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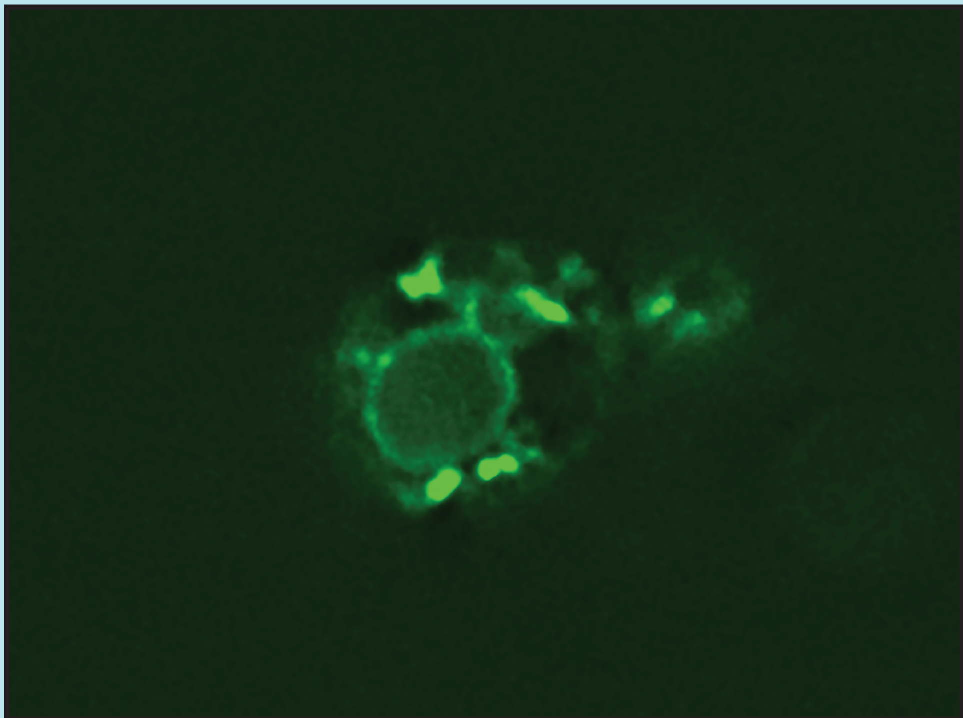

Volume 5, 2023 eBio No. 7

eBio

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Cover Photograph: A snapshot of a live budding yeast cell expressing fluorescently-tagged wildtype Atg27 protein. Micrograph generated by Hannah M. Smith.

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2023 *eBio* 7:1–6

Generation of a Fluorescent Atg27p Mutant Abrogated for its Putative Mannose-6-Phosphate Receptor Homology (MRH) Domain

Hannah M. Smith[1,2], Brenna J. Ivory[1], Kathleen M. Haesemeyer[3], Stacey O. Brito[3], Kyani A. Quarles[3], Elizabeth Cabrera[1], Meaghan R. Robinson[1], Nicholas Zanghi[1,4], Taylor Cunningham[1,5], Molly C. Holbrook[1,6], Deanna C. Clemmer[1,7] Thomas Moss[1,8], Isabel Moreno Vazquez[3], and Verónica A. Segarra[3, 9*]

Abstract - While the identified function of the C-terminus of Atg27p, a transmembrane protein that contributes to cellular self-eating or autophagy, is to control the localization of the protein throughout the endosomal/vacuolar system, the function of its lumenal domain remains unknown. Using bioinformatics tools, we confirm that Atg27 has a predicted mannose-6-phosphate receptor domain (MRH) and identify key conserved tyrosine (Y31) and arginine (R44) residues that, when mutated, would likely abrogate the putative MRH function of Atg27p. Mutating these two residues in Atg27p yields protein molecules that are stably expressed in cells as determined by fluorescence microscopy.

Summary

Autophagy is a conserved process by which eukaryotic cells recycle damaged or unnecessary cellular components. This process enables cells to survive through periods of stress such as starvation. A unique hallmark of autophagy is the formation of autophagosomes, temporary and large double-membraned vesicles that sequester cellular components to be digested and recycled in the degradative organelle of the cell, the vacuole in yeast.

