

Disruption of Endocytic Pathway Regulatory Genes Activates Autophagy in *C. elegans*

Meenakshi Dwivedi^{1,6}, Hyun Sung^{2,6}, Haihong Shen³, Byung-Jae Park^{4,*}, and Sangho Lee^{5,*}

Autophagy and endocytic pathway are highly regulated catabolic processes. Both processes are crucial for cell growth, development, differentiation, disease and homeostasis and exhibit membrane rearrangement for their function. Autophagy and endocytic pathway represent branches of the lysosomal digestive system, autophagy being responsible for degradation of cytoplasmic components and endocytic pathway for degradation of exogenous substances. Here we report that autophagy is activated when endocytic pathway regulatory genes such as *rab-5* and *rabx-5* are disrupted. Defects in the ubiquitin binding domain of RABX-5 are critical in activating autophagy. We also observed that the elevated autophagy level does not contribute to lifespan extension of *rabx-5* mutant. Our results suggest that autophagy may compensate for the endocytic pathway when regulatory genes for the endocytic pathway malfunction, providing a case of complementation between two functionally related cellular processes.

INTRODUCTION

Autophagy is a ubiquitous cellular process that involves a process of self-cannibalization through the lysosomal degradation pathway and is evolutionarily conserved in all eukaryotic organisms. Autophagy is a complex catabolic process that digests various cytosolic proteins and organelles through autophagosomal-lysosomal pathway (Klionsky, 2005). Autophagy allows cell survival under starvation conditions by the bulk degradation of cytoplasmic constituents generally by non-selective sequestration. In *C. elegans* autophagy is essential for lifespan extension, reproductive development, programmed cell death, necrotic cell death, neurodegenerative diseases, cell size regulation, neurotransmitter receptor trafficking and dauer development (Melendez and Levine, 2009). Earlier reports have suggested the positive correlation between the elevated autophagy and extended lifespan in various model organisms (Dwivedi and Ahn, 2009).

Endocytic pathway resembles autophagy in a way as it is re-

sponsible for uptake of extracellular constituents and its degradation. Endocytic pathway is regulated by Rab GTPases and their associated factors. RAB-5, a small GTPase, plays a crucial role in regulating early endocytic pathway (Bucci et al., 1992; Chavrier et al., 1990; Christoforidis et al., 1999; De Reniz et al., 2002). RABX-5 is a guanine nucleotide exchange factor (GEF) for RAB-5 (Horiuchi et al., 1997). RABX-5 contains an ubiquitin binding domain at its N-terminus followed by helical bundle and Vps9 domains functioning as catalytic core. Another GEF for RAB-5 is RME-6 that is conserved from worms to human and also includes Vps9 domain but lacks ubiquitin binding domain. RME-6 is distinct from other RAB-5-associated proteins in that RME-6 acts at the clathrin-coated pit (Sato et al., 2005).

Growing evidence suggests that there may be a crosstalk between autophagy and endocytic pathway. Inactivation of certain *C. elegans* Vps genes lead to the accumulation of enlarged endosomal structures and an increase in autophagy (Michelet et al., 2009; Roudier et al., 2005). In addition, certain regulators in autophagy and endocytic pathway are reportedly involved in both processes. BEC-1, the *C. elegans* ortholog of Atg6/Vps30/Beclin1 and a key regulator of the autophagic machinery, also contributes to endosome function (Ruck et al., 2011). UVRAG, the human ortholog of Vps38 and a Beclin1-binding protein, has a role in endocytosis and autophagy (Liang et al., 2008).

To study inter-dependence of autophagy and endocytic pathway in *C. elegans*, we used GFP::LGG-1 as a reporter gene for autophagy to investigate the effects of RNAi for endocytic pathway genes such as *rab-5*, *rabx-5*, *rme-6* and double mutant of *rabx-5* and *rme-6*. We also used *dyn-1* mutant to check the level of autophagy. Finally, we studied the lifespan of endocytic mutants showing elevated autophagy. Our data suggest that autophagy may compensate endocytic pathway at early endosomes and that the elevated autophagy does not contribute to extending the lifespan of endocytic mutants.

MATERIALS AND METHODS

C. elegans strains and maintenance

The following strains were obtained from the *Caenorhabditis*

¹Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi, India, ²Department of Biological Sciences, Purdue University, USA, ³School of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea, ⁴Department of Life Science, Hallym University, Chunchon 200-702, Korea, ⁵Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Korea, ⁶These authors contributed equally to this work.

