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Autophagy is a necessary recycling process that occurs in cells, but the functions of the proteins that carry this out are not completely understood. The protein ATG10 has a distinct role in forming autophagosomes, large double membrane vesicles necessary for this process. ATG10 forms a covalent bond between the proteins ATG12 and ATG5 but its final effect on autophagosome formation is still unclear. We are using DNA manipulation techniques to add a myc epitope tag to yeast ATG5 which will allow us to measure the effect of atg10 mutants on ATG12-5 conjugation via Western Blot. These results will help us correlate ATG10 activity to autophagosome size and number.

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Tagging ATG5 with an Epitope Tag to Measure the Amount of ATG12-5 Conjugation in atg10 Mutants

By

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Abstract

Autophagy is a necessary recycling process that occurs in cells, but the functions of the proteins that carry this out are not completely understood. The protein ATG10 has a distinct role in forming autophagosomes, large double membrane vesicles necessary for this process. ATG10 forms a covalent bond between the proteins ATG12 and ATG5 but its final effect on autophagosome formation is still unclear. We are using DNA manipulation techniques to add a myc epitope tag to yeast ATG5 which will allow us to measure the effect of atg10 mutants on ATG12-5 conjugation via Western Blot. These results will help us correlate ATG10 activity to autophagosome size and number.

Introduction

Autophagy is necessary for cell longevity and function (Wu et al., 2018). This process allows cells to dispose of unwanted items and reuse protein materials (Wu et al., 2018). Many autophagic proteins help with the formation of autophagosomes, large double membrane vesicles necessary for this process (Lőrincz & Juhász, 2020). Autophagosomes are transported to the vacuole (the lysosome in animals) where the membranes fuse together, and the cargo carried is then degraded (Lőrincz & Juhász, 2020). The contents are broken down into reusable molecules such as amino acids through the use of hydrolases (Lőrincz & Juhász, 2020). Autophagy is important to keeping cells healthy because it is necessary to break down old or harmful materials such as aggregates or mitochondria (Son et al., 2012). This is especially necessary for neurons since they are unable to replicate and must maintain functional parts for

proper brain function (Son et al., 2012). This is why autophagy is important in studying neurodegenerative diseases such as Alzheimer's (Wu et al., 2018).

The type of autophagy we will be studying is macroautophagy, a process that can take on one of two forms, selective or nonselective (Gatica et al., 2018). Selective autophagy targets a specific type of cargo in the cytoplasm such as pathogens, damaged mitochondria, protein aggregate, and more. Nonselective autophagy, however, targets a random section of the cytoplasm and encapsulates anything in the area (Gatica et al., 2018). This version is induced by starvation in order to produce enough materials to make proteins more necessary for survival at the moment (Gatica et al., 2018). Since we can force starvation on the cells and create a large increase in autophagic activity we use this pathway in order to measure and quantitative differences in autophagic flux which is the amount of degradation due to autophagy.

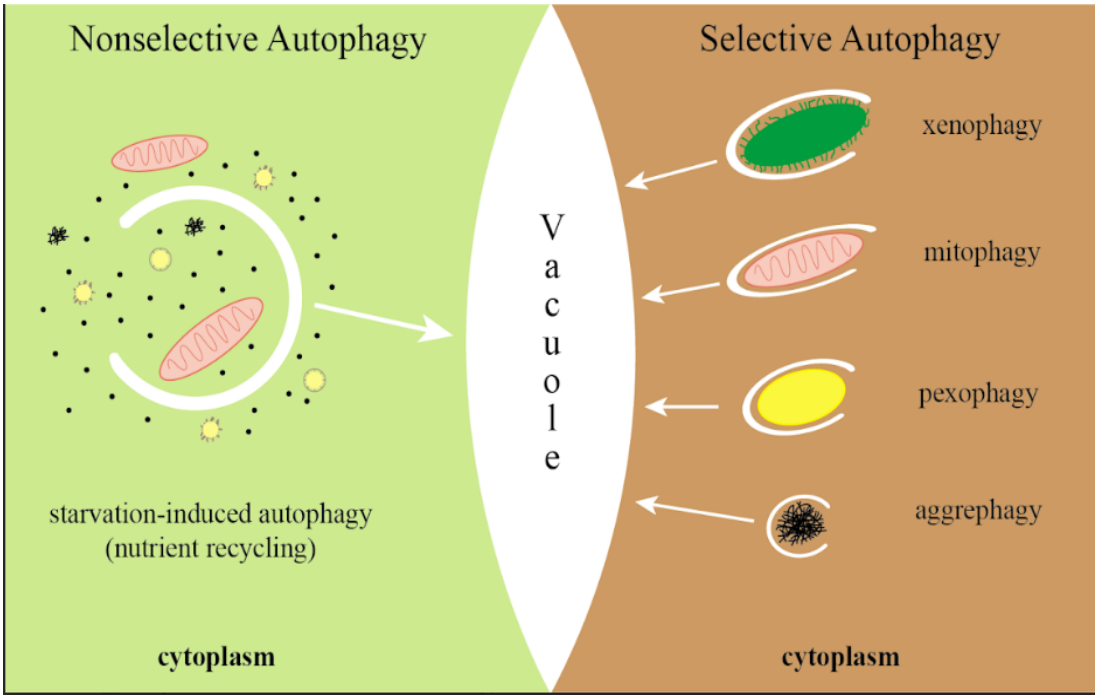

Figure 1: Nonselective and Selective Autophagy. The left shows a random portion of cytoplasm being encased in a double membrane vesicle due to non-selective autophagy. The right shows smaller specific cargo similarly being encased. Diagram made by Steven Backues.

