Genome-wide screen identifies signaling pathways that regulate autophagy during *Caenorhabditis elegans* development

Bin Guo¹²³†, Xinxin Huang³†, Peipei Zhang², Linxiang Qi², Qianqian Liang², Xuebo Zhang³, Jie Huang², Bin Fang², Wenru Hou², Jinghua Han² & Hong Zhang²* 

Abstract
The mechanisms that coordinate the regulation of autophagy with developmental signaling during multicellular organism development remain largely unknown. Here, we show that impaired function of ribosomal protein RPL-43 causes an accumulation of SQST-1 aggregates in the larval intestine, which are removed upon autophagy induction. Using this model to screen for autophagy regulators, we identify 139 genes that promote autophagy activity upon inactivation. Various signaling pathways, including Smad/Mab TGF-β signaling, lin-35/Rb signaling, the XBP-1-mediated ER stress response, and the ATFS-1-mediated mitochondrial stress response, regulate the expression of autophagy genes independently of the TFEB homolog HLH-30. Our study thus provides a framework for understanding the role of signaling pathways in regulating autophagy under physiological conditions.

Keywords autophagy; *Caenorhabditis elegans*; ER stress; mitochondrial stress; Rb

Introduction
Autophagy is an evolutionarily conserved lysosome-mediated degradation process. It involves the formation of a cup-shaped membrane sac, known as the isolation membrane, which expands and seals to form an enclosed double-membrane autophagosome. In higher eukaryotes, autophagosomes mature by fusing with endosomes before fusing with lysosomes to form degradative autolysosomes [1–3]. Distinct steps of autophagosome formation require different sets of genes [2,4]. The Atg1/Atg13 kinase complex and the Vps34/Atg6 class III PI(3)K complex are required for the induction and nucleation of isolation membranes. Expansion and closure of the autophagosome is regulated by the Atg8-phosphatidylethanolamine (PE) and Atg12-Atg5 ubiquitin-like conjugation systems [2]. Progression of autophagosomes into autolysosomes requires components of the endocytic pathways and the SNARE complex [3,5].

Autophagy is activated by a variety of stress conditions, including nutrient starvation, energy deprivation, and reactive oxygen species [6]. The mammalian target of rapamycin serine/threonine kinase complex 1 (mTORC1) and the Vps34 PI(3)K complex are major nodes for integrating various signaling pathways with autophagy regulation [7–9]. Transcriptional regulation of autophagy genes confers another layer of regulation. The master positive regulator TFEB and the master repressor ZKSCAN3 transcriptionally regulate a network of genes involved in autophagosome and lysosome biogenesis [10,11]. The forkhead transcription factor FoxO also activates the transcription of multiple autophagy genes and promotes autophagy activity [6]. Although numerous factors have been uncovered that regulate autophagy activity, the mechanisms that integrate developmental signals into the autophagic machinery during multicellular organism development are poorly understood.

Here, we established *Caenorhabditis elegans* as a genetic model to study autophagy regulation and identified 139 genes which, when inactivated, enhance autophagy activity. We showed that autophagy activity is activated by various stress-induced conditions and is tightly controlled by various developmental signaling pathways.

Results and Discussion
Loss of *rpl-43* activity results in accumulation of SQST-1 aggregates in the larval intestine
The *C. elegans* p62 homolog SQST-1 is removed by autophagy during development. In wild-type animals, SQST-1::GFP is weakly and diffusely expressed in the cytoplasm from embryonic to adult...
In autophagy mutants, numerous SQST-1::GFP aggregates accumulate in almost all cells during embryogenesis and in multiple larval tissues, including hypodermis, intestine, and neurons (Supplementary Fig S1C and E–L). Intestinal SQST-1 aggregates, which are spherical and dispersed in the cytoplasm, accumulate throughout larval
Starvation or inactivation of Tor signaling resulted in the degradation of SQST-1::GFP (Fig 1C–E). Unlike autophagy mutants, bp399 mutants showed the accumulation of SQST-1 aggregates strictly in the intestine in a distinct temporal pattern. SQST-1::GFP aggregates were absent in bp399 embryos, but started to form in L1 larvae and increased in number and size throughout larval development (Fig 1C and D; Supplementary Fig S1D and M–T). SQST-1::GFP aggregates in bp399 were heterogeneous in size and some were much larger than those in autophagy mutants (Supplementary Fig S1E–T). Endogenous SQST-1 also accumulated in the intestine in bp399 mutants (Supplementary Fig S1U–X). SQST-1::GFP aggregates in bp399 mutants were co-stained by anti-ubiquitin, suggesting the accumulation of ubiquitinated proteins (Supplementary Fig S1Y–B2).

