internalized by a classic clathrin-coated pit mechanism that uses β-arrestin as an adaptor to link to various elements of the endocytic machinery (*14*). To compare the mechanisms of Fz4 and β_2-AR internalization, we expressed Fz4-GFP and β_2-AR–red fluorescent protein (*15*) in the same HEK293 cells and stimulated them for 30 min with, simultaneously, Wnt5A (in the presence of PMA) and isoproterenol. Both receptors were internalized and appeared in the same population of intracellular vesicles (fig. S1). Moreover, treatment of Fz4-GFP–expressing cells with the clathrin inhibitors sucrose (0.45 M) or monodansylcadaverine (200 μM) inhibited Wnt5A- and PMA-induced internalization of Fz4 (*16*). Taken together, these data suggest that Fz4 internalization is mediated by a clathrin-mediated process similar to that used for internalization of the β_2-AR.

Because the β_2-AR and numerous other 7TMS receptors use β-arrrestins as essential adaptors in clathrin-mediated endocytosis (*14*), we tested whether β-arrestins were involved in Fz4 internalization. We used small interfering RNA (siRNA) to reduce the expression of βarr2 in HEK293

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Autophagy Genes Are Essential for Dauer Development and Life-Span Extension in *C. elegans*

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