Atg27p is one of the autophagy-related proteins responsible for bringing about the process of autophagy in *Saccharomyces cerevisiae* (Baker's or Budding yeast). Atg27p is a single-pass, Type-I (C-terminus cytoplasmic) transmembrane protein that is thought to facilitate the mobilization of membrane material to the pre-autophagosomal structure (PAS), the location in the cell where autophagosomes form. Atg27p localizes to the late Golgi, vacuole, endosomes, PAS, and clathrin-positive structures (Ma et al. 2017; Segarra et al. 2015; Segarra et.al. 2021a, b; Suzuki and Emr 2018). Cells missing the *ATG27* gene have delayed autophagy, produce fewer autophagosomes, and display altered trafficking of Atg9p, a highly conserved core autophagy transmembrane protein that facilitates the mobilization of membrane material for early autophagosome formation (Legakis et al. 2007, Mari et al. 2010, Segarra et al. 2015, Yamamoto et al. 2012, Yen et al. 2007).

[1]High Point University, Department of Biology, High Point, NC 27268. [2]Current Affiliation: Department of Biodiversity, Earth, and Environmental Science, Drexel University, Philadelphia, PA 19104 [3]Department of Biological Sciences, Goucher College, Baltimore, MD 21204. [4]Current affiliation: Rowan-Virtua School of Osteopathic Medicine, Stratford, New Jersey, 08084. [5]Current Affiliation: West Virginia University School of Medicine, Department of Pediatrics, Morgantown, WV 26506. [6]Current Affiliation: Department of Biological Sciences, University of North Carolina, Charlotte, USA 28223. [7]Current Affiliation: Department of Microbiology and Immunology, State University of New York (SUNY) Upstate Medical University, Syracuse, NY 13210. [8]Current Affiliation: Department of Internal Medicine, Edward Via College of Osteopathic Medicine, Spartanburg, SC 29303. [9]Department of Chemistry, Goucher College, Baltimore, MD 21204. *Corresponding author

Associate Editor: Christoper Gissendanner, University of Louisiana Monroe

While the ~50-residue cytoplasmic C-terminus of Atg27p contains sorting signals that mediate its endo-vacuolar localization and trafficking (Segarra et al. 2015, Suzuki and Emr 2018), the function of its ~179-residue N-terminal lumenal domain has yet to be empirically studied. *In silico* methods predict that the lumenal domain of Atg27p contains a mannose-6-phosphate receptor homology (MRH) domain (Kelley et al. 2015, Segarra et al. 2015, Suzuki and Emr 2018). In mammalian cells, MRH domains are known to recognize and bind to protein cargoes modified with mannose-6-phosphate, such as lysosomal hydrolases, which are destined for transport to the lysosome (Castonguay et al. 2011). While MRH domain containing proteins are not common in yeast, both Mrl1p and Yos9p have been identified as MRH-like based on a combination of sequence and function conservation (Hosokawa et al. 2010, Whyte and Munro 2001). Mrl1p is thought to act as a sorting receptor in the delivery of vacuolar hydrolases while Yos9 is a lumenal membrane-associated ER protein required for endoplasmic reticulum-associated degradation (ERAD) of glycoproteins. If Atg27p contains a lumenal MRH-like domain, it might function to recognize modified vacuolar hydrolases or other protein cargo and facilitate delivery to the vacuole.

It is known that MRH domains bind mannose in a manner that is dependent upon critical glutamine (Q), arginine (R), glutamate (E), and tyrosine (Y) residues (Castonguay et al. 2011). Y31 and R44 of Atg27p stood out as the only tyrosine and arginine within the N-terminal domain. Amino acid sequence alignment of the indicated N-terminal regions of the MRH-containing proteins Yos9p, Mrl1p, and Atg27p revealed that Y31 and R44 in the predicted MRH domain of Atg27p align with their equivalent residues in Yos9p and Mrl1p, with identical spacing exactly 13 residues apart in all the three proteins (Fig. 1A).

To begin to characterize the putative MRH domain of Atg27p, site-directed mutagenesis of a functional *ATG27-GFP* reporter (Carrigan et al. 2011, Segarra et al. 2015) was used to express mutant proteins that contain alanines, singly and/or in combination, instead of tyrosine 31 and arginine 44. In our study, we mutated tyrosine (Y) 31 and arginine (R) 44 (Fig. 1A) to alanine (A). Y31 and R44 are the only Y and R residues in the putative MRH domain of Atg27p. By mutating these residues to A, singly and in combination, we aimed to abrogate the putative MRH domain of Atg27p. Once the desired mutations were confirmed by sequencing, live cell fluorescence microscopy was used to ascertain the mutant Atg27p proteins were stable and successfully expressed by cells (Fig. 1B). The general localization profile of the mutant Atg27p molecules is what we would expect given what we know about the wildtype protein. For example, N-terminal MRH mutants of Atg27p were able to localize to the vacuolar membrane. This is synergistic with what is known about Atg27p vacuolar localization as previous findings have shown that vacuolar localization of Atg27p is dependent on a sorting signal on its C-terminus (Segarra et al. 2015).