Our lab is currently studying how the autophagic proteins shown in figure 2 each affect autophagosome size and number. The protein ATG7 starts off the cascade and was previously shown to affect both size and number of autophagosomes while ATG8 was shown to only affect size (Xie et al., 2008; Cawthon et al., 2018). The E1 enzyme ATG7 activates the E2 enzymes ATG10 and ATG3. ATG3 attaches ATG8 to PE which is critical for the formation of the autophagosome membrane (Phillips et al., 2008). This process occurs at a very low level naturally and needs to be amplified by the ATG12-5 complex, which is why ATG10 is important in maintaining significant levels of autophagy (Phillips et al., 2008). The hypothesis for this project is that one of the other proteins in the cascade has an additional function that affects autophagosome number that isn't understood yet.

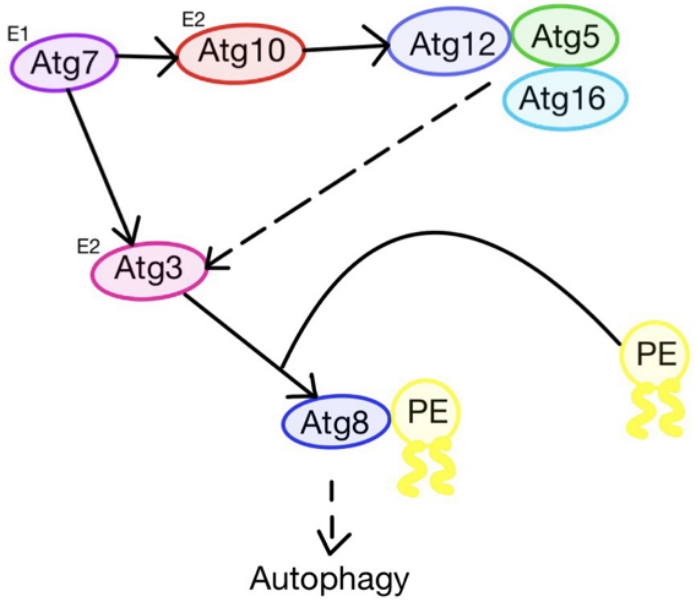

Figure 2: The autophagic pathway by which ATG7 attaches ATG8 to PE via protein of interest ATG10. Diagram made by Nadia Silvia.

To test this we decided to focus on studying ATG10 and its role by looking at mutants that have a partial loss of function. This means that our ATG10 mutants conjugate significantly less ATG12-5 which in turn results in a measurable decrease in autophagic flux. This is so we can see the difference in the autophagosome size and number when autophagic flux is decreased. Previous progress was made on creating and verifying successful atg10 mutants that were significantly different from WT ATG10 and atg10Δ. The ideas for the mutations came from a structural study of ATG10 (Yamaguchi 2012) which showed promising catalytic mutants in the active site that reduced the amount of 12-5 conjugate made. The mutation H131A showed 70% of original 12-5 conjugation, while Y73Q showed 30% (Yamaguchi 2012). These were the metrics we were looking for because we want two mutants that are both significantly different from the WT, atg10Δ, as well as each other. They, however, were all tested *in vitro*, so we had to make sure the results held up *in vivo* within complex yeast cells. We conducted an ALP assay to show the levels of autophagic flux based on a colorimetric enzyme assay of phoΔ80 peptidase that shows rate at which it entered the vacuole for activation via autophagy. The results show that H131A is significantly different from WT but Y73Q was not (figure 3). We conducted further tests and created a double mutant, Y73QH131A, that fits these parameters and was significantly different so we had two successful clones.

