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Investigation of Atg11 and Its Interaction withYpt1 in Autophagy

Abstract

Autophagy is a cellular process conserved in eukaryotes that involves the trafficking of intracellular material from the cytosol to the vacuole/lysosome. This material is wrapped in a double membraned vesicle called an autophagosome, which ls constructed at the pre-autophagosomal structure. Many proteins are Involved In autophagy, one of them being Atgll. This protein is crucial in the selective autophagy pathway, and is responsible for proper formation of the pre-autophagosomal structure as well as recruiting the material intended for degradation to the autophagosome. Atgl 1 interacts with many other autophagy proteins, but it Is unknown whether it interacts with these binding partners spatially or temporally. To better understand the mechanism of Atg11' s interactions, it was tested against Yptl, an upstream regulator whose Interaction with Atgll is crucial for selective autophagy. Yptl with an N-terminal 6-histidine tag was expressed and purified using Rosetta cells, yielding a concentration of 0. 242 mg/ml seems low. This protein was then used in an *in vitro* binding test with pure GST ·Atgl1CC2·3 and imaged via Western blot using primary antibodies rabbit antihistidine {raHis) and rabbit anti-GST (raGST}, and secondary antibody goat anti-rabbit (Gar). The blot showed no Interaction between the proteins *in vitro*, so the interaction was tested *in vivo* using a yeast 2-hybrid screen with a multiple-knockout strain. BO-Yptl was tested against AD-Atg11CC2-3, pseudo-positive control AD-Atgll, and negative control AD-empty. Contrary to previous literature, i no interaction showed between the two proteins in any of the strains. This indicates that another type of selection must be used, or there might be another protein involved in the interaction. 3

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Investigation of Atg11 and its Interaction with Ypt1 in Autophagy

By

Hayley Cawthon

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

In Partial Fulfillment of the Requirements for Graduation

with Honors in Chemistry

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0.0 Abstract

Autophagy is a cellular process conserved in eukaryotes that involves the trafficking of intracellular material from the cytosol to the vacuole/lysosome. This material is wrapped in a double membraned vesicle called an autophagosome, which is constructed at the pre-autophagosomal structure. Many proteins are involved in autophagy, one of them being Atg11. This protein is crucial in the selective autophagy pathway, and is responsible for proper formation of the pre-autophagosomal structure as well as recruiting the material intended for degradation to the autophagosome. Atg11 interacts with many other autophagy proteins, but it is unknown whether it interacts with these binding partners spatially or temporally. To better understand the mechanism of Atg11's interactions, it was tested against Ypt1, an upstream regulator whose interaction with Atg11 is crucial for selective autophagy. Ypt1 with an N-terminal 6-histidine tag was expressed and purified using Rosetta cells, yielding a concentration of 0.242 mg/mL seems low. This protein was then used in an *in vitro* binding test with pure GST-Atg11CC2-3 and imaged via Western blot using primary antibodies rabbit anti-histidine (rαHis) and rabbit anti-GST (rαGST), and secondary antibody goat anti-rabbit (Gαr). The blot showed no interaction between the proteins *in vitro*, so the interaction was tested *in vivo* using a yeast 2-hybrid screen with a multiple-knockout strain. BD-Ypt1 was tested against AD-Atg11CC2-3, pseudo-positive control AD-Atg11, and negative control AD-empty. Contrary to previous literature,[2] no interaction showed between the two proteins in any of the strains. This indicates that another type of selection must be used, or there might be another protein involved in the interaction.

1.0 Introduction

Macroautophagy, hereafter referred to as autophagy[1], is a process of intracellular degradation. Translating to "self-eating", autophagy involves the sequestration of cellular material intended for degradation (referred to as cargo) into double-membraned vesicles known as autophagosomes.[2] These autophagosomes are built and organized at the pre-autophagosomal structure (PAS)[3], and upon completion are directed toward the vacuole (in yeast, for example) or the lysosome (in animals). Here, the outer membrane fuses with the vacuole/lysosome, and the inner membrane and cargo are degraded and recycled for future use within the cell.[2]