A detailed examination of the localization of these mutants using co-localization standards is beyond the scope of this short communication and will be the focus of future studies.

Materials and Methods

Yeast and plasmid methods. Standard methods were used for genetic manipulations and growth of yeast (Guthrie and Fink 1991). *S. cerevisiae* strains used in this study are listed in Table 1 below. Yeast strains were constructed using the Longtine method and their genotypes confirmed using polymerase chain reaction (PCR) (Longtine et al. 1998). Cells were transformed with the indicated plasmids (Table 2) using the standard lithium acetate and single-stranded DNA (ssDNA) carrier/polyethylene glycol (PEG) method (Gietz and Schiestl 2007).

Site-directed mutagenesis. Standard site-directed mutagenesis (Carrigan et al. 2011) was used to generate MRH abrogated mutants. In short, the plasmid containing a functional *ATG27-GFP* reporter (Segarra et al. 2015) was amplified with mutagenic primers that would change codons 31 and 44 of *ATG27* to code for alanine instead of tyrosine and arginine, respectively, singly and/or in combination. The mutagenic primers used to modify codon 31 are: 5'-GATGTATTGAAAAAGGCTCAGGTGGGAAAATT-3' (anneals to bottom strand) and 5'-AATTTTCCCACCTGAGCCTTTTTCAATACATC-3' (anneals to top strand). The mutagenic primers used to modify codon 44 has the following sequence: 5'-CTAACTTC-TACGGAAGCGGATACTCCGCCAAG-3' (anneals to bottom strand) and 5'-CTTGGCG-GAGTATCCGCTTCCGTAGAAGTTAG-3' (anneals to top strand). After the mutagenized plasmid was generated, successful mutation of the desired positions was confirmed by

