Research Article

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Autophagy in *Saccharomyces cerevisiae*: A Study of Arl1p's Role in Heat and Starvation-Induced Cell Stress

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Abstract

ADP-ribosylation factor-like protein (Arl1p) is a component of autophagy pathways within *Saccharomyces cerevisiae* (*S. cerevisiae*), a model organism used in studying human autophagy pathways and related diseases. In the non-specialized macroautophagy and specialized Cytoplasm-to-vacuole (Cvt) trafficking pathways, Arl1p facilitates three main processes: synthesis of acidic phagophores, facilitation of the docking and tethering of Atg9p mediated autophagosomes, and fusion of autophagosomes with the vacuole. The current understanding of autophagy emphasizes the importance of cellular recycling for cellular maintenance during growth. We used fluorescence microscopy to visualize acridine orange (AO) stained acidic compartments and DAPI stained nuclei, and analyzed AO/DAPI quantity in cells incubated in conditions of both nitrogen starvation and non-permissive temperatures. We found significant increases in autophagosomes within the cytoplasm of *arl1Δ* mutants after incubation in nitrogen starvation conditions, thus determining that *arl1Δ* mutants are unable to fuse autophagosome content. We conducted western blots by antibody tagging aminopeptidase 1 (Ape1) to determine Arl1p's role in the Cvt pathway. We found starvation conditions resulted in significantly more Ape1 translocation, and no significant difference between *arl1Δ* mutant and wildtype cells. Thus, Arl1p maintains a supporting role in autophagy.

Introduction

The macroautophagy (autophagy) biochemical pathway in *Saccharomyces cerevisiae* involving the ADP-ribosylation factor-like protein 1 (Arl1p), a GTPase within the Arf/Sal/Sar family (Yang & Rosenwald, 2018), is an essential and conserved process of cellular recycling (Zhang et al., 2007). The autophagy pathway, when non-functioning, has detrimental impacts on mitochondrial function and cellular maintenance. This is due to its inability to recycle mitochondrial components resulting in growth limitations for the cell (Zhang et al., 2007). Activation of a starvation response within S. cerevisiae, outlined in Figure 1, initiated by the inhibition of TOR protein complex 1 (TORC1p) by Rapamycin, induces autophagy (Wang et al., 2017). This starvation response activates signal transduction pathways and negative regulation by Ras proteins, RhoAp and ROCKp, Rac/Rho/Cdc42 protein family (Yang & Rosenwald, 2018), and PKAp (Inoue & Klionsky, 2010). The complete biochemical pathway for the initiation of the starvation response is not known. Arl1p is activated upon induction of the

starvation response by the activation of Arl3p and the transmembrane protein Sytp at the trans-Golgi Network (TGN) (Wang et al., 2017). The guanine nucleotide exchange factors (GEFs) involved in the activation of the Arl3p and Arl1p pathway are not known. Arl1p facilitates a synonymous secondary pathway to the protein Ypt6p with independent effectors and signal cascades (Benjamin et al., 2011).

Arl1p facilitates the synthesis of phagophores, a double-membrane acidic vesicle, at the phagophore assembly site (PAS) (Yang & Rosenwald, 2016). Phagophores mature in the cytoplasm to autophagosomes containing an Atg9 transmembrane protein (Atg9p) (Kakuta et al., 2012) to collect proteins and organelles from the cytoplasm for cellular recycling (Yang & Rosenwald, 2016). Atg9p is necessary for autophagosome fusion into the vacuole and the subsequent hydrolysis and release of proteins and organelles back into the cytosol (Inoue & Klionsky, 2010). Anterograde trafficking of Atg9p (Inoue & Klionsky, 2010) mediates the tethering of autophagosomes with the t-SNARE protein (Tlg2p) (Yang & Rosenwald, 2016). Activation of Tlg2p and facilitation of the fusion of Atg9p containing autophagosomes is mediated by the Arl1p and "Golgi-associated retrograde protein" (GARP) Vps53p complex (Yang & Rosenwald, 2016). The cytoplasm-to-vacuole trafficking (Cvt) pathway is a specialized autophagy pathway mediated by the anterograde trafficking of aminopeptidase 1 (Ape1) receptors, using many of the same mechanisms as the macroautophagy pathway (Abeliovich, 1999). Specifically, Arl1p facilitated Tlg2p is required for the fusion of autophagosomes with the vacuole (Abeliovich, 1999).