A transgene containing the single gene rpl-43 rescued the SQST-1::GFP accumulation phenotype in bp399 mutants (Fig 1F). The glycine at position 15 of RPL-43 was mutated to arginine in bp399 mutants (Fig 1G). rpl-43 encodes the 60S ribosomal protein L37. RNAi inactivation of other ribosomal subunits, ribosomal maturation factors, and translation initiation factors resulted in the same phenotype as rpl-43(bp399) (Supplementary Table S1; Supplementary Fig S1C2–T). This suggests that impaired protein synthesis causes the accumulation of SQST-1 aggregates in the larval intestine.

Autophagic degradation of other autophagy substrates was unaffected in rpl-43 mutants (Supplementary Fig S2A–D) [13]. Levels of the C. elegans Atg8 homolog LGG-1, which has been widely used to measure autophagy activity, were unaltered in rpl-43 mutants and no intestinal LGG-1 puncta were detected (Supplementary Fig S2Q–T). Thus, rpl-43 is not an essential component of the autophagy pathway.

**SQST-1 aggregates in rpl-43 mutants are degraded by elevated autophagy activity**

Starvation or inactivation of Tor signaling resulted in the degradation of SQST-1 aggregates in rpl-43 mutants, but not in autophagy mutants (Fig 1E, J, and K; Supplementary Fig S3A–N). Intestinal SQST-1 aggregates persisted in rpl-43; atg-3 mutants after starvation or let-363/mTOR inactivation (Supplementary Fig S3O and P). We then followed the dynamic distribution of SQST-1::GFP aggregates and lysosomes. SQST-1::GFP aggregates and LAAT-1::cherry-labeled lysosomes were separable in rpl-43 mutants (Fig 1H). Upon starvation, SQST-1::GFP aggregates in rpl-43 mutants fused and became encircled by lysosomes (Fig 1I). Thus, the SQST-1 aggregates in rpl-43 mutants are degraded upon autophagy induction.

**Genome-wide RNAi screen for gene inactivation elevating autophagy activity**

We screened a C. elegans RNAi feeding library targeting 16,749 genes (~87% of C. elegans ORFs) to identify RNAi clones that significantly suppressed the SQST-1::GFP aggregate accumulation phenotype in rpl-43 mutants (Fig 1L–R). The RNAi clones that reproducibly suppressed rpl-43 were subjected to several additional screens. First, a GFP reporter driven by the sqst-1 promoter was screened to exclude genes that transcriptionally repress sqst-1 (Supplementary Fig S3Q–T). Second, to exclude RNAi inactivations that cause disappearance of SQST-1 aggregates by mechanisms independent of autophagy induction, we screened for RNAi clones that did not affect the accumulation of intestinal SQST-1 aggregates in atg-9 mutants. Third, we identified gene inactivations that promoted the degradation of another autophagy substrate W07G4.5::GFP in the intestine (Supplementary Fig S3U–X) [14]. After these screens, 139 genes were identified as suppressors of rpl-43 (Table 1; Supplementary Table S2).

Based on annotated biological function, the 139 genes were enriched for signal transduction, protein turnover, intracellular trafficking, and cellular metabolism processes (Fig 1S). According to gene ontology, 111 of the genes have clear human homologs.

We identified genes encoding proteins involved in the ubiquitin-proteasome degradation system (UPS), including components of the proteasome and the Cdc48/Ufd1/Npl4 complex involved in ER-associated degradation (ERAD), and also genes encoding transcription factors, metabolic enzymes, and transporters (Table 1; Fig 1L, M, and R). Genes encoding factors involved in intracellular trafficking included the central and lobe A subunits of the conserved oligomeric Golgi (COG) complex, clathrin adaptor protein complex AP-2, and components of the endosomal sorting ESCRT complex (Fig 1N–Q). Our screen identified genes involved in developmental signaling pathways, including TGF-β signaling (sma-3, sma-4), Hippo signaling (orts-1), PKC1-MPK1 signaling (gls-1), Notch signaling (gfp-1), and the ERK MAPK pathway (mek-2).

