

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

Verónica A. Segarra<sup>1§</sup>, Anupam Sharma<sup>2</sup> and Sandra K. Lemmon<sup>3</sup>

<sup>1</sup>Department of Biology, High Point University, High Point, NC, USA 27268

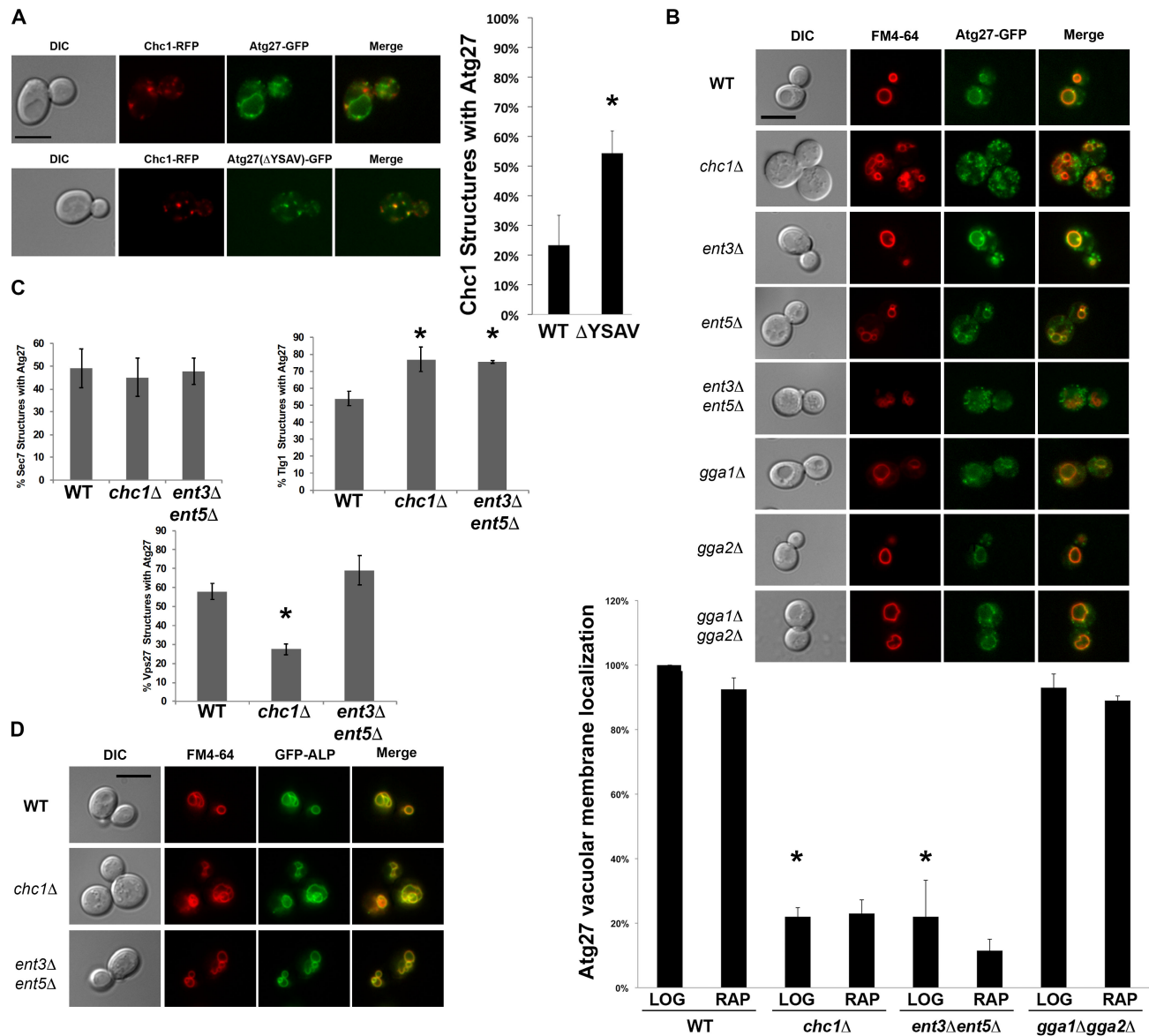
<sup>2</sup>Department of Microbiology, University of Georgia, Athens, GA, USA 30602