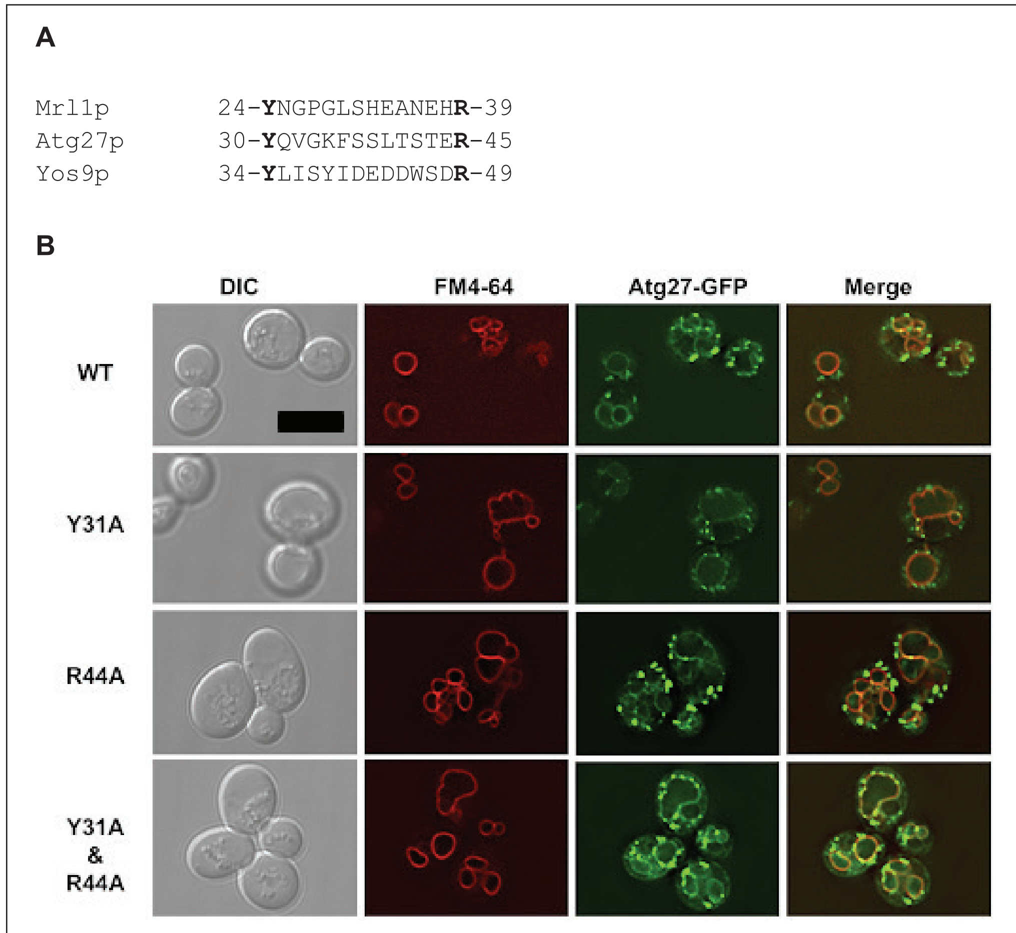

Figure 1. Atg27p contains a putative mannose-6-phosphate receptor (MRH) domain. (A) Amino acid sequence alignment (Clustal Omega; Sievers et al. 2011) of the indicated regions of the MRH-containing proteins Yos9p, Mrl1p, and Atg27p. (B) Putative mannose-6-phosphate receptor domain Atg27p Y31A and/or R44A mutants are stably expressed in yeast cells. Yeast cells deleted for endogenous ATG27 were transformed with plasmids containing either the wild type ATG27-GFP or mutant ATG27-GFP reporter gene of interest (Y31A, R44A, singly or in combination). The vital dye FM4-64 is used to mark the vacuolar membrane. Scale bar = 5 microns.

Table 1. Yeast strains used in this study.

Name	Alias	Genotype	Reference
VS92	Atg27	MATα leu2 ura3-52 trp1 his3-Δ200 atg27Δ::HISMX6 pRS416-ATG27-GFP	
VS93	(Y31A)ATG27	MATα leu2 ura3-52 trp1 his3-Δ200 atg27Δ::HISMX6 pRS416-(Y31A)ATG27-GFP	This study
VS94	(R44A)ATG27	MATα leu2 ura3-52 trp1 his3-Δ200 atg27Δ::HISMX6 pRS416-(R44A)ATG27-GFP	
VS95	(Y31A/R44A)ATG27	MATα leu2 ura3-52 trp1 his3-Δ200 atg27Δ::HISMX6 pRS416-(Y31A/R44A)ATG27-GFP	

Table 2. Plasmids used in this study.

Name	Gene Expressed	Promoter	Type	Selectable Marker	Parent Vector	Reference
pRS416	None	None				Sikorski and Hieter, 1989
p*ATG27*	*ATG27-GFP*					Nunnari Lab, see Segarra et al., 2015
p*(Y31A)ATG27*	*(Y31A)ATG27-GFP*	*ATG27*	*CEN*	*URA3*	pRS416	This study
p*(R44A)ATG27*	*(R44A)ATG27-GFP*					This study
p*(Y31A/R44A)ATG27*	*(Y31A/R44A)ATG27-GFP*					This study

DNA sequencing. Mutagenized [*(Y31A)ATG27, (R44A)ATG27, (Y31A/R44A)ATG27*] or wild-type *ATG27* plasmids (Table 2) were then transformed into strains of yeast deleted for genomic *ATG27* (Table 1), allowing the plasmid-borne *ATG27* gene to be the sole source of information for cells to express the protein.

Microscopy methods. Yeast cells in logarithmic growth were imaged in selective growth medium, and z-stacks were collected at 0.25-μm increments on a DeltaVision elite workstation (Cytiva) based on an inverted microscope (IX-70; Olympus) using a 100×1.4NA oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) and deconvolved using the iterative-constrained algorithm and the measured point spread function. Image analysis and preparation was done using Softworx 6.5 (Cytiva). FM4-64 was used to stain vacuolar membranes as described previously (Segarra et al. 2015).

Acknowledgements

The authors would like to thank the Department of Biology and the Wanek School of Natural Sciences at High Point University (HPU) for initial resources and Goucher College and its Departments of Biological Sciences and Chemistry for the resources that allowed the project to be completed. Additional support was provided by HPU through Summer Undergraduate Research Program fellowships to HMS, BJI, and DCC. HMS and NZ would like to thank the Natural Sciences Fellows Program at HPU for additional resources and mentorship. The authors would like to thank Sarah Edmark and Emily K. Davis for assistance with some of the site-directed mutagenesis and writing, respectively.

Funding. This work was supported by an internal HPU Research Advancement Grant to VAS (17-065). Additional support was provided by HPU through Summer Undergraduate Research Program fellowships to MH, HMS and BJI. KMH, SOB, KAQ, IMV, and VAS additionally thank the Departments of Biological Sciences and Chemistry at Goucher College for the funding to complete and publish the work.

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