Ypt6p's synonymous pathway transcribes protein expression to compensate for the lack of expression of Arl1p at permissive temperatures of 30°C, but is unable to compensate at non-permissive temperatures of 37°C (Yang & Rosenwald, 2018). Optimal temperatures for yeast growth have been found to range from 30°C to 35°C, supporting why the non-permissive temperatures used (37°C) would be suboptimal (Walsh & Martin, 1977). However, studies by Yang & Rosenwald (2016) have found that the Arl1p protein conformation, Arl1 Q72Lp, aids in the mediation of docking and fusion of autophagosomes with the vacuole at non-permissive temperatures. Without the expression of Arl1p at non-permissive temperatures, *S. cerevisiae* would be unable to fuse autophagosomes at the vacuole.

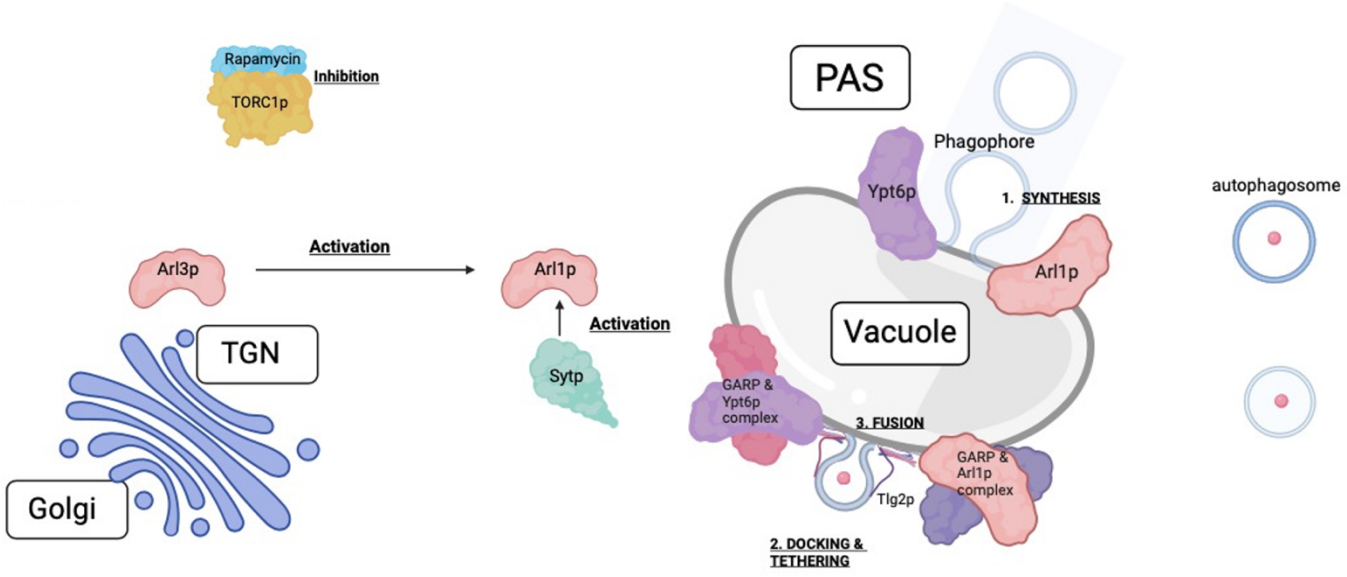

Figure 1. Biochemical Pathway of Yeast Starvation Response and Autophagy Initiation. Rapamycin inhibits TORC1p. Inhibition leads to the activation of Arl1p via Arl3p and Sytp at the trans Golgi network (TGN). Arl1p facilitates phagophore synthesis at the phagophore assembly site (PAS). Autophagosomes containing Atg9p (pink) dock and fuse with Arl1p and GARP complex mediated t-SNARE Tlg2 at the vacuole. The synonymous biochemical pathway for synthesis of phagophores, docking, and fusion is shown of Ypt6p and the corresponding GARP complex. Figure adapted from BioRender.com.

To observe autophagosome formation and fusion, we stained the acidic compartments of wild type (WT-a) and *arl1Δ* mutant cells subjected to non-permissive temperatures and nitrogen starvation with acridine orange (AO). Due to *arl1Δ* mutants lacking expression of Arl1p Q72L, we predicted it would show less fluorescence of acridine orange at non-permissive temperatures in comparison to the wildtype cells during autophagy induced conditions through nitrogen starvation. We investigated Ape1 concentration by Western blot in wildtype and *arl1Δ* mutant cells subjected to the same conditions from our fluorescence staining. We anticipated that less Ape1 would be translocated within the cell during periods of nitrogen starvation in *arl1Δ* mutants in comparison to wildtype cells, due to the Cvt pathway's accelerated induction in starved conditions.