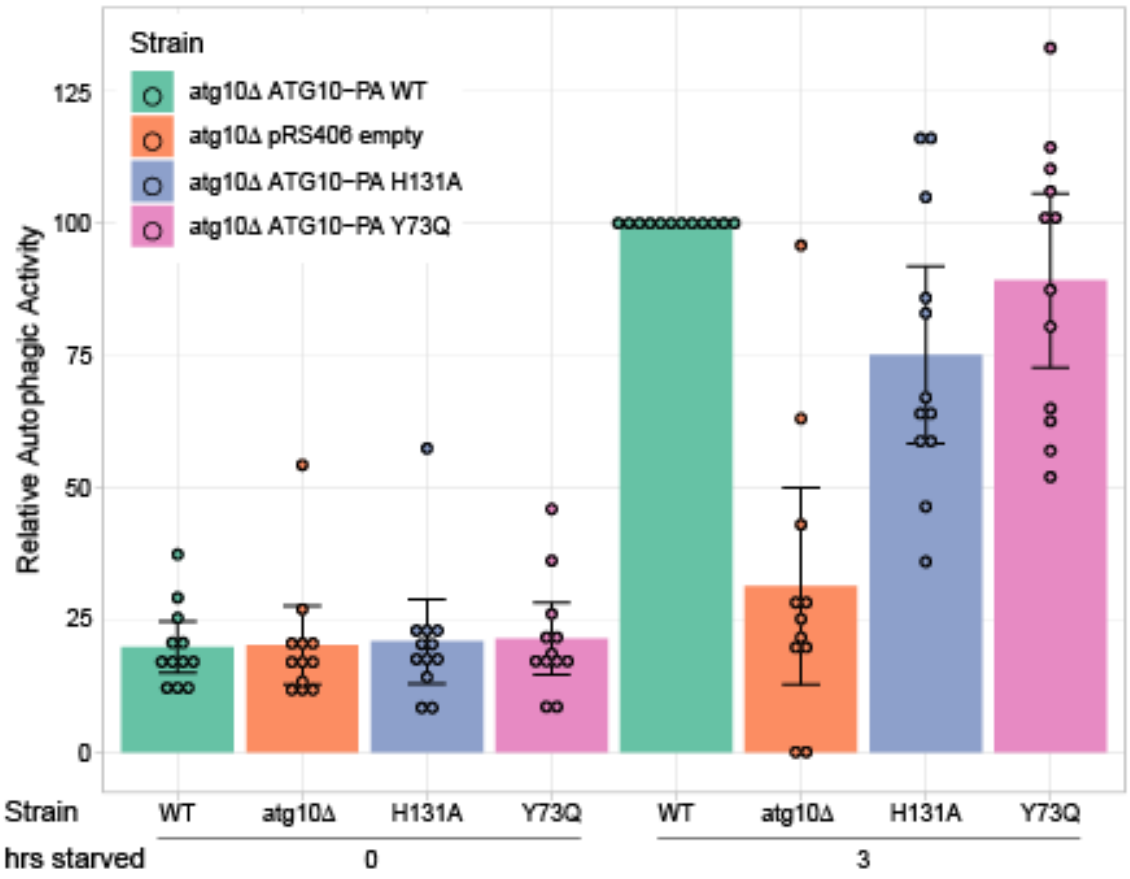

Figure 3: ALP assay data. This shows the amount of autophagic activity in relation to the WT and determines that H131A is significantly different from the WT and atg10Δ based on a 95% confidence interval. Y73Q is not significantly different so can't be used.

The purpose of the current study is to create a system for the measurement of Atg5-12 conjugation for further testing of these mutants. First, we must remove the ATG5 gene already present in our strains of interest. Then we make a myc-tagged version of ATG5 so that the anti-myc antibodies can detect the presence of ATG5 and the size. We then add this back into the strains as the only copy of ATG5 and verify that the tag works. We hypothesize that this will help us quantify the 12-5 conjugation when we perform Western blots to test the direct change that our atg10 mutants have on 12-5 conjugation compared to the WT. Ultimately, these results will help us correlate ATG10 activity to autophagosome size and number.

Methodology

Strains

Table 1: Strains used in this study

Number	Name	Description	Selection
SKB1000	WLY176 atg10D::Kan clone 1	atg10D::kan transformed into WLY176 and verified by PCR (Primers 444 and 482) - Verified by ALP, Ape1	kan
SKB1008	WLY176 atg10D::Kan clone 1	atg10D::kan transformed into WLY176 and verified by PCR (Primers 444 and 482).	kan
SKB1027	atg10D 406E	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 empty linearized with NcoI.	kan, ura
SKB1028	atg10D ATG10-PA(2)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA clone 2 linearized with StuI.	kan, ura
SKB1029	atg10D ATG10-PA(4)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA clone 2 linearized with StuI.	kan, ura
SKB1030	atg10D ATG10-PA Y73Q(1)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA Y73Q clone 1 linearized with StuI.	kan, ura
SKB1031	atg10D ATG10-PA Y73Q(2)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA Y73Q clone 2 linearized with StuI.	kan, ura
SKB1032	atg10D ATG10-PA H131A(2)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA H131A clone 2 linearized with StuI.	kan, ura
SKB1033	atg10D ATG10-PA H131A(4)	WLY176 atg10D::Kan clone 1 (SKB1000) was transformed with pRS406 ATG10-PA H131A clone 4 linearized with StuI.	kan, ura
SKB1051	atg10D::kan Atg10-PA H131A Y73Q Clone 5	WLY176 atg10D::kan (SKB1008) transformed with stu1 digested pRS406 Atg10-PA H131A Y73Q (Clone 5) PCR product.	kan, ura
SKB1052	atg10D::kan Atg10-PA H131A Y73Q Clone 6	WLY176 atg10D::kan (SKB1008) transformed with stu1 digested pRS406 Atg10-PA H131A Y73Q (Clone 6) PCR product.	kan, ura
SKB1059	WLY176 WT pug72 C2	FRY143 FLO5p-ATG7-PA clone 1 (SKB442) transformed with Atg5 delta ura, pug72 pcr product	ura
SKB1060	WLY176 WT pug72	FRY143 FLO5p-ATG7-PA clone 1 (SKB442) transformed with	ura

	C3	Atg5 delta ura, pug72 pcr product	
SKB1063	WLY176 10D pug72 C2	WLY176 atg10D::Kan clone 1 (SKB1008) transformed with Atg5 delta ura, pug72 pcr product	ura
SKB1064	WLY176 10D pug72 C3	WLY176 atg10D::Kan clone 1 (SKB1008) transformed with Atg5 delta ura, pug72 pcr product	ura
SKB1069	pRS414-Atg5-9xmyc C1	Verified by sequencing	amp
SKB1070	pRS414-Atg5-9xmyc C2	Verified by sequencing	amp
SKB1071	WLY176 5D 10D C11	SKB1008 transformed with Atg5 delta, ura marker removed with Cre-Gal and verified by plating. Verified by PCR against atg5 and atg10, and Atg5-myc western blot (no conjugation)	kan
SKB1072	WLY176 5D 10D C18	SKB1008 transformed with Atg5 delta, ura marker removed with Cre-Gal and verified by plating	kan
SKB1073	WLY176 5D C3	SKB442 transformed with Atg5 delta, ura marker removed with Cre-Gal and verified by plating	n/a
SKB1074	WLY176 5D C2	SKB442 transformed with Atg5 delta, ura marker removed with Cre-Gal and verified by plating	n/a
SKB1078	WLY176 Atg10d5d atg5-myc PRS406 E	Verified by PAP and myc WB	kan, -ura, -trp
SKB1079	WLY176 Atg10d5d atg5-myc PRS406 10-PA C1	Verified by PAP and myc WB	kan, -ura, -trp
SKB1080	WLY176 Atg10d5d atg5-myc PRS406 10-PA C2	Verified by PAP and myc WB	kan, -ura, -trp
SKB1081	WLY176 Atg10d5d atg5-myc PRS406 H131A C1	Verified by PAP and myc WB	kan, -ura, -trp
SKB1082	WLY176 Atg10d5d atg5-myc PRS406 H131A C2	Verified by PAP and myc WB	kan, -ura, -trp
SKB1083	WLY176 Atg10d5d	Verified by PAP and myc WB	kan, -ura, -