*Correspondence: bjparkkk@hallym.ac.kr (BJP); sangholee@skku.edu (SL)

Genetics Center (CGC) at University of Minnesota, USA: Bristol N2, CX51 *dyn-1(ky51)* X, CB1370 *daf-2(e1370)* III, *rabx-5(tm1512)* III, and *rme-6(b1014)*. Worm breeding and handling were conducted as previously described (Brenner, 1974).

Analysis of autophagic events

The level of autophagy in various mutants was assessed using GFP::LGG-1 translational reporter characterized previously (Melendez et al., 2003). The wild type worms carrying the extrachromosomal array GFP::LGG-1 were kindly provided by Alicia Melendez. The extrachromosomal array GFP::LGG-1 was introduced into *rabx-5(tm1512)* and *dyn-1(ky51)* mutants by crossing. GFP-positive puncta were counted (using 1,000-fold magnification on a Zeiss Axioplan II microscope equipped with fluorescent optics) in the seam (lateral epidermal) cells of L3 transgenic animals. When performing RNAi experiments to count GFP::LGG-1 positive foci, young adults (P0) were fed with RNAi bacteria, and the L3 progeny of the F1 generation were examined. All RNAi clones used were obtained from gene service constructed by Julie Ahringer's group at the The Wellcome CRC Institute, University of Cambridge, Cambridge, England. At least 3 to 10 seam cells were examined in each of 10 to 20 animals from at least two independent trials and averaged. Statistical data analysis was done using unpaired, two-tailed *t*-test. We have checked the level of GFP::LGG-1 in *dyn-1* mutants as dynamin is the key component in clathrin-mediated endocytosis and is involved in pinching off the coated pit from the plasma membrane to form a clathrin-coated vesicles (CCVs) (Grant and Sato, 2006).

Sequence alignment and homology modeling

Pairwise protein sequence alignment was performed using ClustalW2 web server hosted by European Bioinformatics Institute (Chenna et al., 2003). Homology modelling was done using SWISS-MODEL web server (Arnold et al., 2006). The resulting homology structural model figure was generated using PyMOL (Schrödinger, LLC).

Lifespan analysis

The lifespan analysis after RNAi treatments at 20°C was done in F1 and F3 generation as standardized previously in the laboratory (Dwivedi et al., 2009). In all experiments, the prefertile period of adulthood (L4 stage) was used as $t = 0$ for lifespan analysis. Animals that ruptured, bagged (exhibited internal progeny hatching), or crawled off the plates were censored from lifespan analysis data. Each lifespan experiment was repeated at least three times with $n = 75$ to 100 animals per experimental group. Kaplan-Meier survival analysis was used to compare the mean lifespan of different treatments, and *p* values were calculated using the logrank test (the Mantel-Cox test) to determine the relative effects on wild-type N2, *rabx-5(tm1512)* and *rme-6(b1014)* mutant animals.

RESULTS AND DISCUSSION

Activation of autophagy by knockdown of RAB-5 and RABX-5

To study whether RABX-5 is required in autophagy, we employed GFP-tagged LGG-1 protein as a reporter for autophagy. LGG-1 is the worm ortholog of yeast Atg8p and localizes to pre- and autophagosomal membranes. During autophagy, GFP::LGG-1 forms fluorescent puncta. To minimize endogenous auto-fluorescence in adult animals, we restricted our observations to the hypodermal seam cells of L3 larvae. GFP::LGG-1 in wild-type worms fed with bacteria expressing L4440 vector