Methods

All materials and equipment were provided by the BIOL 340 laboratory. All lab protocols were sourced from Moussavi (2023).

Inoculating cells in liquid yeast peptone dextrose (YPD) and in yeast nitrogen base (YNB – without amino acids)

We prepared and labeled 8 sterile cell culture tubes, 4 for WT-a and 4 for *arl1Δ* mutants. We inoculated 4 WT-a colonies and 4 *arl1Δ* mutant colonies in Yeast Peptone Dextrose (YPD). All 8 tubes were incubated at a permissive temperature of 30°C and stored overnight. Approximately 24 hours later, we added 200 µL of acridine orange (AO), an acidic fluorescent stain, to each of these samples and incubated them for an additional hour at 30 °C. For half of our samples (2 WT-a colonies and 2 *arl1Δ* mutants) we discarded the remaining YPD and added 1 mL of Yeast Nitrogen Base (YNB without amino acids). We then vortexed each tube briefly to resuspend the formed pellets.

Exposing grown cells to non-permissive temperatures of 37°C

After inoculation, half of our samples were incubated at a non-permissive temperature of 37 °C for 1 hour to induce autophagy (Yang & Rosenwald, 2016).

Prepping cells and staining with DAPI

We prepped our cells for staining by resuspending them with 500 µL of Phosphate Buffer Saline (PBS) and vortexing them gently. We then pipetted 10 µL of each sample, and 0.5 µL of DAPI onto a microscope slide, ensuring that each sample was incubated in a dark box for approximately 5-10 minutes prior to placing the cover slip. Since AO has been found to stain the nucleus (Thomé et al., 2016), and DAPI is used to stain the nucleus (*DAPI (4',6-Diamidino-2-Phenylindole)*, n.d.), we used DAPI to correct for false positives and remove the possibility of counting the nucleus as an acidic compartment. One drop of oil was added to the cover slip to allow for visualization at 100x magnification.

Extracting and quantifying protein from Wt-a and *arl1Δ mutant cells*

For crude protein extraction from all 8 cell cultures, 100 µL of beads and 200 µL of LSB were added to each culture and vortexed for 5 minutes. Cultures were then flashed on ice for 5 minutes, placed in heat at 95°C for 10 minutes, and placed directly back on ice. 1 µL of the resulting crude protein was then diluted tenfold with 1X LSB. The protein concentrations were quantified with spectrophotometry.

Running SDS-PAGE gels and transferring to PVDF membranes

We prepared and diluted all protein samples in LSB to an identical concentration of 1.034 µg/µL, based on spectrophotometer concentrations and our standard curve (R^2 = 0.988). We ran two technical replicates on two 12% polyacrylamide gels using the BioRad MiniProtean Tetra setup at 210 volts for 40 minutes. All 8 diluted protein samples made up one technical replicate. We transferred the proteins to polyvinylidene fluoride (PVDF) membranes using the Bio-Rad Transblot unit at 25 volts for 15 minutes. To verify the success of our protein transfer, we stained our membranes with Ponceau Red for 5 minutes.

Antibody tagging for aminopeptidase 1 and α/β tubulin detection via Western blot

We incubated both PVDF membranes for 2 hours with the primary α-aminopeptidase I (yH16) goat poly antibody and the secondary donkey α-goat IgG HRP antibody, each diluted with TBS-T by a factor of 1:200. We then incubated both PVDF membranes with the primary anti a/b tubulin sheep poly antibody and the secondary donkey α sheep HRP antibody for 1.5 hours, each diluted with TBS-T by a factor of 1:1000. For both incubations, we washed the membrane with one 10-minute rinse and three 5-minute rinses. After the antibody tagging process, we visualized our PVDF membranes with the ChemiDoc MP Imaging System.

Stripping and prepping PVDF membranes

Refer to Abcam protocol (Western Blot Membrane Stripping for Restaining Protocol, n.d.). We used a mild stripping buffer composed of glycine, sodium dodecyl sulfate, Tween 20, hydrochloric acid, and distilled water to remove primary and secondary antibodies from the PVDF membranes in between the aminopeptidase 1 and α/β tubulin antibody tagging. The buffer was prepared by Cassandra Sgarbi and Hannah Naysmith.