	atg5-myc PRS406 Y73Q/H131A C1		trp
SKB1084	WLY176 Atg10d5d atg5-myc PRS406 Y73Q/H131A C2	Verified by PAP and myc WB	kan, -ura, -trp

Primers

Table 2: Primers used in this study

Number	Name	Description	Sequence
442	Atg10 flanking R	Sequencing primer (reverse) starting 100bp downstream of Atg10 stop codon	cagacaagaaaaagaaacaaataatatagactga
444	Pringle Internal Checking R	Primer annealing to the pFA6A (pringle) fragment (reverse of F1 sequence). Use with a forwards primer upstream of a gene to check for deletion of that gene via pringle method)	TTAATTAACCCGGGGATCCG
451	Atg12 pUG KO 5'	Cre-mediated atg12 deletion reverse primer (as described in Gueldener paper)	acatccctaactgtatattctacagtagagtgaaccaatgacagtCAGCTGAAGCTTCGTACGC
452	Atg12 pUG KO 3'	Cre-mediated atg12 deletion reverse primer (as described in Gueldener paper)	atcgactgtaggttttcttcttagaccattccagcgcccgggtatGCATAGGCCACTAGTGGATCTG
481	Atg10 seq +200 bp rev	Internal reverse Atg10 sequencing primer	GGGCTCGTTATATACTTTTGAATATGTA
482	Atg10 -324 new seq flanking Forward	Sequencing primer starting 325 bp upstream of Atg10 start codon, reading forwards	GGCGACAAGGTGTTCTAGT
543	Atg5 pUG KO 5’	Forwards primer for knocking out Atg5 using the pUG system of vectors	ggttctagaagaacggagataggaaacctatgatgtaagtCAGCTGAAGCTTCGTACGC
544	Atg5 pUG KO 3’	Reverse primer for knocking out Atg5 using the pUG system of vectors	tatttgaatgacacttttaaatgcgtatataacagctcGCATAGGCCACTAGTGGATCTG

545	Atg5 flanking 5’	500 bp upstream of ATG5 start, reading forwards	CTAAACGTTCGTTGAGCGAAGG
546	Atg5 flanking 3’	500 bp downstream of ATG5 stop, reading reverse. Can be used with SKB545 to check for or transfer an ATG5 KO	GAGAAATATTCCCCATTCGACCC
547	ATG5pro cloning F	Forwards primer for amplifying 800 bp promoter of Atg5 for cloning into pRS414, EcoRI side, by Infusion	ataagcttgatatcgaattcATAACTTCTTCAAATACAGACTCTATTATTCAAGAA
548	Cyc1 term cloning R	Reverse primer for amplifying Cyc1 terminator for cloning into pRS414, BamHI side, by Infusion. Use with SKB547 to amplify ATG5pro-ATG5-9xmyc-cyc1term from Dr. Sascha Martens	gctctagaactagtggatccGCAAATTAAAGCCTTCGAGCG

Plasmids

Table 3: Plasmids used in this study

Insert/Selection	Name	Details
myc/ura	pRS316-Atg5-9xmyc	pRS316-longAtg5-9xmyc-cyc1term (SMc270) from Sascha Martens
ura	PRS406E	n/a
PA/ura	PRS406E 10-PA	Made by Nadia Silvia using in-fusion
PA/ura	PRS406E 10-PA H131A	Made by Nadia Silvia using in-fusion
PA/ura	PRS406E 10-PA Y73Q/H131A	Made by Konrad Lautenschlager by Infusion
NA/trp	CreGal 414	Klionsky lab

Polymerase Chain Reaction (PCR)

All the PCRs done used the Phusion recipe shown below and then were put in the PCR machine. During this reaction the DNA is denatured with excess heat to break the hydrogen bonds between the strands and make them single stranded and exposed. The temperature is then decreased so that the DNA primers can anneal to the single stranded DNA. The temperature is then increased slightly so that the polymerase can work to elongate the strands and add nucleotides to fit the DNA sequence. This is done about 35 times and the DNA is replicated over and over to create over 34 billion strands of DNA with the exact sequence desired.

Table 4: Phusion PCR recipe

ddH2O	66 ul
5x HF buffer	20 ul
dNTP 10mM	2 ul
Forward Primer	5 ul
Reverse primer	5 ul
plasmid template	1 ul
Phusion polymerase	1 ul

Agarose Gel Electrophoresis

The agarose gel was first made by microwaving 50 mg of agarose powder in a flask with 50 mL of 1x TAE until fully dissolved. The product was then cooled before adding 1.5 µL of Gel red dye and then it is poured into a mold and left to set. The 5x orange dye was added to the PCR product in a 1:5 ratio before adding to the wells. We added 5 µL of final product into the

wells, including a DNA ladder, and ran the gel at 100 V for 30-50 minutes until the bands reached the bottom. The gels were then imaged with a BioRad Gel Doc using UV light and the images were analyzed.