Genome-wide functional interaction maps have been created for C. elegans based on gene expression, physical and genetic interactions, and functional annotation [15,16]. Among the 139 genes, 72 genes (nodes) formed at least one interaction (edge) with other genes, and a total of 103 edges were identified (Supplementary Fig S3Y). As a control, we randomly selected four groups of 139 genes from the same library, which showed 3, 0, 4, and 5 interactions, respectively.

**Sma TGF-β signaling regulates autophagy**

The Sma/Mab and Dauer TGF-β signaling pathways in C. elegans, consisting of distinct components, regulate different developmental processes (Supplementary Fig S4A) [17]. In addition to sma-3 and sma-4 (Smads) identified in our screen (Fig 2A–D), knockdown of other components in the Sma TGF-β pathway, including dbl-1 (ligand), sma-6 (type I receptor), sma-2 (Smad), and sma-9 (cofactor), also suppressed SQST-1 aggregates in rpl-43 mutants (Supplementary Fig S4B). Inactivation of Sma/Mab TGF-β signaling suppresses the rpl-43 mutant phenotype independent of its role in regulating body size (Supplementary Fig S4B–D). Loss of function of components in the Dauer TGF-β signaling pathway, includingdaf-7 (ligand), daf-1 (type I receptor), daf-8 (Smad), and daf-3 (Smad), did not suppress SQST-1 aggregates in rpl-43 mutants (Supplementary Fig S4B, E and F).

Expression of endogenous LGG-1 and GFP::LGG-1 was greatly increased and a large number of GFP::LGG-1 puncta formed in dbl-1 and sma-3 mutants compared to wild-type animals (Fig 2E; Supplementary Fig S4G and H). mRNA levels of lgg-1 and other autophagy genes, includingbec-1, epg-8, and atg-7, were increased in dbl-1, sma-2, and sma-3 mutants (Fig 2F), indicating that Sma TGF-β signaling regulates autophagy at least partially through transcriptional control of autophagy genes.
Table 1. List of some RNAi inactivations that lead to suppression of the accumulation of SQST-1::GFP aggregates in the intestine in rpl-43 mutants