Autophagy consists of two major categories: selective and non-selective. Selective autophagy involves the targeting of specific cellular components for degradation.[4] The breakdown of protein aggregates (aggrephagy), excess or nonfunctioning mitochondria (mitophagy)[4], and invading microorganisms (xenophagy) are all example of selective autophagy.[5] Non-selective autophagy, as the name implies, refers to the process of non-specific cargo being sequestered into autophagosomes and sent for degradation.[4]

Both processes are vital for proper cellular function and homeostasis[6]: non-selective autophagy allows the cell to survive under starvation conditions, while selective autophagy clears away unwanted organelles and other debris, thereby prolonging cellular health. Because of this, autophagy is a medically-relevant function. Reduced autophagy activity, or a lack of it altogether, can be seen in neurological diseases such as Alzheimer's and Parkinson's, as well as some cancers.[7] For example, the lack of

aggrephagy prevents the clearance of protein aggregates within the cell, leading to the appearance of neuronal plaques that can be found in the brains of Alzheimer's patients. A better understanding of autophagy and the proteins involved could lead to the creation of drugs that can control autophagy, and thereby combat these diseases.

As with any biochemical process, autophagy relies on the interaction of various proteins. Models in baker's yeast (*Saccharomyces cerevisiae*) have been developed to study these proteins and their roles in autophagy. Over thirty autophagy-related (Atg) proteins have been identified in yeast[5], each with a specific role in the mechanism and analogs to autophagy proteins in humans.[8] One important autophagy protein is Atg11, a scaffolding protein with interaction sites composed of three coiled-coil (CC) domains, which is critical for selective autophagy to occur.[9] It is responsible for proper formation of the PAS, for sending cargo to the PAS to be sequestered into vesicles, and directing other Atg proteins to this site to aid in autophagosome formation. In cells without Atg11, these other Atg proteins, such as Atg20[9] andAtg32[4], are not successfully recruited to the PAS, causing autophagy activity to be severely decreased.[1] In order to recruit the targeted cargo to the PAS, Atg11 must interact with their respective receptors during specific selective autophagy pathways. For example, during mitophagy, Atg11 interacts with phosphorylated Atg32 to bring mitochondria to the PAS and to be further degraded in the vacuole/lysosome.[4]

Atg11 also interacts with Ypt1, a small soluble GTPase that belongs to the Rab family of proteins.[2] It has been reported that Ypt1-GTP interacts with the CC2 and CC3 regions of Atg11, and that this interaction is necessary for PAS formation.[2]

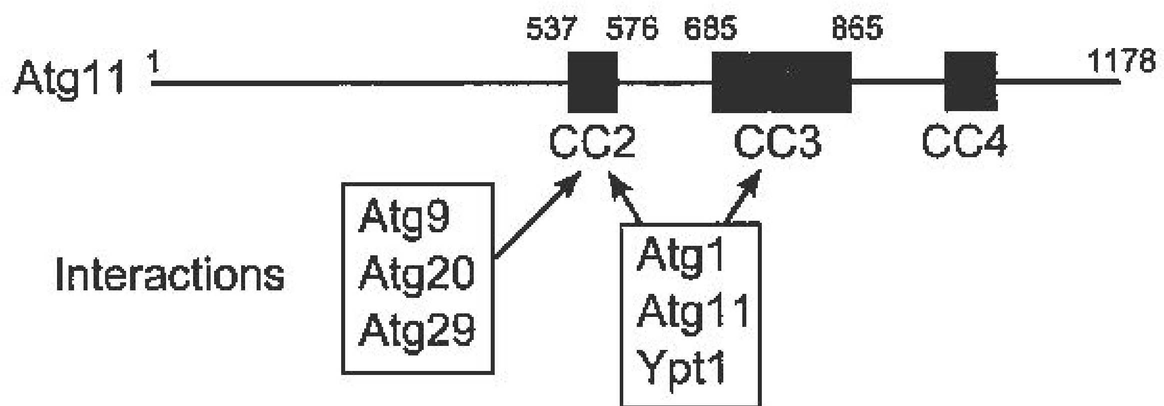

Figure 1: Interaction sites of Atg11. Most protein interactions with Atg11 occur at its coiled-coil (CC) regions. Ypt1 interacts at the CC2 and CC3 region. Figure courtesy of Dr. Steven Backues.