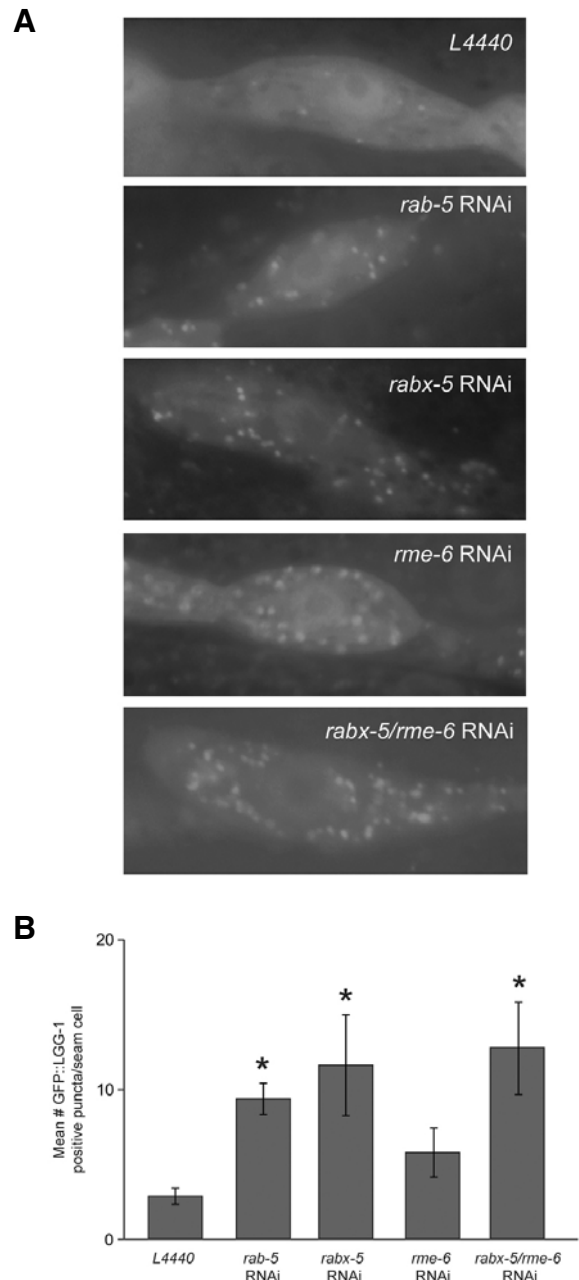


Fig. 1. (A) Representative images of GFP::LGG-1 expression in the seam cells (F1 generation) fed with bacteria containing L4440 (vector) or expressing *rab-5*, *rabx-5*, *rme-6* and *rabx-5/rme-6* dsRNA, respectively. (B) Quantification of GFP::LGG-1 containing puncta in each case described in (A). Magnification of the images is 1,000.

shows diffused expression pattern (Fig. 1A). However, worms treated with RNAi targeted against *rab-5*, *rabx-5*, *rme-6* and *rabx-5/rme-6* genes, GFP::LGG-1 puncta were observed (Fig. 1A), establishing a crosstalk between autophagy and the endocytic pathway. Quantitation of the number of GFP::LGG-1 puncta reveal that the average number of GFP::LGG-1 puncta was significantly increased for all three RNAi treated cases (Fig. 1B). These results indicate that autophagy can be activated upon disruption in the endocytic pathway.

Among three genes we tested, *rab-5* and *rabx-5* RNAi showed

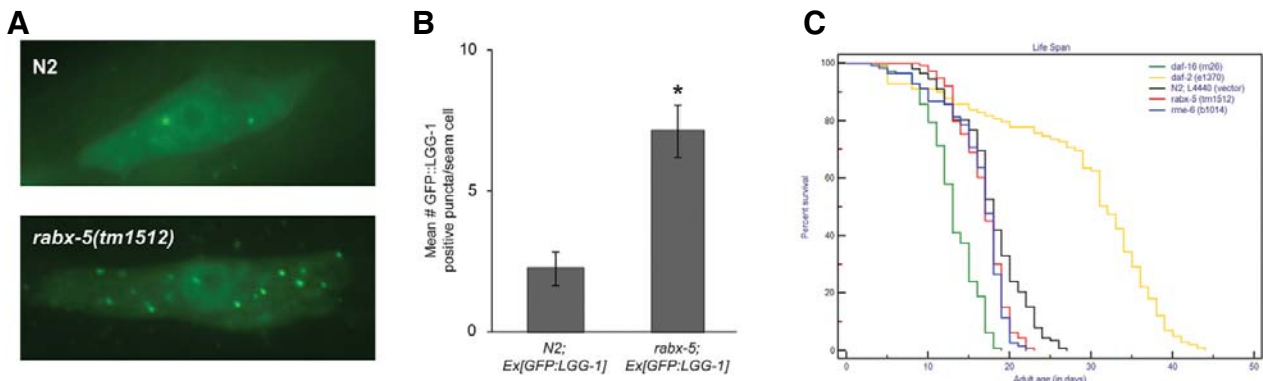


Fig. 2. (A) Representative images of GFP::LGG-1 expression in the seam cells in N2 strains or *rabx-5(tm1512)* mutant. (B) Quantification of GFP::LGG-1 containing puncta in each case described in (A). (C) Kaplan-Meier survival curve showing the life-span of N2 wild-type, *daf-16(m26)*, *daf-2(e1370)*, *rabx-5(tm1512)* and *rme-6(b1014)* mutants. Magnification of the images is 1,000.