<table>
<thead>
<tr>
<th>Process</th>
<th>Sequence name</th>
<th>Gene name</th>
<th>Brief description of gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signaling</td>
<td>F54C8.5</td>
<td>rbe-1</td>
<td>Orthologous to the mammalian Rheb and Rheb1 GTPases</td>
</tr>
<tr>
<td>Signaling</td>
<td>R13F6.9</td>
<td>smo-3</td>
<td>A Smad protein</td>
</tr>
<tr>
<td>Signaling</td>
<td>R12B2.1</td>
<td>smo-4</td>
<td>A Smad protein</td>
</tr>
<tr>
<td>Signaling</td>
<td>F02A9.6</td>
<td>gip-1</td>
<td>N-glycosylated transmembrane protein</td>
</tr>
<tr>
<td>Signaling</td>
<td>T20F10.1</td>
<td>uts-1</td>
<td>Warts/lats-like serine threonine kinase</td>
</tr>
<tr>
<td>Signaling</td>
<td>F42G8.8</td>
<td>gis-1</td>
<td>Transducer of the stress-activated PKC1-MPK1 signaling pathway</td>
</tr>
<tr>
<td>Signaling</td>
<td>Y54E10BL6</td>
<td>mek-2</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>Protein turnover</td>
<td>F23F12.6</td>
<td>rpl-3</td>
<td>An AAA ATPase subunit of the 19S regulatory particle of the 26S proteasome</td>
</tr>
<tr>
<td>Protein turnover</td>
<td>F19B6.2</td>
<td>ufd-1</td>
<td>Protein involved in the recognition of polyubiquitinated proteins</td>
</tr>
<tr>
<td>Protein turnover</td>
<td>F59E12.5</td>
<td>npl-4.2</td>
<td>Ubiquitin-binding protein involved in protein degradation</td>
</tr>
<tr>
<td>Protein turnover</td>
<td>C06A1.1</td>
<td>cdc-48.1</td>
<td>An AAA ATPase homologous to yeast Cdc48 and mammalian p97/VCP</td>
</tr>
<tr>
<td>Transcription</td>
<td>F47D12.4</td>
<td>hmg-12</td>
<td>HMG box-containing protein</td>
</tr>
<tr>
<td>Transcription</td>
<td>F31E3.1</td>
<td>ceh-20</td>
<td>Homeobox family member</td>
</tr>
<tr>
<td>Transcription</td>
<td>C01B7.1</td>
<td>ztf-12</td>
<td>Zinc finger transcription factor family</td>
</tr>
<tr>
<td>Transcription</td>
<td>R06C7.7</td>
<td>lin-61</td>
<td>Polycomb group protein SCM/L3/M8T</td>
</tr>
<tr>
<td>Transcription</td>
<td>C32F10.2</td>
<td>lin-35</td>
<td>The Caenorhabditis elegans retinoblastoma protein (Rb) ortholog</td>
</tr>
<tr>
<td>Transcription</td>
<td>JCB6.6</td>
<td>lin-54</td>
<td>Metallothionein-like protein</td>
</tr>
<tr>
<td>Transcription</td>
<td>F52C12.5</td>
<td>elt-6</td>
<td>GATA-4/5/6 transcription factor</td>
</tr>
<tr>
<td>Transcription</td>
<td>F44C4.2</td>
<td>nhr-37</td>
<td>Nuclear hormone receptor</td>
</tr>
<tr>
<td>Transcription</td>
<td>D2021.1</td>
<td>utx-1</td>
<td>General transcriptional co-repressor</td>
</tr>
<tr>
<td>Transcription</td>
<td>C33D3.1</td>
<td>elt-2</td>
<td>GATA-type transcription factor</td>
</tr>
<tr>
<td>Protein trafficking</td>
<td>C07G1.5</td>
<td>hgr-1</td>
<td>Membrane trafficking and cell signaling protein HRS</td>
</tr>
<tr>
<td>Protein trafficking</td>
<td>ZK652.2</td>
<td>tomm-7</td>
<td>Translocase of outer mitochondrial membrane complex</td>
</tr>
<tr>
<td>Protein trafficking</td>
<td>E04A4.5</td>
<td></td>
<td>Mitochondrial import inner membrane translocase</td>
</tr>
<tr>
<td>Protein trafficking</td>
<td>F35H10.4</td>
<td>uha-5</td>
<td>Subunit a of the vacuolar H + -ATPase V0 sector</td>
</tr>
<tr>
<td>Protein trafficking</td>
<td>CD4.4</td>
<td>ups-37</td>
<td>A member of the endosomal sorting ESCRT-I complex</td>
</tr>
<tr>
<td>Metabolism</td>
<td>F54D8.3</td>
<td>alh-1</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>Metabolism</td>
<td>C28H8.11</td>
<td></td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>Metabolism</td>
<td>F37E3.1</td>
<td>ncbp-1</td>
<td>Nuclear cap-binding protein complex</td>
</tr>
<tr>
<td>Metabolism</td>
<td>K04E7.2</td>
<td>pep-2</td>
<td>A low-affinity/high-capacity oligopeptide transporter</td>
</tr>
</tbody>
</table>

The LIN-35/Rb pathway regulates autophagy

Our screen identified several class B synthetic multivulva (SynMuvB) genes, including lin-35 (the Rb homolog), lin-54, and lin-61 (Fig 2C, D, G, and H; Supplementary Fig S5A–D). Endogenous SQST-1 aggregates were absent in rpl-43; lin-35 mutants (Supplementary Fig S5E and F). SynMuvB genes function redundantly with class A or class C SynMuv genes to antagonize specification of the vulva cell lineage [18]. RNAi knockdown of other SynMuvB genes, including lin-15B and lin-36, components of a Rb-containing DRM complex (dpl-1, lin-9, lin-37, lin-53), a SynMuvB heterochromatin complex (hpl-2, lin-13), and a SUMO-recruited Mec complex (mep-1 and let-418), all suppressed the accumulation of SQST-1::GFP aggregates in rpl-43 mutants (Supplementary Fig S5G). However, RNAi inactivation of class A (lin-15A, lin-8, and lin-56) and class C (trr-1 and mys-1) SynMuv genes had no effect (Fig 2J and J; Supplementary Fig S5G). Knockdown of genes essential for SynMuvB function in vulval development, RNAi, and soma-germ fate determination did not cause reappearance of SQST-1 aggregates in lin-35; rpl-43 mutants (Supplementary Fig S5H–J). Thus, SynMuvB genes regulate autophagy activity independent of their role in other processes.