In normal cells, GFP-Atg11 localizes to one spot in the cell, the PAS.[2] In mutant cells without Ypt1, GFP-Atg11 localizes in several spots throughout the cell, showing that without this GTPase, Atg11 is not trafficked to the PAS.[2] GST-Ypt1 has been previously purified[2], and a protein module containing Ypt1 acts at the endoplasmic reticulum to regulate the commencement of selective autophagy in yeast.[3] Ypt1 also regulates the delivery to Atg1 to the PAS, thereby regulating Atg9 vesicle binding.[10] By being involved in many crucial interactions in the autophagy pathway, Ypt1 and its homolog Rab1 are key players in autophagy.

We proposed that purified His_6-Ypt1 would interact with purified GST-Atg11CC2-3s *in vitro* and *in vivo*. The focus of my thesis is to express and purifying Ypt1, and test its interaction with Atg11 *in vitro* and *in vivo*. The goal of the project is to reproduce reported results of this interaction, to eventually use Ypt1 to test the functionality of purified Atg11 and as a control for future projects with mutated Atg11. I hypothesized that I would be able to successfully purify the protein and show its interaction with purified Atg11CC2-3 *in vitro* and *in vivo*. This information would be crucial in future

experimentation with mutated Atg11, and would aid in elucidating the mechanisms of the protein-protein interactions Atg11 is involved with in selective autophagy.

2.0 Materials and Methods

2.1 Apparatus

PCR was performed using the BioRad MyCycler™ thermal cycler. Gel electrophoresis was done using a Horizontal Electrophoresis System from Fisher Biotech, and an EC135 power supply from E-C Apparatus Corporation. Absorbance was read using a Beckman Coulter DU 800 UV/Visible Spectrophotometer at a wavelength of 600 nm, and protein concentrations were measured using a Nanodrop 2000c Spectrophotometer from Fischer Scientific and a Synergy 2 microplate reader by Biotek. Plates containing yeast were incubated in a Lab-Line/CS&E Imperial II incubator, while bacteria plates were incubated in a Model 1545 incubator from VWR Scientific. Shaking of cultures was done in an innova 4300 floor-model incubator shaker from New Brunswick Scientific. Centrifugation of small volumes was done with a Sorvall Biofuge pico centrifuge, and large yeast cultures were spun down using a Clay Adams brand Dynac II Centrifuge by Becton Dickinson. Sonication was performed using the VirSonic 100 by Virtis. SDS-PAGE was performed using a power supply, cables, glass plates, and container from BioRad. Protein dialysis was done with Slide-A-Lyzer cassettes from Pierce Chemical Company. All micropipetting was performed using Denville Ultra EZpettes, ranging from 1.0-1000 µL.

2.2 Reagents

All genomic yeast DNA was purified from wild-type *Saccharomyces cerevisiae* yeast. The Ypt1 used in *in vitro* studies had a His_6 tag on it, and the Atg11CC2-3 used in the *in vitro* studies had a GST tag. This GST-Atg11CC2-3 was expressed and purified by Carson Zois. Plasmid minipreps were performed using a Purelink ™ Quick Plasmid Miniprep Kit purchased from Invitrogen. The Q5 DNA polymerase used in PCR amplification and dNTPs were purchased from New England Biolabs. T4 DNA polymerase and Generuler 1 kB ladder were purchased from Fischer Scientific. GTP and GTP-γ-s were purchased from Sigma Aldrich, and the nickel resin used for large-scale purification was Novagen Ni-NTA His Bind® Resin. The primary antibodies used in running the Western blot were rabbit anti-histidine (rαHis) and rabbit anti-GST (rαGST), and the secondary antibody was goat anti-rabbit (Gαr). These blots were imaged using Luminata Forte Western HRP substrate from Millipore. The primers used in PCR are described in Table 1 below:

Table 1: Primers used for PCR.