<sup>3</sup>Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL, USA 33101

<sup>§</sup>To whom correspondence should be addressed: vsegarra@highpoint.edu

### 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 ( $n \geq 100$ , 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 co-localization of Atg27p-GFP with Golgi (Sec7p) and endosomal (Tlg1p early; Vps27p late) markers in clathrin and *ent3/ent5* mutants. Bar graphs indicate the percentage of indicated Golgi/endosomal structures containing Atg27p-GFP ( $n \geq 100$ , 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 ( $\Delta$ YSAV). 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  $\Delta$ YSAV 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 vacuolar 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 organellar 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  $\mu$ g/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**

Name	Alias	Genotype	Panel	Reference
SL5970	Chc1-RFP Atg27-GFP	<i>MATa leu2 ura3 trp1 his3 lys2 CHC1-RFP::KANMX6 ATG27-GFP::HISMX</i>	1A	This Paper
SL6154	Chc1-RFP Atg27( $\Delta$ YSAV)-GFP	<i>MATa leu2 ura3 trp1 his3 lys2 CHC1-RFP::KANMX6 ATG27(-YSAV)-GFP::HISMX</i>		
SL5837	Atg27-GFP	<i>MAT<math>\alpha</math> leu2 ura3-52 trp1 his3-<math>\Delta</math>200 ATG27-GFP::HISMX6</i>	1B	Segarra <i>et al.</i> 2015
SL6240	<i>chc1<math>\Delta</math></i> Atg27-GFP	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>chc1<math>\Delta</math></i>::LEU2 ATG27-GFP::HISMX</i>		This paper
SL5945	<i>ent3<math>\Delta</math></i> Atg27-GFP	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>ent3<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6</i>		
SL5946	<i>ent5<math>\Delta</math></i> Atg27-GFP	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>ent5<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6</i>		
SL5947	<i>ent3<math>\Delta</math> ent5<math>\Delta</math></i> Atg27-GFP	<i>MAT<math>\alpha</math> leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>ent3<math>\Delta</math></i>::TRP1 <i>ent5<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6</i>		
SL6214	<i>gga1<math>\Delta</math></i> Atg27-GFP	<i>MAT<math>\alpha</math> leu2 ura3-52 trp his3-<math>\Delta</math>200 <i>lys2-801 gga1<math>\Delta</math></i>::HIS3 ATG27-GFP::HISMX</i>		
SL6215	<i>gga2<math>\Delta</math></i> Atg27-GFP	<i>MAT<math>\alpha</math> leu2 ura3-52 trp his3-<math>\Delta</math>200 <i>lys2-801 gga2<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX</i>		
SL6222	<i>gga1<math>\Delta</math> gga2<math>\Delta</math></i> Atg27-GFP	<i>MAT<math>\alpha</math> leu2 ura3-52 trp his3-<math>\Delta</math>200 <i>lys2-801 gga1<math>\Delta</math></i>::HIS3 <i>gga2<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX</i>		
SL7575	<i>chc1<math>\Delta</math></i> Atg27-GFP Sec7-dsRed	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>scd1-v chc1<math>\Delta</math></i>::LEU2 ATG27-GFP::HISMX6 <i>pSEC7-dsRed (316)</i></i>	1C	
SL7576	<i>chc1<math>\Delta</math></i> Atg27-GFP mcherry-Tlg1	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>scd1-v chc1<math>\Delta</math></i>::LEU2 ATG27-GFP::HISMX6 <i>pRS316-mcherry-TLG1</i></i>		
SL7577	<i>chc1<math>\Delta</math></i> Atg27-GFP Vps27-RFP	<i>MATa leu2 ura3-52 trp1 his3-<math>\Delta</math>200 <i>scd1-v chc1<math>\Delta</math></i>::LEU2 ATG27-GFP::HISMX6 <i>pRS316-VPS27-RFP</i></i>		
SL7572	<i>ent3<math>\Delta</math> ent5<math>\Delta</math></i> Atg27-GFP Sec7-dsRed	<i>MAT<math>\alpha</math> ura3-52 his3 <math>\Delta</math> -200 <i>trp1901 leu2-3,112 lys2-801 suc2- <math>\Delta</math>9 <i>ent3<math>\Delta</math></i>::TRP1 <i>ent5<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6 <i>pSEC7-dsRed (316)</i></i></i>		
SL7573	<i>ent3<math>\Delta</math> ent5<math>\Delta</math></i> Atg27GFP mcherry-TLG1	<i>MAT<math>\alpha</math> ura3-52 his3 <math>\Delta</math> -200 <i>trp1901 leu2-3,112 lys2-801 suc2- <math>\Delta</math>9 <i>ent3<math>\Delta</math></i>::TRP1 <i>ent5<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6 <i>pRS316-mcherry-TLG1</i></i></i>		
SL7574	<i>ent3<math>\Delta</math> ent5<math>\Delta</math></i> Atg27GFP Vps27-RFP	<i>MAT<math>\alpha</math> ura3-52 his3 <math>\Delta</math> -200 <i>trp1901 leu2-3,112 lys2-801 suc2- <math>\Delta</math>9 <i>ent3<math>\Delta</math></i>::TRP1 <i>ent5<math>\Delta</math></i>::TRP1 ATG27-GFP::HISMX6 <i>pRS316-VPS27-RFP</i></i></i>		