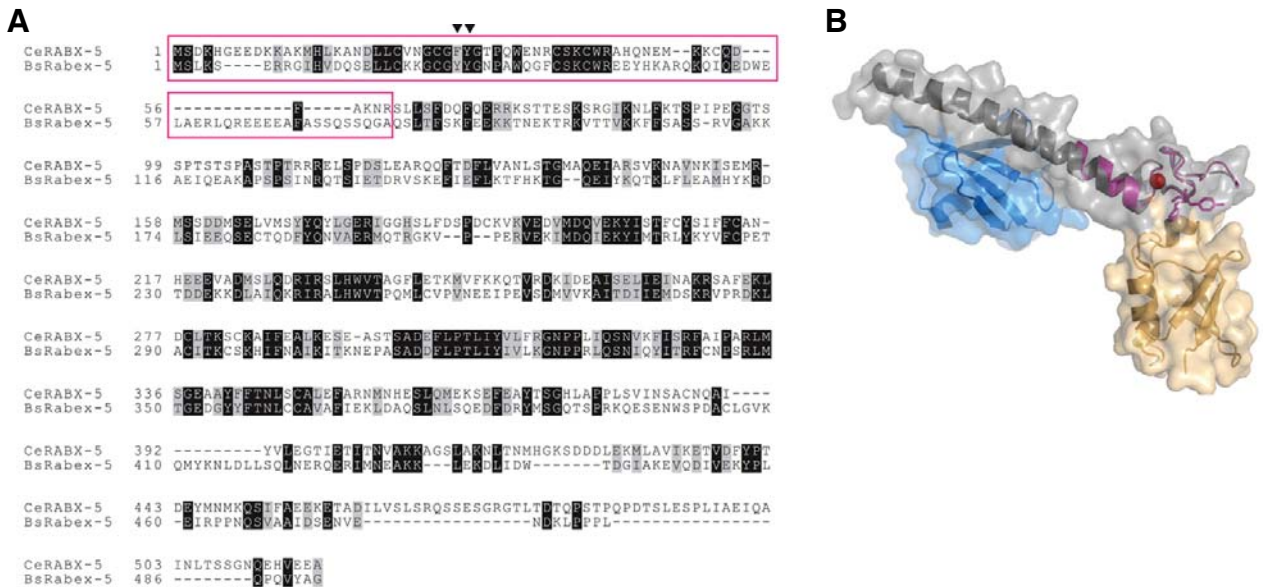


Fig. 3. (A) Sequence alignment between *C. elegans* RABX-5 and bovine Rabex-5. The region which is disrupted in the *C. elegans rabx-5(tm1512)* mutant is shown as a purple box. Black inverted triangles refer to the key residues in recognizing ubiquitin in bovine Rabex-5. (B) Homology modeling of *C. elegans* RABX-5 A20 zinc finger domain using SwissModel Server. *C. elegans* RABX-5 is colored in purple with zinc ion in red. Bovine Rabex-5 spanning A20 zinc finger and motif-interacting with ubiquitin (MIU) is depicted in gray with two bound ubiquitin molecules in blue and yellow.

more increases in the number of puncta than *rme-6* RNAi (Fig. 1B), implying that crosstalk between autophagy and the endocytic pathway may occur in early endosomes predominantly because RME-6 is known to regulate RAB-5 in clathrin-coated pits. Consistent with such idea, double RNAi for *rabx-5* and *rme-6* did not indicate any synergistic effect on the number of GFP::LGG-1 puncta (Fig. 1B). Taken together, our data suggest that there is crosstalk between autophagy and the endocytic pathway and that the disruption of endocytic pathway at early endosomal may result in the activation of autophagic pathway.

Lifespan extension by RABX-5

The increased autophagic puncta in worms treated with RNAi targeted against endocytic pathway genes motivated us to study the lifespan in these mutants. We first analyzed the autophagic puncta in *rabx-5(tm1512)* mutants and found similar

increase in GFP::LGG-1 puncta as we observed after the treatment with RNAi clone when compared to wild-type worms (Figs. 2A and 2B). Interestingly the lifespans of *rme-6(b1014)* and *rabx-5(tm1512)* mutants were comparable to that of wild-type (Fig. 2C). The *daf-2(e1370)* and *daf-16(m26)* were taken as positive and negative controls for lifespan, respectively. The comparable lifespan studies of the endocytic mutants to wild-type worms suggest that all pathways leading to extended lifespan may converge to autophagy (Toth et al., 2008) but all pathways showing activated autophagy may not necessarily extend lifespan.

