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Different effects of *Atg2* and *Atg18* mutations on Atg8a and Atg9 trafficking during starvation in Drosophila ☆

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ABSTRACT

The Atg2–Atg18 complex acts in parallel to Atg8 and regulates Atg9 recycling from phagophore assembly site (PAS) during autophagy in yeast. Here we show that in Drosophila, both Atg9 and Atg18 are required for Atg8a puncta formation, unlike Atg2. Selective autophagic degradation of ubiquitinated proteins is mediated by Ref(2)P/p62. The transmembrane protein Atg9 accumulates on refractory to Sigma P (Ref(2)P) aggregates in *Atg7*, *Atg8a* and *Atg2* mutants. No accumulation of Atg9 is seen on Ref(2)P in cells lacking Atg18 or Vps34 lipid kinase function, while the Atg1 complex subunit FIP200 is recruited. The simultaneous interaction of Atg18 with both Atg9 and Ref(2)P raises the possibility that Atg18 may facilitate selective degradation of ubiquitinated protein aggregates by autophagy.

Structured summary of protein interactions:
Ref(2)P physically interacts with **Atg18** by anti tag coimmunoprecipitation (View interaction) **Atg18** physically interacts with **Atg2** by anti tag coimmunoprecipitation (View interaction) **CG8678** physically interacts with **Atg2** by anti tag coimmunoprecipitation (View interaction) **Atg18** physically interacts with **atg9** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Autophagy is a major catabolic pathway capable of degrading all kinds of intracellular material including proteins, lipids, polysaccharides, and nucleic acids. During its main pathway, phagophores capture cytosol and organelles to form autophagosomes, followed by the fusion of these double-membrane vesicles with lysosomes [1]. Autophagy was initially considered to be a non-specific, bulk degradation system, in contrast with the ubiquitin–proteasome pathway, in which individual polyubiquitinated proteins are recognized, unfolded and degraded in the inner proteolytic chamber of proteasomes. More recently, multiple studies showed that ubiquitination also signals for selective autophagic degradation, and characterization of ubiquitin-specific autophagy receptors revealed the molecular mechanism involved [2,3]. Proteins such as p62 contain distinct domains mediating multimerization, ubiquitin binding and interaction with Atg8 family proteins. Atg8 is a ubiquitin-like protein bound to phagophores and autophagosomes through a lipid anchor [1,4,5]. Ubiquitinated proteins are captured into aggregates by binding to p62 multimers, and the interaction of p62 with Atg8-positive phagophores is considered to be responsible for their elimination by autophagy [2,3,6–8]. In contrast with this simple model, p62 was found to colocalize with proteins involved in the initiation of phagophores independent of the presence of mammalian Atg8 homologs such as LC3 [9]. These results suggest that additional factors also contribute to the recognition of p62 aggregates by phagophores.

Atg9 is the only transmembrane protein of core autophagy factors, and it likely supplies initial vesicles for phagophore nucleation from multiple membrane sources including endosomes, plasma membrane and Golgi [10–12]. Atg9 is considered to be an upstream factor in the hierarchy of autophagy-related (Atg) proteins in yeast, but the molecular determinants of Atg9

Abbreviations: Atg, autophagy-related; PAS, phagophore assembly site; PI3P, phosphatidylinositol 3-phosphate; Ref(2)P, refractory to Sigma P; ULK, uncoordinated-51 like autophagy kinase; Vps, vacuolar protein sorting; WIPI, WD40 repeat domain phosphoinositide-interacting protein

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recruitment to the phagophore assembly site (PAS) are incompletely characterized [13]. A recent study shows that mammalian Atg9 is recruited to damaged mitochondria independent of lipidated Atg8 homologs and the upstream kinase Atg1/ULK1 during selective autophagic degradation of mitochondria [14]. In yeast, the Atg2–Atg18 protein complex is thought to act in parallel to the Atg8 system, and regulates Atg9 recycling from PAS [13,15]. Diverse Atg18-like proteins are found in eukaryotes. The four mammalian homologs fall into two groups based on bioinformatic analysis: WIPI1/2 and WIPI3/4 [16,17]. Of these, both WIPI2 and WIPI4 were suggested to promote autophagosome formation based on siRNA experiments in cultured cells [16,18]. Yeast Atg18 and its paralog Atg21 belong to the WIPI1/2 group [16,17]. Atg21 only functions in an autophagy-related biosynthetic pathway called cytoplasm to vacuole targeting (cvt) which delivers a subset of hydrolases to the vacuole under growth conditions, whereas Atg18 is required for both autophagy and cvt in yeast [19]. Ygr223c, the third Atg18 family protein in yeast, belongs to the WIPI3/4 group and regulates micronucleophagy, a selective autophagy pathway for degradation of nuclear components [16,20].

Clear orthologs of most Atg proteins are found in the popular metazoan model Drosophila, and the p62 ortholog refractory to Sigma P (Ref(2)P) also promotes ubiquitinated protein aggregation [21,22]. Single genes code for Atg9 and Atg2, and the WIPI1/2 homolog CG7986 is referred to as Atg18, as it is required for autophagy in Drosophila [23–26]. The roles of another WIPI1/2 family Drosophila protein, CG8678, and of the WIPI3/4-like CG11975 are unknown [16,17].

Here we show that loss of Atg2 or Atg18 have different consequences on Atg8a puncta formation and Atg9 recruitment to Ref(2)P aggregates during starvation in Drosophila, raising the possibility that these proteins act differently during autophagosome formation in Drosophila.