SL7575	<i>chc1Δ</i> Atg27-GFP Sec7-dsRed	<i>MATa leu2 ura3-52 trp1 his3- Δ200 scd1-v chc1Δ::LEU2 ATG27-GFP::HISMX6 pSEC7-dsRed (316)</i>		
SL7576	<i>chc1Δ</i> Atg27-GFP mcherry-Tlg1	<i>MATa leu2 ura3-52 trp1 his3- Δ 200 scd1-v GAL2? chc1Δ::LEU2 ATG27-GFP::HISMX6 pRS316-mcherry-TLG1</i>		
SL7577	<i>chc1Δ</i> Atg27-GFP Vps27-RFP	<i>MATa leu2 ura3-52 trp1 his3- Δ 200 scd1-v GAL2? chc1Δ::LEU2 ATG27-GFP::HISMX6 pRS316-VPS27-RFP</i>		
SL6679	<i>pGFP-ALP</i> ( <i>SL6610</i> )	<i>MATα leu2 ura3-52 trp1 his3-Δ200 pGFP-ALP</i>	1D	This paper
SL6681	<i>chc1Δ</i> GFP-ALP	<i>MATα leu2 ura3-52 trp1 his3-Δ200 chc1Δ::LEU2 pGFP-ALP</i>		
SL6680	<i>ent3Δ ent5Δ</i> GFP-ALP	<i>MATα leu2 ura3-52 trp1 his3-Δ200 ent3Δ::TRP1 ent5Δ::TRP1 pGFP-ALP</i>		

### 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

- Casler JC, Glick BS. A microscopy-based kinetic analysis of yeast vacuolar protein sorting. *Elife*. 2020 Jun 25;9:e56844. DOI: 10.7554/eLife.56844 | PMID: 32584255.
- Costaguta G, Duncan MC, Fernández GE, Huang GH, Payne GS. 2006. Distinct roles for TGN/endosome epsin-like adaptors Ent3p and Ent5p. *Mol Biol Cell* 17: 3907-20. PMID: 16790491.
- Cowles CR, Odorizzi G, Payne GS, Emr SD. 1997. The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell* 91: 109-18. PMID: 9335339.
- Day KJ, Casler JC, Glick BS. 2018. Budding Yeast Has a Minimal Endomembrane System. *Dev Cell* 44: 56-72.e4. PMID: 29316441.
- Ding J, Segarra VA, Chen S, Cai H, Lemmon SK, Ferro-Novick S. 2016. Auxilin facilitates membrane traffic in the early secretory pathway. *Mol Biol Cell* 27: 127-36. PMID: 26538028.
- Grissom JH, Segarra VA, Chi RJ. 2020. New Perspectives on SNARE Function in the Yeast Minimal Endomembrane System. *Genes (Basel)* 11: 899. PMID: 32781543.
- Guthrie C, Fink GR. 1991. Guide to yeast genetics and molecular biology. *Methods Enzymol* 194:1–863. PMID: 2005781.
- Huang KM, D'Hondt K, Riezman H, Lemmon SK. 1999. Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO J* 18: 3897-908. PMID: 10406795.

Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14: 953-61. PMID: 9717241.

Segarra VA, Boettner DR, Lemmon SK. 2015. Atg27 tyrosine sorting motif is important for its trafficking and Atg9 localization. *Traffic*. 16(4):365-378. PMID: 25557545.

Segarra, VA; Sharma, A; Lemmon, SK. 2021. Atg27p co-fractionates with clathrin-coated vesicles in budding yeast. *microPublication Biology*. DOI: 10.17912/micropub.biology.000380.

Stepp JD, Huang K, Lemmon SK. 1997. The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. *J Cell Biol* 139: 1761-74. PMID: 9412470.

Suzuki SW, Emr SD. 2018. Membrane protein recycling from the vacuole/lysosome membrane. *J Cell Biol* 217: 1623-1632. PMID: 29511122.

Vowels JJ, Payne GS. 1998. A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. *EMBO J* 17: 2482-93. PMID: 9564031.

**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

**Copyright:** © 2021 by the authors. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**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>