RABX-5 ubiquitin binding domain and autophagy

To analyze which region of RABX-5 may be responsible for the activation of autophagy, we verified the precise region in RABX-5 disrupted in the *rabx-5(tm1512)* mutants. We found that the region disrupted in the *rabx-5(tm1512)* mutant encodes A20

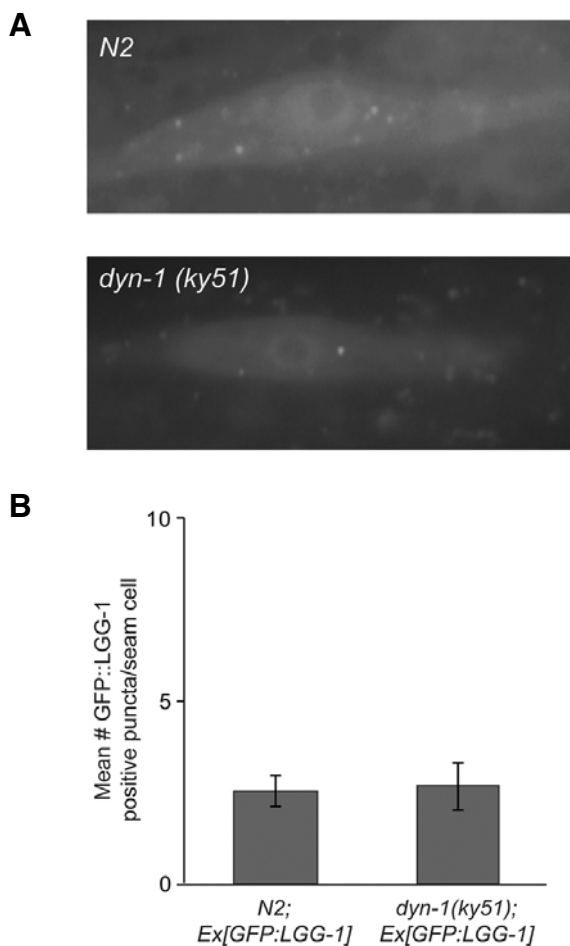


Fig. 4. (A) Representative images of GFP::LGG-1 expression in the seam cells in N2 strains or *dyn-1(ky51)* mutant. (B) Quantification of GFP::LGG-1 containing puncta in each case described in (A). Magnification of the images is 1,000.

zinc finger, a ubiquitin binding domain (Fig. 3A). Sequence alignment between RABX-5 and bovine Rabex-5, a mammalian ortholog, reveals that RABX-5 contains a single ubiquitin binding domain while bovine Rabex-5 revealed two such domains arranged in tandem: an A20 zinc finger domain and a motif-interacting with ubiquitin (MIU) domain. Biochemical and structural studies have established that the two tandem ubiquitin binding domains of Rabex-5 recognize ubiquitin as an intracellular trafficking signal with modest affinities (Lee et al., 2006; Penengo et al., 2006). Homology modeling of the A20 zinc finger domain of RABX-5 suggests that RABX-5 would be able to bind ubiquitin (Fig. 3B). These data suggest that the activation of autophagy by RABX-5 may be regulated by its ubiquitin recognition that in turn provides a signal for the endocytic pathway.

Autophagy in *dyn-1* mutants

To check the sites where the levels of autophagy in *rab-5*, *rabx-5* and *rme-6* mutants are elevated, we investigated the expression pattern of GFP::LGG-1 in *dyn-1* mutants as dynamin acts at the early stages of clathrin-mediated endocytosis. We found that the number of GFP::LGG-1 puncta in *dyn-1(ky51)* mutants were comparable to that of wild-type worms (Figs. 4A and 4B).

This result suggests that autophagy can compensate the endocytic pathway only after the formation of mature CCV. It is well established that CCVs are pinched off by dynamin, actively uncoated and transported to early endosomes. We postulate that autophagic machinery compensates the function for the degradation of cargo and clathrin molecules assembled in mature CCVs if the endocytic pathway is blocked and/or defective.

Summary

Our results establish that genes involved in the endocytic pathway such as *rab-5*, *rabx-5* and *rme-6* can activate autophagy when the endocytic pathway is disrupted. Among those genes investigated, *rab-5* and *rabx-5* RNAi predominantly activate autophagy. Disruption of the ubiquitin binding domain in RABX-5 is responsible for autophagy activation, suggesting that ubiquitin recognition regulates switching the involvement of RABX-5 between endocytic pathway and autophagy. However, lifespan of worms are not affected by RNAi targeted against *rabx-5*. Activation of autophagy by defective RABX-5 precisely appears to occur subsequent to CCV maturation. Our findings reveal for the first time the involvement of RABX-5 in autophagy.

ACKNOWLEDGMENTS

We thank Alicia Melendez for providing the wild-type worms carrying the GFP::LGG-1 extrachromosomal array. One of the authors, M. Dwivedi, is thankful to CSIR for providing financial assistance. This work was supported by the National Research Foundation Grant funded by the Korean Government (KRF-2008-314-C00224).

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