The number of GFP::LGG-1 puncta and levels of endogenous LGG-1 were dramatically elevated in lin-35, lin-15B, and lin-9 mutants (Fig 2K; Supplementary Fig S5N and O). Levels of bec-1, epg-8, lgg-1, and atg-7 mRNAs were increased in lin-35, lin-15B, lin-9, and mep-1 mutants (Fig 2L and data not shown). Thus, the lin-35/ Rb pathway transcriptionally regulates autophagy genes.

daf-2 insulin/IGF signaling regulates autophagy activity

Reduction of daf-2 signaling elevates autophagy activity [19]. Accumulation of SQST-1 aggregates in rpl-43 mutants was
suppressed by the loss of function of *daf-2* and *pdk-1* (Fig 2C, D, M, and N; Supplementary Fig S6A and B). Reduced *daf-2* activity results in nuclear translocation of the forkhead transcription factor DAF-16, which further activates the expression of downstream target genes. SQST-1 aggregates were partially restored in *daf-16; rpl-43; daf-2* mutants (Fig 2C, D, O, and P), indicating that *daf-2* regulates autophagy activity partially through DAF-16. *daf-16* is not required for suppression of *rpl-43* by inactivation of *let-363*, *sma-3*, and *lin-35* (Supplementary Fig S6C–H).
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Published online: April 24, 2014

EMBO reports

**daf-2** mutants did not show elevated LGG-1 levels by immunoblotting (Supplementary Fig S6I). mRNA levels of lgg-1, but not of other autophagy genes, were slightly increased in daf-2 mutants (Supplementary Fig S6J). Uregulation of lgg-1 was suppressed by the loss of daf-16 activity (Supplementary Fig S6J). DAF-16 negatively regulates daf-15 transcription [20]. Knockdown of mTOR or raptor did not increase mRNA levels of autophagy genes (Supplementary Fig S6K). These results suggest that daf-2 may regulate autophagy by converging on DAF-15, as in the regulation of dauer formation and fat accumulation [20].

**ER stress activates autophagy activity**

Accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER) triggers the ER unfolded protein response (UPR) to ameliorate the stress [21]. 

bp399 did not cause evident ER stress, as shown by very low expression of the ER stress marker *P*hs4::GFP (Fig 3A and B; Supplementary Fig S7A and B). Treatment of rpl-43 mutants with tunicamycin and DTT induced ER stress and strongly suppressed the accumulation of SQST-1 aggregates (Fig 3C; Supplementary Fig S7E-H). *P*hs4::GFP expression was also greatly increased by RNAi inactivation of 24 genes identified in our screen, including components of the proteasome and the ERAD complex (Supplementary Table S3; Fig 3D and E; Supplementary Fig S7I and J). Among the 24 ER stress-inducing genes, 10 are functionally connected and share 19 interactions (edges) in total (Fig 3L).

In *C. elegans*, IRE-1-XBP-1, the PERK kinase homolog PEK-1, and activating transcription factor-6 (ATF-6) sense ER stress and activate the UPR. To determine whether UPR activation plays a causative role in autophagy induction, we examined the role of *xbp-1*, *pek-1*, and *atf-6* in suppression of the rpl-43 phenotype by the 24 identified genes. Loss of activity of *ire-1*, *xbp-1*, *pek-1*, or *atf-6* had no evident effect on the expression of *P*hs4::GFP or the number of SQST-1 aggregates in rpl-43 mutants (Supplementary Fig S7C, D, M, and N).

**Figure 3.** XBP-1-mediated ER stress suppresses the rpl-43 phenotype.

A, B *P*hs4::GFP is very weakly expressed in wild-type animals. C Percentage of indicated animals with different levels of SQST-1::GFP aggregates. S: strong. M: medium. N: none. >30 animals were examined for each genotype. D, E *P*hs4::GFP expression is much higher in rpt-3(RNAi) animals. F, G Uregulation of *P*hs4::GFP expression in rpt-3(RNAi) animals is suppressed by the *xbp-1*(pc12) mutation. H, I SQST-1::GFP aggregates are absent in rpl-43(bp399); rpt-3(RNAi) animals. J, K Loss of function of *xbp-1* restores SQST-1::GFP aggregates in rpl-43(bp399); rpt-3(RNAi) animals. L Functional interaction of ER stress-induced genes. M The increase in bec-1, epg-8, lgg-1, and atg-7 mRNA levels in rpl-43, rpt-3, and pep-2 RNAi animals is dependent on *xbp-1*. *P* < 0.05, **P** < 0.01. 500 young adult animals were collected for analysis. Error bars indicate s.d. from three experiments.