Marker removal

To remove the pUG insert from our cells we transformed the cells with the Gal Cre expression vector CreGal414 using a standard method (Gueldener et al., 2002). We then inoculated out colonies in 5 mL of YP-GAL media and let it grow for 2-3 days. We then calculated what amount was needed to plate 200 cells and diluted the cultures to get that amount. Finally, these were plated on YPD and restruck on YPD, SMD-ura, and SMD-trp plates. If the pUG was successfully removed and no CreGal414 is present the strains would only grow on the YPD plates.

Yeast growth and starvation

In order to induce autophagy, the samples are starved and compared to unstarved versions of the samples. For this process, the cultures first were inoculated into 5 mL of preferred media then grown overnight in 30 °C incubation shaking at 300 bpm. In the morning they were then measured using a 1:4 dilution with water to get an accurate measure of the concentration of yeast cells in OD (optical density). The amount of saturated culture needed was calculated and then diluted to 5 mL with media down to 0.1-0.2 OD and grown in the same conditions until they reach 0.8-1.0 OD. Then, the samples were spun down in the centrifuge at 1000 rpm for 2 minutes and the media was poured into a waste flask leaving the sample pellet. This was then washed by adding 5 mL of ddH2O, centrifuging it in the same way and then

pouring out the water. The sample was then resuspended in 5 mL of SD-N which is a growth medium that doesn't contain nitrogen or amino acids. The sample was then put in the shaker for 3 hours at 30 °C to induce starvation.

TCA precipitation

The volume of culture containing one OD of cells was calculated and placed into a tube and then centrifuged. The supernatant was removed, and the pellet was resuspended in 1 mL of ice-cold TCA and incubated for 20 minutes on ice. The TCA was then removed carefully, and the pellet was washed with 1 mL ice-cold acetone. This was removed after 1 minute of centrifugation and the samples were left to air dry for 15 minutes so the remaining acetone can evaporate. This process leaves only the proteins left and they are isolated so they can be run on a gel.

SDS-Page

The protein (TCA pellet) was resuspended in SSB+Tris (0.05 M Tris-HCl pH 6.8, 0.2% SDS, 5% glycerol,0.2% β-mercaptoethanol, 0.2 ppm bromophenol blue) in a 50 µL to 1 OD ratio. Glass beads were added, and the samples are vortexed to break up the pellet before loading. This was done in the cold room and then the samples were incubated at 95 °C and centrifuged for 1 minute max speed. We added 10 µL of the samples to the gel along with 5 µL of the protein ladder. We used 12.5% resolving and 5% stacking following the ingredients in table 5. We filled the gel box with a 1x SDS page buffer making sure the level inside was higher than outside and it wasn't leaking. Then we ran the gel at 120 V until the dye reached the bottom.

Table 5: SDS page gel recipe (for 2 gels)

Ingredient	Amount for 5% stacking gel (ml)	Amount for 12.5% resolving gel (ml)
ddH20	2.916	2.347
Acrylamide 30%	0.667	3.125
Tris 1.5 M	0.333 (pH 6.8)	1.875 (pH 8.8)
SDS 10%	0.04	0.075
APS 10%	0.04	0.075
TEMED 100%	0.004	0.003

Immunoblotting

The gel was then transferred to 0.45 uM Immobilon-P transfer membranes by sandwiching the gel next to the membrane and running it at 120 V for 1 hour in a cold transfer buffer. The blot was then stained with ponceau and rinsed off with TBST for 20 minutes. For the myc blot we blocked for 30 min in 4% milk block (in TBST) and probed with 1:1000 4AG primary overnight and 1:5000 of goat anti mouse secondary for 2 hours the next day. For the PA blot we blocked for 30 min in 4% milk block (in TBST) and probed with 1:5000 PAP primary overnight. These were then rinsed in TBST, and the detection reagent was added in order to image using the gel doc.

Results

Knocking ATG5 out of the genome

Since ATG10 is directly upstream of ATG12-5 conjugation, the readout of ATG12-5 conjugation protein levels reflects how much activity atg10 mutants have, which can then be compared to how this affects the autophagic process as a whole. To accurately quantify the amount of ATG12-5 in the cells via western blot, all the ATG5 present needs to be tagged. To achieve this, the original gene that encodes for ATG5 must be removed from the DNA. We used homologous recombination to replace the ATG5 DNA segment with pUG72 Ura DNA that we made by amplifying with PCR (figure 4). We chose pUG72 even though we would have to later remove the ura marker because the PCR for pUG73 wasn't successful after multiple attempts and pUG66 would require the use of bleomycin which is expensive and can give a higher rate of false positive transformants. The verification PCR used ATG5 flanking primers to replicate the genomic locus where ATG5 should be to see if it is ATG5 DNA or Ura DNA. Expected band sizes are 2.7 kB if ATG5 is replaced with Ura (knockout) and 1.9 if ATG5 is still present. Wild type (WT) Clone 2 (C2) and Clone 3 (C3) were successful, atg10Δ C2 and C3 were also successful.

