Atg27p localization is clathrin- and Ent3p/5p-dependent
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Abstract

The autophagy-related protein Atg27p has been previously shown to localize to the autophagy-specific pre-autophagosomal structure (PAS) as well as to several organelles, including the late Golgi, the vacuolar membrane, and the endosome. Given that Atg27p localization to the vacuolar membrane in particular has been shown to be dependent on both its C-terminal tyrosine sorting motif and the AP-3 adaptor, and that Atg27p can be found in clathrin-coated vesicles, we set out to determine whether Atg27p localization inside cells is dependent on clathrin or on any of its cargo adaptors. We report that Atg27p localization is clathrin- and Ent3p/5p-dependent.

Figure 1. Atg27p displays clathrin- and Ent3p/5p-dependent localization in budding yeast: (A) Atg27p co-localizes with Chc1p with or without its C-terminal tyrosine sorting motif (YSAV). The percentage of Chc1p structures co-localizing with Atg27p (with or without its YSAV motif) was quantified ($n \geq 200$, combined from three independent experiments). (B) Vacuolar membrane localization of Atg27p-GFP, shown previously to be an AP-3 cargo, is abrogated in...
cells deleted for CHC1 and in the clathrin adaptor double mutant ent3Δ ent5Δ, but is still present in the gga1Δ gga2Δ clathrin adaptor double mutant with or without rapamycin. Individual deletion of the ENT3, ENT5, GGA1, or GGA2 clathrin adaptor genes has no effect on Atg27p localization. Bar graphs indicate the percentage of FM4-64-stained vacuolar membranes containing Atg27p-GFP in the indicated mutant backgrounds (n ≥ 100, combined from three independent experiments) during logarithmic growth or after induction of autophagy by treatment with rapamycin. All micrographs presented were captured during logarithmic growth. Error bars indicate standard deviation (SD). p values less than 0.05, relative to the wildtype/logarithmic growth conditions are indicated by an asterik (*). (C) Quantification of colocalization of Atg27p-GFP with Golgi (Sec7p) and endosomal (Tlg1p early; Vps27p late) markers in clathrin and ent3Δ/ent5 mutants. Bar graphs indicate the percentage of indicated Golgi/endosomal structures containing Atg27p-GFP (n ≥ 100, combined from two independent experiments for the wild type strain and three independent experiments for all other strains). Error bars indicate standard deviation (SD). p values less than 0.05, relative to the wildtype/logarithmic growth conditions are indicated by an asterik (*). (D) Classical AP-3 cargo alkaline phosphatase (ALP) displays traditional clathrin-independent, as well as ent3Δ ent5Δ-independent localization to the vacuolar membrane. Scale bars = 5 microns.

**Description**

The autophagy-related protein Atg27p has been previously shown to localize to the autophagy-specific pre-autophagosomal structure (PAS) as well as to several organelles, including the late Golgi or trans-Golgi network (TGN), the vacuolar membrane, and the early and late endosomes (Segarra et al. 2015). Moreover, Atg27p localization to the vacuolar membrane, in particular, is dependent on both its C-terminal tyrosine sorting motif and the AP-3 adaptor (Segarra et al. 2015; Suzuki and Emr 2019), but not other yeast AP-related adaptors (Segarra et al. 2015). We recently reported that Atg27p can be found in clathrin-coated vesicles (CCVs; Ding, Segarra et al. 2016, See accompanying micropublication). For this reason, we set out to determine whether Atg27p localization is dependent on clathrin or on any other of its cargo adaptors.

We found that Atg27p co-localizes with Chc1p (Figure 1A), even in the absence of its C-terminal tyrosine sorting motif YSAV (ΔYSAV). Thus, the presence of Atg27p in CCVs does not seem to require the anterograde transport of Atg27p to the vacuolar membrane. Atg27p mutant molecules deleted for their YSAV motif display increased colocalization with clathrin (Figure 1A). AP-3 sorting to the vacuole of these ΔYSAV mutants is most likely impaired, leading to increased amounts of Atg27p entering the traditional TGN/endosomal pathway.

To examine the overall localization pattern of Atg27p in cells lacking clathrin, we imaged cells expressing GFP-tagged Atg27p that were deleted for CHC1. In chc1Δ, Atg27p localizes to small punctate structures throughout the cell and its vacuolar membrane localization is lost (Figure 1B). This was surprising because Atg27p has been shown to be an AP-3 cargo (Segarra et al. 2015) and the trafficking of AP-3 cargo, such as alkaline phosphatase (ALP; Stepp et al. 1997; Cowles et al. 1997), has traditionally been considered to be clathrin-independent (Vowels and Payne 1998). Similarly, we examined vacular membrane localization of Atg27p in cells missing the other TGN/endosomal clathrin adaptors, epsins or ggas. Double deletion of the ENT3 and ENT5 adaptor genes appear to recapitulate the vacuolar membrane localization defect seen in the clathrin-null cells, suggesting that transport of Atg27p is also dependent on the Ent3p and Ent5p adaptor proteins (Figure 1B). Ent5p was also identified in the mass spectrometry screen for CCV components, similar to Atg27p (WT screen, Ding, Segarra et al. 2016). The Ent3p and Ent5p epsin-like proteins have been shown previously to have separate roles in clathrin-mediated TGN/endosomal traffic (Costaguta, Duncan et al. 2006). Our finding that the Atg27p localization phenotype in clathrin null cells can only be recapitulated by combined deletion of ENT3 and ENT5 may indicate that clathrin is acting at more than one step in Atg27p TGN/endosomal transport. It is also interesting that the vacuolar membrane localization of Atg27p is not affected in any of the GGA adaptor mutants, as GGA clathrin adaptors were shown by Casler and Glick to be required for traffic of vacuolar cargo from the maturing Golgi (2020).