Data information: (A), (D), (F), (H), and (J): DIC images of the animals in (B), (E), (G), (I), and (K), respectively. Scale bars: 100 μm (A, B, D–G), 20 μm (H–K).
Simultaneous inactivation of xbp-1 blocked upregulation of Phsp-40::GFP in ER stress-induced mutant animals (Fig 3F and G; Supplementary Fig S7K and L). In rpl-43; xbp-1 double mutants, SQST-1 aggregate persisted upon DTT treatment or knockdown of identified ER stress-induced genes except unc-89 (Fig 3H–K; Supplementary Fig S7O–R). SQST-1::GFP aggregates were restored in unc-89; rpl-43 mutants by atf-6 inactivation (Supplementary Fig S7S–V). Inactivation of pek-1 or atf-6 did not affect other identified ER stress-induced genes. Thus, XBP-1 mediates the induction of autophagy by ER stress. SQST-1 aggregates in rpl-43; xbp-1 mutants were still suppressed by the inactivation of TOR signaling, daf-2, sma-3, and lin-35 (Supplementary Fig S7W–Z), indicating that impaired ER stress did not block autophagy induction by other signaling pathways.

We determined the expression of autophagy genes in ER stress-induced mutants. GFP::LGG-1 forms a large number of puncta in the intestine after knockdown of ufd-1, rpl-4.2, and pep-2 (Supplementary Fig S7A and B). Levels of bec-1, epg-8, lgg-1, and atg-7 mRNA were increased in rpl-4.1-, rpl-3-, and pep-2-knockdown animals (Fig 3M). Simultaneously depleting xbp-1 abolished the upregulation of autophagy genes in these same mutants (Fig 3M).

Mitochondrial stress upregulates autophagy activity

Perturbation of the protein-folding environment in the mitochondrial matrix activates the mitochondrial unfolded protein response (UPRmt) to re-establish protein homeostasis [22]. Inactivation of sqg-7 (not included in the RNAi feeding library, encoding C. elegans paraplegin, induced mitochondrial stress and suppressed SQST-1::GFP aggregates in rpl-43 mutants (Supplementary Fig S8A, B, J and K). RNAi inactivation of 6 genes identified in our screen, namely egl-46, gfi-1, vha-15, tomm-7, tomm-22, and ED4A4.5, increased the expression of the mitochondrial stress marker Phsp-60::GFP (Fig 4A and B; Supplementary Fig S8C and D).

The bZip transcription factor ATFS-1 is essential for UPRmt activation in C. elegans [22]. atfs-1(RNAi) suppressed the elevated expression of Phsp-60::GFP in sqg-7 and 6 other identified mutants and also reduced the suppression effect of SQST-1 aggregates in rpl-43 mutants (Fig 4C–H; Supplementary Fig S8E–P). atfs-1 is not required for the suppression effect caused by the loss of activity of sma-3, lin-35, and daf-2 (Supplementary Fig S8Q–V). Inactivation of daf-16 and xbp-1 had no effect on mitochondrial stress-induced autophagy (Supplementary Fig S8W–Z). LGG-1 levels and LGG-1::GFP puncta accumulated in egl-46 and gfi-1 mutants (Fig 4I and J; Supplementary Fig S8A2). Transcription of bec-1, epg-8, lgg-1, and atg-7 was upregulated in egl-46, gfi-1, and ED4A4.5 mutants and reduced by simultaneous loss of atfs-1 activity (Supplementary Fig S8B2). Thus, mitochondrial stress activates autophagy through ATFS-1-mediated upregulation of autophagy genes.