2. Materials and methods

2.1. Molecular cloning, immunoprecipitation and antibody production

Atg18 coding sequences were PCR amplified from LD38705 (DGRC), and cloned into appropriate vectors to generate R4-mCherry-Atg18, UAS-3xHA-Atg18, and UAS-3xFLAG-Atg18, respectively. Coding sequences were amplified from genomic DNA or GH07816 (DGRC) to generate UAS-3xFLAG-Atg2 or UAS-3xHA-CG8678, respectively. These UAS plasmids, together with UAS-3xHA-Atg9, UAS-3xFLAG-Ref(2)P and mt-Gal4, were used to transfect D.Mel-2 cells (Invitrogen), followed by processing for immunoprecipitation as described [25,27]. His-tagged recombinant Atg9 protein fragment (amino acids 541–845) was purified from bacteria and used for immunization of rats as before [27].

2.2. Drosophila genetics

Flies were reared on standard cornmeal-yeast-agar diet, and well-fed mid-third instar larvae were floated in a 20% sucrose solution for 3 h in starvation experiments. Genotypes used in this study are RNAi lines *Atg9*^{GD10045} (VDRC), *Atg9*^{HMS01246}, *Atg9*^{JF02891}, *Atg18*^{JF02898}, *Atg8a*^{KK109654}, *Atg2*^{JF02786}, and *w*^{1118} used as wild type (all from BDSC) [26], mutants *Atg7*^{d77} [28], *Atg2*^{EP3697}*/Df(3L)BSC119*, *Atg18*^{KG03090}*/Df(3L)Exel6112*, *Atg8a*^{d4} [23,26], *cg-Gal4, UAS-Vps34*^{KD} [29], and stocks for clonal analysis *hs-Flp; UAS-Dcr2; Act > CD2 > Gal4, UAS-GFPnls, R4-mCherry-Atg8a* and *hs-Flp; UAS-Lamp1-GFP; Act > CD2 > Gal4, UAS-Dcr2* [25–27]. *R4-mCherry-Atg18* transgenics were generated by Bestgene.

2.3. Western blots and histology

Western blots were carried out as described previously [25–27]. Dissected larval carcasses were processed for LysoTracker Red (Invitrogen) or immunostaining as before, using rabbit anti-Ref(2)P (1:2000), rat anti-Atg8a (1:300), rat anti-Atg9 (1:300), rat anti-FIP200 (1:300) and rat anti-mCherry (1:300) primary antibodies [25–27]. Preparations were photographed on an Axioimager M2 with Apotome2 (Zeiss), and original unmodified images were processed for statistical evaluation using ImageJ (NIH) and SPSS Statistics (IBM), as described [25–27]. Colocalizations were calculated either by the colocalization tool in ImageJ to obtain Mander's colocalization coefficients for Atg8a-Ref(2)P, or by manual counting for Atg9-Ref(2)P. Immunogold labeling for ubiquitin was done as described [28]. Anti-Atg9 (1:30) and anti-Ref(2)P (1:60) double immunogold labeling was carried out on adult brains embedded into LR White resin (Sigma) to increase antigenicity, using secondary antibodies anti-rat conjugated to 10 nm gold (1:20, Sigma) and anti-rabbit conjugated to 18 nm gold particles (1:60, Jackson Immunoresearch).

3. Results

p62 aggregates are selectively captured into autophagosomes under both basal or starvation conditions in mammalian cells, as more than 90% of LC3-positive autophagosomes are also positive for p62 [6]. In line with that, most Atg8a-positive autophagosomes colocalized with Ref(2)P in fat bodies of well-fed, starved or wandering Drosophila larvae, respectively (Figs. 1A and S1). As expected, the number of Atg8a dots increased in response to autophagy induction (Fig. 1B). Strikingly, larger Ref(2)P aggregates seen under low autophagy level in fed cells disappeared during

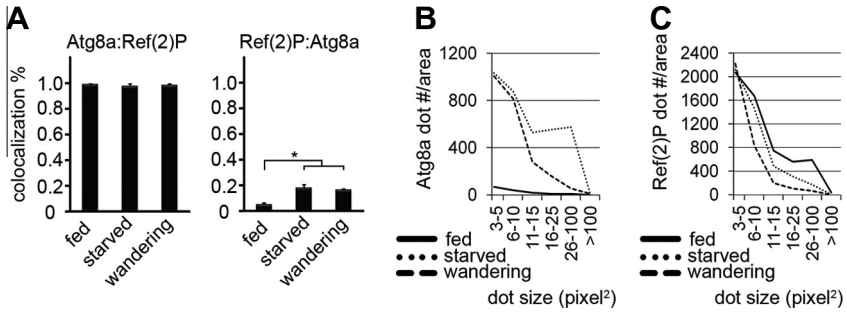

Fig. 1. (A) Atg8a colocalizes with Ref(2)P in Drosophila fat body cells of fed, starved and wandering wild type L3 larvae. The colocalization of Ref(2)P with Atg8a increases during starvation or developmental autophagy, as much more autophagosomes are generated under these circumstances. $N = 6–15$ per stage, $^{*}P < 0.001$, ANOVA, errors bars: S.D. (B) Atg8a-positive autophagosomes are induced by starvation or during the wandering stage. (C) Larger Ref(2)P aggregates observed in fat body cells of well-fed animals are eliminated during starvation or wandering.