To determine the cellular location of the small Atg27p puncta in the chc1Δ and ent3Δ ent5Δ mutants, we quantified their co-localization with traditional Golgi/endosomal markers Sec7p, Tlg1p, and Vps27p. Atg27p puncta in the clathrin-null and the ent3Δ ent5Δ double deletion mutants partially localize to the Sec7p- and Tlg1p-marked TGN/recycling endosome and the Vps27p-marked pre-vacuolar endosome (Figure 1C). The budding yeast TGN has been shown to serve as the cell’s early/recycling endosome (Day et al. 2018), potentially explaining why certain organelar markers such as Sec7p and Tlg1p are sometimes reported to co-localize (Grissom et al. 2020). All in all, we can conclude that the Atg27p puncta in the clathrin null and in the ent3Δ ent5Δ cells partially localize to TGN/recycling endosome and the pre-vacuolar endosome.

The observed loss of vacuolar membrane localization was specific to Atg27p and not to all AP-3 cargoes, since ALP was able to localize to the vacuolar membrane in the clathrin and ent3 ent5 mutants (Figure 1D). This suggests that Atg27p is a non-canonical AP-3 cargo in that its vacuolar membrane localization is both AP-3 and clathrin- and epsin-dependent. Recent studies showed that Atg27p is recycled from the vacuolar membrane to endosomes and then the TGN (Suzuki and Emr 2018). Possibly, in the absence of clathrin or the epsins, Atg27p recycling to the TGN is impaired, leading to the loss of Atg27p from the vacuolar membrane and accumulation in TGN/endosomal compartments. Of interest, in chc1Δ cells,
Atg27p was decreased on Vps27p-positive structures (Figure 1C), suggesting that clathrin’s major role is in a later retrieval step from the TGN.

**Methods**

**Request a detailed protocol**

**Yeast and plasmid methods**

Standard methods and media were used for genetic manipulations, growth, and transformation of yeast (Guthrie and Fink 1991). To induce autophagy, log phase cells were treated with rapamycin (LC Laboratories, R-5000) at 0.2 µg/mL for at least 2 hours at 30°C. *Saccharomyces cerevisiae* strains used in this study are listed in the table below. Unless otherwise indicated, the Longtine method was used for yeast construction (Longtine et al. 1998). pGFP-ALP was used as an ALP localization reporter (Cowles et al. 1997).

**Yeast strains used in this study**

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### Microscopy methods

Vacuolar membrane staining with FM4-64 was carried out as described previously (Segarra et al. 2015). Microscopy was performed on an Olympus fluorescence BX61 upright microscope equipped with Nomarski differential interference contrast (DIC) optics, a Uplan S Apo 100x objective (NA 1.4), a Roper Cool-Snap HQ camera, and Sutter Lambda 10–2 excitation and emission filter wheels, and a 175 watt Xenon remote source with liquid light guide. Image capture was automated using Intelligent Imaging Innovations Slidebook 4.01 for Mac. A series of optical Z-sections (0.25 μm) was captured for each cell analyzed. Prior to analysis, the stacks were deconvolved using the nearest neighbor algorithm. Representative single-plane micrographs from cells at log phase were chosen to be included in the figures.

To quantify co-localization of Atg27p with Chc1p or organellar markers, deconvolved Z-stacks were examined to confirm that both fluorescent signals were in the same plane, and that peak fluorescence overlapped in corresponding sections. Co-localization was expressed as the percent of structures of interest that contained the GFP-tagged Atg27p construct. To determine statistical significance, two-tailed Student’s *t*-tests were performed to compare each condition of interest to the WT control.

### Acknowledgments:
VAS thanks the Department of Biology and the Wanek School of Natural Sciences at High Point University for resources that allowed for the writing of this manuscript. We thank Greg Odorizzi for the GFP-ALP and SEC7-dsRed plasmids. We also thank David Katzmann for the mcherry-TLG1 and VPS27-RFP plasmids.

### References


Funding: This work was supported by National Institutes of Health grant R01-GM055796 to SKL and T32-HL07188 to VAS.

Author Contributions: Verónica A. Segarra: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review and editing. Anupam Sharma: Investigation, Validation, Conceptualization, Visualization, Writing - review and editing. Sandra K. Lemmon: Conceptualization, Resources, Project administration, Writing - review and editing.

Reviewed By: Anonymous

History: Received January 16, 2021 Revision received March 21, 2021 Accepted March 24, 2021 Published March 29, 2021

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Citation: Segarra, VA; Sharma, A; Lemmon, SK (2021). Atg27p localization is clathrin- and Ent3p/5p-dependent. microPublication Biology. https://doi.org/10.17912/micropub.biology.000381