The TGF-β, lin-35/Rb, ER stress, and mitochondrial stress pathways activate autophagy activity independent of HLH-30

The C. elegans TFEB homolog HLH-30 activates the expression of autophagy genes during starvation [23,24]. Suppression of the rpl-43 mutant phenotype by ER stress, by mitochondrial stress, or by the loss of activity of lin-35 and sma-3 was not affected by simultaneous depletion of hlh-30 (Supplementary Fig S9A–H). The transcription factor MXL-3 acts antagonistically to HLH-30 to repress the expression of lysosomal lipases [23]. Inactivation of mxl-3 did not affect the accumulation of SQST-1::GFP aggregates in rpl-43 mutants (Supplementary Fig S9I and J), indicating that autophagy activity was not elevated in the mxl-3 mutant intestine. Thus, TGF-β, lin-35/Rb, ER stress, and mitochondrial stress signaling function in parallel to HLH-30 to control autophagy gene expression.

Here, we established the degradation of SQST-1 aggregates in the rpl-43 mutant intestine as a genetic model system to investigate autophagy regulation. We demonstrated that autophagy activity is elevated by various stresses. ER stress and mitochondrial stress activate autophagy activity by transcriptional upregulation of autophagy genes in C. elegans. Components of the endocytic pathway, including the AP2 complex, the COG complex, and the ESCRT complex, were identified in our screen probably because their partial loss of function may not impair their normal function in the autophagy pathway [3], but impose a stress on the intestine which in turn activates autophagy.

In addition to these stress-induced gene inactivations, we found that the loss of activity of several developmental signaling pathways, including TGF-β, lin-35/Rb, glp-1, daf-2, mTOR, and LIN-45/MEK-2/MAPK-1 MAPK signaling, promotes autophagy activity. TGF-β lin-35/Rb and glp-1 signaling specify distinct biological processes by transcriptionally regulating distinct target genes and also transcriptionally promote autophagy activity [24]. Therefore, various developmental signaling factors integrate with the autophagy machinery during animal development.

Materials and Methods

See Supplementary Materials and Methods for strains used and additional protocols.

Identification, mapping, and cloning of rpl-43

bp399 was isolated in a genetic screen for mutants with ectopic accumulation of SQST-1::GFP aggregates. bp399 was mapped between the polymorphic markers pkp2118 (II: +21.2) and pkp2113 (II: +22.9). Fosmids in this region were used for transformation rescue. Accumulation of SQST-1::GFP aggregates in rpl-43(bp399) animals was rescued by a transgene expressing the single gene y48b6d.2 (including an approximately 1.5-kb promoter region, the entire ORF, and the approximately 1-kb 3' UTR).

Preparation and induction of RNAi bacterial clones

The RNAi feeding library was purchased from Geneservice. The library contains bacterial clones expressing dsRNA designed to individually inactivate 16,749 genes (targeting about 87% of the predicted genes) [25]. RNAi bacterial clones were grown on LB agar plates supplemented with 100 mg/ml ampicillin and 30 mg/ml tetracycline and then inoculated into LB medium containing 50 mg/ml ampicillin and cultured for 6 h at 37°C. 300 μl of each bacterial culture was dispensed onto 10 cm NGM agar plates containing 5 mM IPTG. dsRNA transcription was induced overnight at 25°C. Synchronized L1 sqst-1::gfp; rpl-43 animals were plated onto RNAi
feeding plates with about 15 worms per plate and were grown at 20°C. The F1 progeny or arrested larvae or sterile adults were examined for expression of the sqst-1::gfp reporter.

**Immunofluorescence staining**

For worm immunofluorescence staining, animals were permeabilized by freeze-cracking and then fixed, blocked, and incubated with diluted antibody at room temperature for 2–4 h. The animals were then washed three times and incubated with rhodamine-conjugated or FITC-conjugated secondary antibody. Fluorescence was examined by a fluorescence microscope (Zeiss Axioplan 2 image) or a confocal microscope (Zeiss LSM 710 Meta plus Zeiss Axiovert zoom).

**Statistical analysis**

Data are shown as mean ± SD. Unpaired t-tests were performed for statistical analysis.

**Supplementary information** for this article is available online: http://embor.embopress.org
Acknowledgements
We are grateful to Dr. Isabel Hanson for editing work. This work was supported by the National Basic Research Program of China (2013CB910100, 2011CB910100) and also a grant from the NSFC (31225018) to H.Z. The research of Hong Zhang was supported in part by an International Early Career Scientist Grant from the Howard Hughes Medical Institute.

Author contributions
BG, XXH, PPZ, and HZ designed the experiments. BC, XXH, QQL, PPZ, LXQ, XBZ, JH, and BF performed the experiments. WRH and JHH performed bioinformatic analyses. BG, XXH, and HZ wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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