Results

Increased levels of autophagosome accumulation in WT-a and *arl1Δ* mutant cells are induced by nitrogen starved conditions

To examine the effects of *arl1*'s deletion on the autophagy pathway in stressful conditions, we measured the presence of acidic compartments through acridine orange staining (Figure 2). We observed a DAPI and acridine orange overlay which indicated stained regions of the cells and, further supported by our brightfield microscopy images which showed the locations of whole cells (Figure 2). To measure the fluorescence of cells in each sample we took three cells from each frame, indicated with a white arrow, and quantified their fluorescence with mean gray value using Image J. Through one-way ANOVA tests we found our data showed a significant difference between AO/DAPI intensity ratios of arl1Δ mutants (2.04) and WT-a (0.81) exposed to a nitrogen deficient environment (f-statistic = 2.526, p-value = 0.0494 < 0.05). AO/DAPI intensity ratios between *arl1Δ* mutant and WT-a cells of the control, and both treatments that included non-permissive temperatures, as well as the ratios between different treatment groups showed no significant differences (p-values > 0.05). These results suggest that there was a significant difference in autophagy induction between WT-a and *arl1Δ* mutant cells when nitrogen starved, where WT-a cells contained fewer acidic compartments than the *arl1Δ* mutant cells. We further visualized this in Figure 3B, which showed that the largest percent significant difference in AO/DAPI intensity between *arl1Δ* mutant and WT-a was in nitrogen starved conditions (f-statistic = 2.526, p-value = 0.0494 < 0.05). In addition, while not statistically significant, *arl1Δ* mutant cells had higher AO/DAPI ratio in all treatments compared to WT-a cells indicating more acidic compartments in *arl1Δ* mutant cells (Figure 3A). This was made evident in Figure 3B as the percent differences in AO/DAPI intensity between WT-a and *arl1Δ* mutant cells in all conditions were positive.

To test the function of the selective autophagic Cvt pathway in *S. cerevisiae*, we used western blotting and tagged our PVDF membranes for Ape1 and α/β tubulin (Figure 4A). We used grey values calculated in ImageJ to quantify the quantity of both proteins. The ratio of the Ape1 to α/β tubulin grey values plotted in Figure 4B describes the relative Ape1 amount found in each of our cells exposed to different treatments. Higher grey values indicate lighter pixels and therefore represent a lower quantity of protein. While we found lower Ape1 protein quantities were present in cells exposed to normal or non-permissive temperatures, we saw higher Ape1 amounts in nitrogen starved cells. To identify significant trends, we performed three two-sample t-tests between permissive and non-permissive temperatures, nitrogen starved and non-starved conditions, and WT-a and *arl1Δ* mutants. We found that there was no significant difference in mean grey value for WT-a and *arl1Δ* mutant cells (SD = 0.049, p-value = 0.372 > 0.05). The same was true when we compared cells treated at non-permissive temperatures to cells at regular temperatures (SD = 0.016, p-value = 0.774 > 0.05). However, we did see a significant difference

between nitrogen starved cells and cells that were supplied with nitrogen (SD = 0.109, p-value = 0.0128 < 0.05). As a result, we found that nitrogen starvation does induce Ape1 translocation and production in the selective autophagy Cvt pathway in yeast. Our data likely suggests that in nitrogen