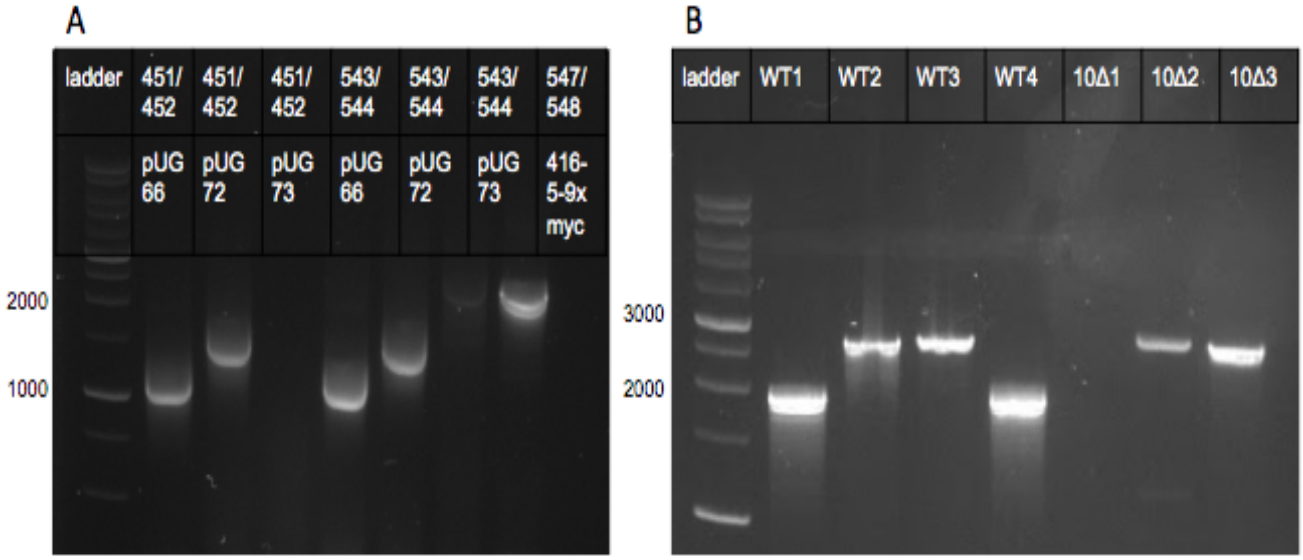

Figure 4: Knocking ATG5 out of the genome. (A) Different primer sets (451/452, 543/544, 547/548) were used to amplify the pUG PCR product and verifying for insertion into DNA to replace ATG5. (B) The WT (which contains ATG10) and 10Δ colonies labeled clones 1-4 and 1-3 respectively. The chosen clones were the colonies that grew on -ura plates and these were verified by PCR using flanking primer (545/546) followed by agarose gel electrophoresis to see if the knockout was successful.

The pUG DNA was then removed by transforming a plasmid expressing Cre recombinase into the cells to remove the ura marker from the deletion using the pUG system. The colonies from this transformation were grown in YP-GAL for three days before being stuck out on non-selective plates (YPD) and selective plates (-ura and -trp) to determine if the DNA with the selective marker was successfully removed (fig. 5). Since this was done on both the atg5Δ and the atg5Δ10Δ strains we chose 2 clones of each to move on with. Clones 2 and 3 were chosen from the atg5Δ strains and clones 11 and 18 were chosen from the atg5Δ10Δ strains.

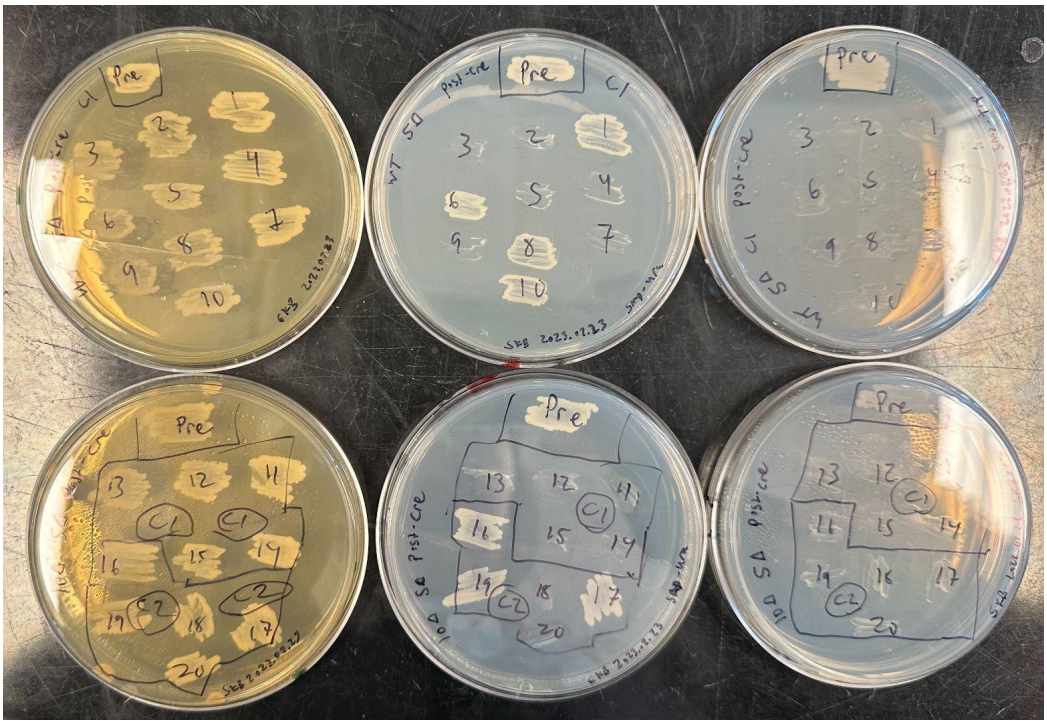

Figure 5: Removal of ura marker from DNA in atg5Δ (top) and atg5Δ10Δ (bottom) strains. Ten colonies from each culture were struck out on YPD (left), SMD -ura (center), and SMD -trp (left). The pre-cre culture served as a negative control.