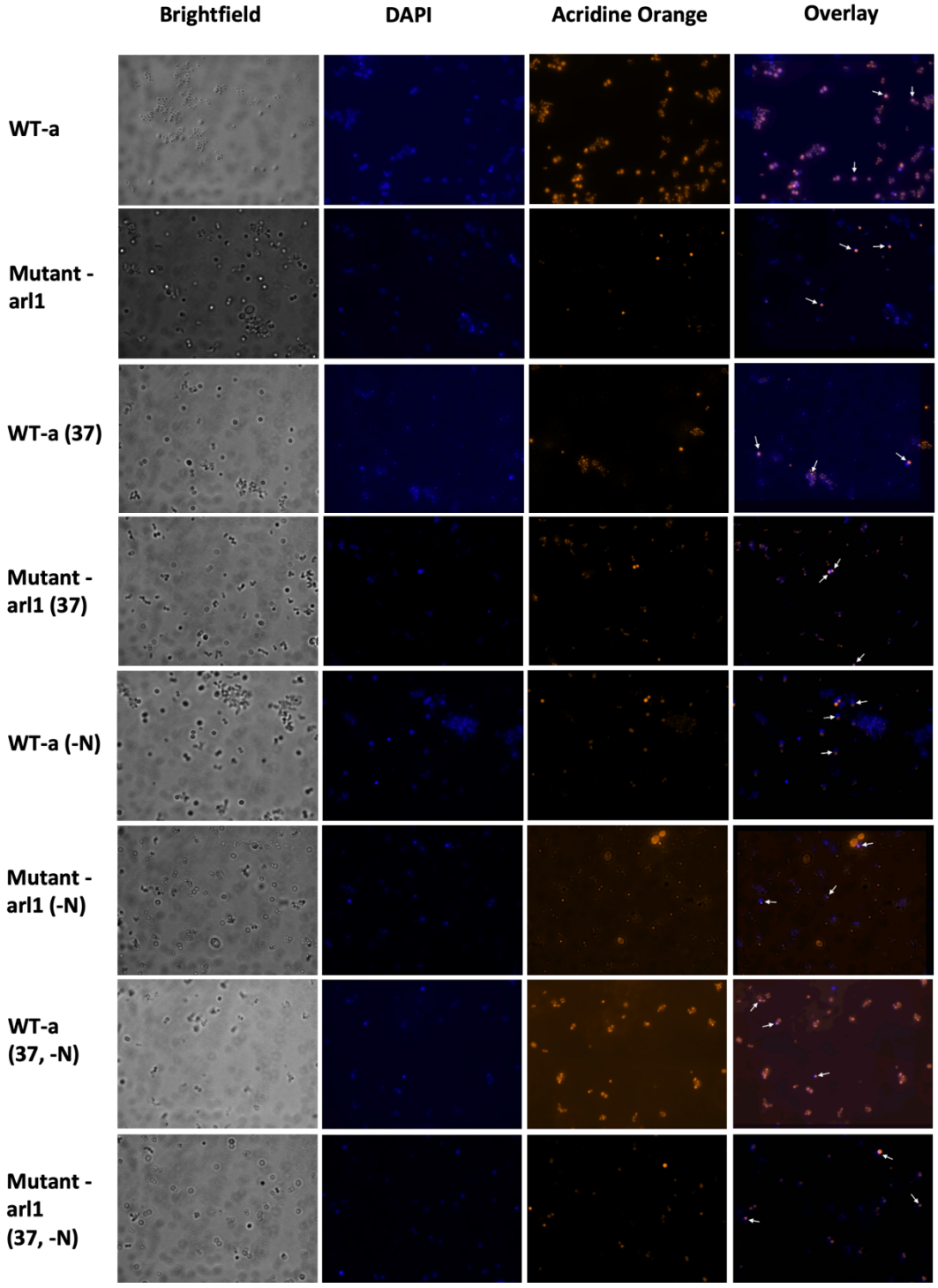

Figure 2. Brightfield and Fluorescence Imaging. DAPI stain (nucleus) and acridine orange stain (nucleus and acidic compartments) were visualized in WT-a and arl1Δ mutant cells under non-permissive temperatures (37°C) and/or nitrogen starved conditions (-N). Arrows represent cells used in quantitative ratio calculations visualized in Figure 3. Overlay images show merged DAPI and acridine orange to further visualize the location of acridine orange in the cells.

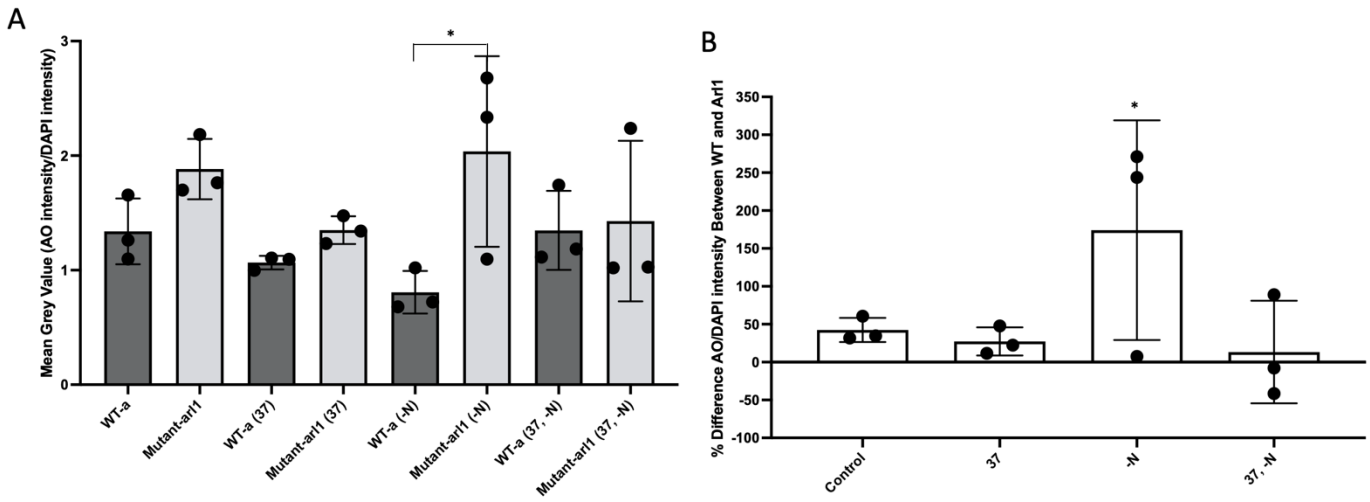

Figure 3. Quantification of AO/DAPI Intensity in WT-a and arl1Δ mutant Cells. **(A)** AO/DAPI ratio cell intensities in cells indicated with an arrow in Figure 2 were quantified with Image J. Dark bands represent WT-a and light bands represent arl1Δ mutant in control, heated (37), starved (-N), and heated+starved (37, -N) conditions. **(B)** Percent difference in AO/DAPI intensity between arl1Δ mutant and WT-a cells in respective treatments. All data are displayed as mean ± SEM. One-way ANOVA performed between groups with p-value < 0.05 indicated by (*).

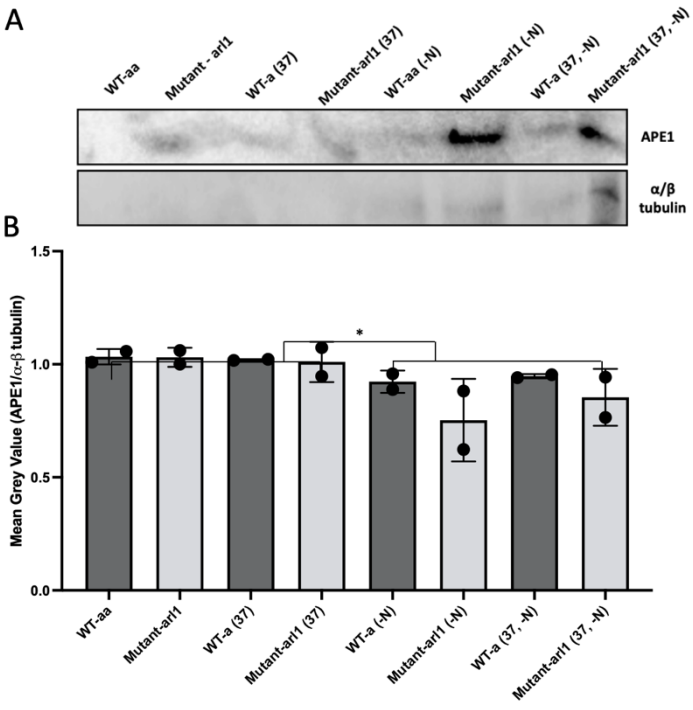

Figure 4. Western blotting for Ape1 quantification. (A) Antibody tagged PVDF membrane for Ape1 and α/β tubulin from WT-a and arl1Δ mutants exposed to permissive temperature and nitrogen deficiency treatments. **(B)** Bar graph showing ratio of Ape1 and α/β tubulin mean grey value along treatments. Dark bands represent WT-a and light bands represent arl1Δ mutants. Mean grey value was quantified with ImageJ. Data are displayed as mean ± SEM. Two-sample t-test indicated *p-value < 0.05.

starved conditions, Arl1p permits increased synthesis, tethering, and fusion of phagophores into the vacuole compared to nitrogen rich conditions.

Discussion

The autophagy pathway is disrupted in *arl1Δ* mutant cells

Previous literature has alluded to Arl1p's role in the autophagic pathway, more specifically its importance in fusing and tethering autophagosomes to the vacuole at non-permissive temperatures (Yang & Rosenwald, 2018). *arl1Δ* mutant cells were found to have higher AO/DAPI intensity than WT-a cells in all treatments, however this was only significant in nitrogen starved conditions. This indicates that in all treatments, *arl1Δ* mutant cells showed higher levels of autophagy than WT-a cells, potentially due to their inability to tether and dock autophagosomes to the vacuole. Yang and Rosenwald (2018) determined that in non-permissive temperatures, Arl1p protein conformation Q72L was necessary for autophagosome docking and fusion. However, in *arl1Δ* mutant cells, this Q72L protein conformation was not synthesized and therefore autophagosomes in *arl1Δ* mutant cells were unable to dock and fuse to the vacuole. This resulted in more autophagosomes in the cytoplasm, which correlates with the high levels of AO stain in arl1Δ mutant cells

(Figure 3A). Our results were not significant however, possibly due to limitations regarding few replications and our restricted view of fusion at the vacuole. While Yang and Rosenwald (2018) explained higher AO intensity in *arl1Δ* mutant cells at non-permissive temperatures, this does not correlate with our main finding; that there was a significant induction of autophagy during nitrogen starved conditions at permissive temperatures.