Creating and inserting atg5-myc DNA

The goal of this project was to replace the original ATG5 DNA with a tagged version so it can be quantified. To accomplish this, a tagged version had to be made and verified. We had previously tried to use a HA tag but could see only a very faint Atg5-HA signal on the western blot and there were a lot of background bands. Since the HA tag didn't work, another option was to attach a myc tag to the genomic copy of ATG5 by using PCR to create a DNA fragment that could insert the myc tag and a selective marker at the end of the genomic copy of ATG5 by homologous recombination. We tried this PCR multiple times with different primers and troubleshooting but were unable to produce enough DNA to transform into our cells. However,

Dr. Sascha Martens had previously created a plasmid where ATG5 was tagged with myc so they sent a sample of this 9xmyc plasmid.

The original atg5-9xmyc they sent was a PRS416 plasmid, which has a Ura selective marker. This marker had already been used in our strains, so the atg5-9xmyc sequence had to be moved to a PRS414 plasmid which has a Trp selective marker. This process was done with PCR and infusion cloning to create pRS414 atg5-9Xmyc clones. This was then sent in for sequencing to verify and check for mutations. The sequencing covered the entire insert including both junctions with only one point mutation in the myc tag. This mutation was seen in all clones so it likely was already included in the original plasmid and therefore not significant.

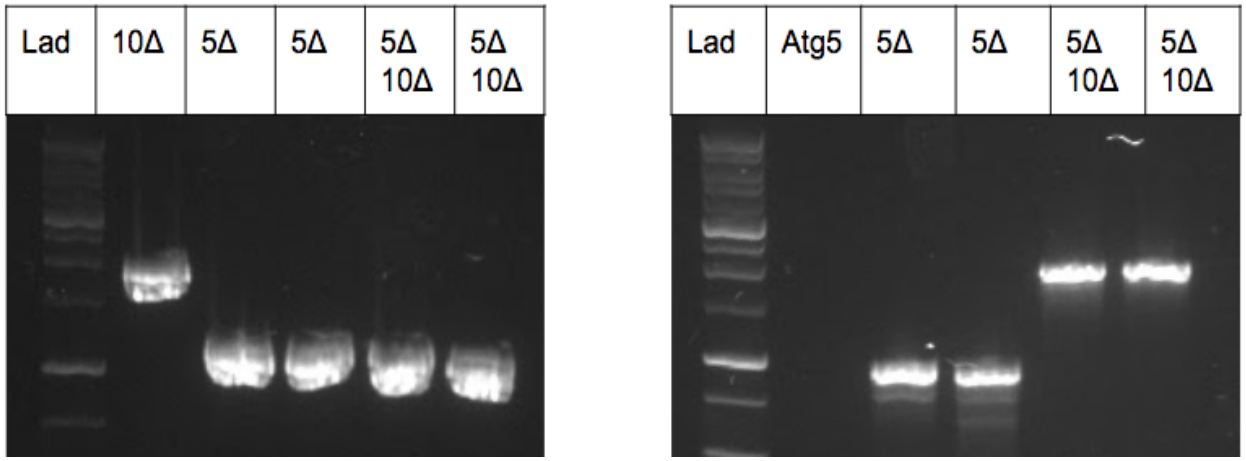

Figure 6: Verifying that the strains used have the correct autophagic genes in their genome. The primers checking for ATG5 (left) shows that the higher band, which is a positive control, has ATG5 but the atg5Δ clones don't, which verifies them. The primers checking for ATG10 (right) shows that the lower band, which is a positive control, has ATG10 but the atg10Δ clones don't, which verifies them.

Before inserting this plasmid, the strains used were verified that they contained the correct genes using flanking primers and PCR to determine if the DNA size was correct (fig. 6). The pRS414 atg5-9Xmyc clones were then transformed into the verified strains and colonies were chosen for analysis. A western blot was performed to view the presence of the myc tag

and the ability to quantify results before moving on. The atg5Δ10Δ clones clearly show bands at 33 kDa, the size of ATG5, and the atg5Δ show bands at 56 kDa, the size of ATG12-5 (fig. 4). This makes sense with what was expected so protein tagging was successful.