Nitrogen starvation induces autophagy in *arl1Δ* mutant cells at permissive temperatures

Our results indicated a significant increase in the induction of autophagy during nitrogen starvation compared to autophagy induced by an increase in temperature in conjunction with nitrogen starvation (Figure 3A). Abudugupur et al. (2002), found *arl1Δ* mutants incubated in nitrogen starved conditions were unable to initiate the formation of a central vacuole. Instead, these cells contained high concentrations of vesicle-like acidic compartments within the cytoplasm. Similarly, our findings in *arl1Δ* mutants showed a significant increase in acidic compartments throughout the cell, which may support this finding (Figure 3A). Our equipment limited our ability to visualize cytoplasm, PAS, and the vacuole, which subsequently restricted our conclusions. Previous studies have also found autophagy being primarily induced as the cell undergoes a stationary growth phase in the growth cycle to maintain cellular processes (Alvers et al., 2009). Our results plausibly support the previously discussed study, Alvers et al. (2009), in which autophagy is induced primarily by cellular maintenance in stationary phase over nitrogen starved conditions, similar levels of autophagy fluorescence at temperatures of permissive, and decreased but similar levels of fluorescence at non-permissive temperatures (Figure 3A). Further, Postmus et al. (2008) found increasing incubation temperature resulted in an increase in the growth rate of *S. cerevisiae*. Thus, we speculate the incubation of our cells at non-permissive temperatures may have increased their growth rate. This would imply we conducted our experiment when autophagy was not fully active, and prompted our results to not be indicative of *arl1Δ* mutants' response to temperature stress when autophagy is induced.

Nitrogen starvation induced autophagy increases Ape1 production at permissive temperatures

Through western blotting we were able to compare Ape1 translocation in *S. cerevisiae* in different conditions. No significant difference in Ape1 translocation was found between WT-a and *arl1Δ* mutant cells, as well as cells exposed to permissive and non-permissive temperature (Figure 1B). We did however find a significant difference between starved cells and those exposed to nitrogen rich conditions. While the lack of difference between *arl1Δ* mutant and WT-a cells does not support our hypothesis, our findings did support that Ape1 is present in higher quantities during nitrogen starvation.

The complicated overlap between the Cvt pathway and macroautophagy is a possible explanation for why *arl1Δ* mutant and WT-a cells showed no difference in Ape1 translocation. Unlike the starvation induced autophagic pathway, the selective autophagic pathway works constitutively during nitrogen rich conditions to transport Ape1, but can also work during starvation conditions (Baba et al., 1997). In nitrogen rich conditions, a Cvt complex is formed in the cytoplasm containing the precursor to Ape1, preApe1 (Suzuki et al., 2002). When the cell is starved, Baba et al. (1997) found that the Cvt complex is engulfed with autophagosomes belonging to the macroautophagy pathway. Abeliovich (1999) further supported this data when they found that mature Ape1 is formed when the Cvt pathway is taken over by macroautophagy induced by nitrogen starvation. Both of these studies support our findings that saw higher levels of Ape1 translocation during nitrogen starved conditions (Figure 2).

WT-a and *arl1Δ* mutant cells translocate the same quantity of Ape1 in all conditions.

Higher Ape1 translocation levels in nitrogen starved conditions does not however explain the lack of difference in *arl1Δ* mutants and WT-a cells that indicates Arl1p has no significant role in the Cvt pathway. Yang & Rosenwald (2016) studied the function of Arl1p and Ypt6p in the Cvt pathway. In contrast to our data, they found that Arl1p was necessary in

the Ape1 translocation in the Cvt pathway, but only during non-starvation conditions, whereas Ypt6p was necessary in all stressful conditions including non-permissive temperatures. A possible reason for similarity between *arl1Δ* mutants and WT-a groups (Figure 2) could be that Ypt6p was able to compensate for Arl1p in all conditions since Arl1p was not necessary. Alternatively, this discrepancy may arise from the fact that Yang & Rosenwald (2016) incubated their cells in starvation conditions three hours longer than we did, as well as in solid media. Our short incubation period may not have induced macroautophagy enough for problems in the Cvt pathway caused by *arl1Δ* mutants to be observed.