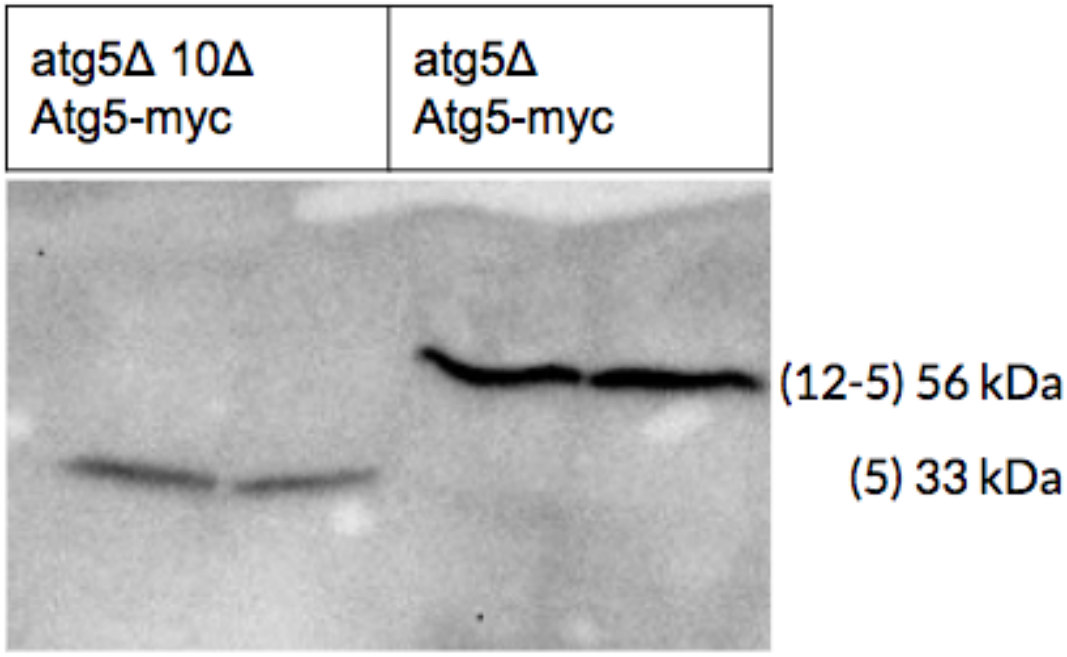

Figure 7: Myc tag used to view ATG5 and ATG12-5 conjugate via western blot. The ATG12-5 conjugate is larger in size compared to ATG5 on its own. As expected, no conjugation is seen in the atg10Δ mutant, while in cells containing ATG10, most of the ATG5 present is in the conjugated form Two clones were blotted for each genotype.

Making and verifying mutants with myc tag

The overall goal of this project is to quantify the difference in ATG12-5 conjugation levels between the WT ATG10 and the atg10 point mutants. For this to be possible the mutant DNA must be added to the atg5myc10Δ clones. This was done by transformation and then selectively plated on SMD-ura plates. The chosen colonies were then grown and processed with half of the samples undergoing a 3 hr nitrogen starvation to induce nonselective autophagy.

The samples were run out on by SDS-PAGE and probed with anti-myc primary. The strain 406E serves as a negative control with no ATG10 while the WT (10-PA) serves as a positive control in which to compare the loss of activity in the mutants. Compared to WT ATG10, the mutant atg10 H131A has significantly less ATG12-5 conjugation and atg10 Y73Q/H131A has even less ATG12-5 conjugation as it is not visible to the naked eye. These show visible signs of decreased activity, as is expected since it correlates to the ALP assays done previously.

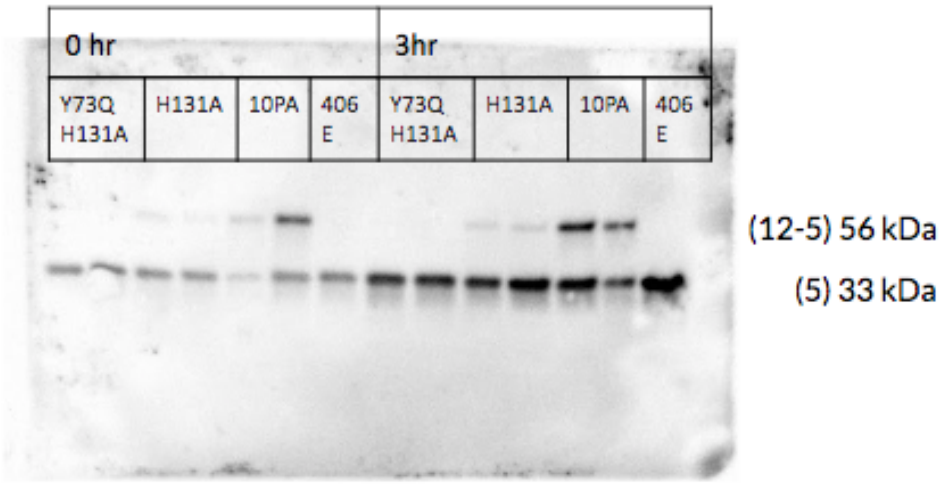

Figure 8: Myc tag used to view ATG5 and ATG12-5 conjugation in atg10 mutants via western blot. The 3 hr samples on the right have been starved to induce autophagy while the 0 hr have not been. There are 2 clones of all strains beside the 406 E.

Discussion

To compare the levels of 12-5 conjugation with the amount of autophagic flux in our mutants, we needed to tag the protein to accurately quantify the amounts. This will help us gain greater understanding of the relationship between 12-5 conjugation amounts and autophagic activity as a whole. The process required the addition of a myc tag to ATG5 so that it will be quantifiable in a western blot. We added this through creating a plasmid that contained

the tag and a proper selection marker. We then transformed this plasmid into yeast cells from which we had removed endogenous ATG5 and added our ATG10 mutants through homologous recombination. The strains were run on a gel and probed with antibody to detect the tag. This process was successful as we are able to see the bands correctly in our sample images. Additionally, the WT ATG10-PA has prominent bands showing the 12-5 conjugation, while the mutants show faint bands demonstrating some conjugation but a reduced activity while the 406 E shows no bands since it has no ATG10 to conjugate the complex at all.

Moving forward we will thoroughly quantify the difference between the mutants by running additional Western blots and ALP assays. The cells will then be prepared for imaging via electron microscope where we can visualize the autophagosomes in the vacuoles. Through using this data, we can calculate the average size and number of autophagosomes present in our strains. This will allow us to determine what role ATG10 has on the formation of the autophagosomes and gain insight into the role of this protein.

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