While Yang & Rosenwald (2016) may have confirmed Arl1p's non necessary role in the Cvt pathway, Abeliovich (1999) demonstrated the complicated nature of Arl1p's role. Tlg2p is an important t-SNARE protein involved in vesicle tethering at the start of the selective Cvt pathway. Yang & Rosenwald (2016) found that Arl1p activates Vsp53p which further binds to Tlg2p initiating tethering in the selective Cvt pathway. However, Abeliovich (1999) findings showed that the transport of preApe1 and the initial mechanisms of the Cvt pathway are controlled independently by Vsp45p, which binds and activates Tgl2p. Since Arl1p has not yet been found to interact with Vsp45p, Arlp is still misunderstood and its complicated role in the Cvt pathway could be the reason for the lack of difference between Ape1 translocation in *arl1Δ* mutants and WT-a cells.

As with cell type, we found no significant difference in Ape1 translocation between cells exposed to permissive and non-permissive temperature. While Suzuki et al. (2002) found that prApe1 and its movement to autophagosomes is temperature dependent, their study exposed cells to non-permissive temperatures for 4 hours. Similar to when we compared our methods of nitrogen starvation, a longer period of exposure to stressful conditions may have been responsible for seeing a significant difference between cell cultures.

We may have been able to gain a better understanding of Ape1 translocation by incubating our cells for longer in stressful conditions if time limitations had not been present. Our lack of replicates and limited gels as well as the discontinued nature of our Ape1 antibodies by Santa Cruz Biotechnology Inc. may also have resulted in a weaker statistical analysis. Additionally, we recognize the quality of our alpha/beta tubulin results was imprecise. This limits our ability to make comprehensive conclusions when comparing our experimental samples.

In an attempt to improve the information we received from our fluorescence microscopy images, we propose using a confocal microscope to image acridine orange in yeast cells. This would provide us with the precise location of acridine orange filled compartments and would allow us to narrow down Arl1p's placement in the autophagy pathway through the extent of autophagosomes fusion to the vacuole. To further investigate the role of Arl1p, we also propose to study the relationship between Ape1 and various degrees of nitrogen starvation. Yang & Rosenwald (2016) found that Arl1p plays a role in both the selective and non-selective autophagy pathways, having grown *S. cerevisiae* cells on nitrogen deficient solid media and incubated for 4 hours. When Wang et al. (2017) rinsed their cells 3 times with nitrogen deficient liquid media before incubating them in that same media for 4 hours, they found that Arl1p was only involved in the non-selective autophagy pathway. Since this difference in stressful conditions may be the reason for contradicting results in literature, conducting a standardized experiment that looks at different methods of nitrogen starvation would help compare current literature discussing the effect of nitrogen starvation on the Cvt pathway.

Yeast as a model for human autophagy

In our study we have been able to better understand the autophagy pathway in *S. cerevisiae*. Autophagy is a process that is highly conserved through the eukaryotic family. As such, certain genes and proteins that control the autophagic pathway in *S. cerevisiae* are similar to those used in human cells and allow for yeast autophagy to serve as a model for human autophagy (Reggiori & Klionsky, 2013). While a homologue for *arl1* has not yet been confirmed in human cells, establishing its role in yeast may help us study the development of certain diseases in humans. Huntington's disease is a neurodegenerative

disorder that may induce neuron death through autophagic processes. Kegel et al. (2000), found that vacuoles carrying the Huntington gene mutant that induces neuron cell death were structurally similar to autophagosomes. Huntington's disease has also been linked to mitochondrial dysfunction (Jakel & Maragos, 2000). Mutations in genes involved in autophagy have been found to induce mitochondrial dysfunction, such as problems with oxygen consumption, in yeast (Zhang et al., 2007). Since Arl1p may be involved in autophagosome formation and fusion, an enhanced understanding of its role could lead us to testing the causes of Huntington's disease.

Conclusion

In this study, we showed Arl1p's integral role in *S. cerevisiae* autophagic pathways, including the selective autophagic Cvt pathway. We observed less fluorescence of acridine orange at non-permissive temperatures in *arl1Δ* mutant cells, lacking Arl1p Q72L expression, in comparison to nitrogen starved wildtype cells. We also observed Ape1 translocation in starved WT-a cells than *arl1Δ* mutant cells due to Arl1p's lack of presence in the Cvt pathway.

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