Primer Number	Description	Sequence
1	Ypt1 LIC cloning, forward	tacttccaatccaatgcaATGAATAGCGAGTACGATTACCTGTTC
2	Ypt1 LIC cloning, reverse	ttatccacttccaatgttaTCAACAGCAGCCCCCACC
3	pMCSG, reverse	TTCCTTTCGGGCTTTGTTAG
4	pMCSG, forward	CTCTTCCGGGCGCTATCATG

The vector used in protein purification was pMCSG7, while the strain used for yeast 2-hybrid was multiple knockout strain YCY149.

2.3 Procedure

2.3.1 DNA Selection and T4 treatment

pMCSG7-Ypt1 was created using Ligation Independent Cloning (LIC) using primers 1 and 2 (Table 1). Ypt1 DNA was amplified using PCR (primers 3 and 4 from Table 1) from both genomic DNA and a plasmid containing Ypt1. One genomic DNA sample was amplified using Q5 polymerase, while other genomic DNA sample was amplified by Dr. Steven Backues, who used Platinum Hot Start 2X Master Mix Polymerase from Invitrogen. Both PCRs were run on an 2% agarose TAE gel in a Horizontal Electrophoresis System from Fisher Biotech. Both genomic samples were T4 treated and annealed with T4 treated linearized pMCSG7 then transformed into *E. Coli* DH5alpha. Four colonies from the transformation were selected and used to inoculate cultures. After incubation, these cultures were spun down and DNA was isolated from the pellets via a plasmid miniprep using the Purelink™ Quick Plasmid Miniprep Kit. The concentration of each pellet was measured using the Nanodrop 2000C Spectrophotometer. PCR was done to verify the presence of the Ypt1 insert (67°C annealing temperature, 20 second extension time, 30 cycles with Q5 polymerase), using primers 3 and 4 (Table 1) and the resulting samples were run on a 2% agarose TAE gel. Sequencing was performed by the University of Michigan Sequencing Core using primers 3 and 4 (Table 1).

2.3.2 Small scale protein expression

The two samples confirmed via sequencing at the University of Michigan Sequencing Core were transformed into the Rosetta strain via heat shock, using SOC for the outgrowth media and plated on LB+gluc+chlor+cam plates. Cultures from this transformation were made in autoinduction media, along with an mTangerine control culture, and were incubated overnight at 30 °C in the innova 4300 floor-model incubator shaker from New Brunswick Scientific. Once they reached the optimal OD range (6-8 OD), they were spun down at 13,000 rpm for 1 minute, and the supernatant removed and discarded. The pellets were sonicated using the VirSonic 100 by Virtis for 3 10 second pulses at power level 4, cooling on ice between pulses. The sonicated samples were heated at 95 °C for 5 minutes before being spun down in the Sorvall Biofuge pico centrifuge at a maximum speed of 13,000 rpm for 5 minutes. 4 µL samples were taken from each tube, separated on a 12% SDS-PAGE gel, visualized by Coomassie staining, and imaged using a ChemiDoc II gel imaging system (BioRad).

2.3.3 Large scale protein purification

Autoinduction cultures were made using 50 mL autoinduction media, and the ODs of each culture were read using a Beckman Coulter DU 800 UV/Visible Spectrophotometer at a wavelength of 600 nm. These cultures were spun down at 6,000 x g for 5 minutes, and the supernatant discarded. Each pellet was weighed, and 5 mL lysis buffer (50mM Tris-HCl, pH =7.5, 300mM NaCl, 5mM $MgCl_2$, 10mM imidazole, 4mM β – mercaptoethanol, 1mM PMSF, 1X protease inhibitor cocktail) was added per gram for

each pellet. Lysozyme was then added to 1 mg/mL, and these samples were incubated for 30 minutes at 4 °C with rotation. Upon completion of incubation, the samples were then sonicated on ice using the VirSonic 100 by Virtis for six 10 second pulses at power setting 4. After collecting a 50 µL sample of this, the total sample was then put into the centrifuge for a clearing spin of 10,000 x g for 10 minutes at 4°C. The supernatant was removed and placed into a new conical tube, after which a 50 µL sample was taken for imaging. After rinsing the nickel resin with a wash buffer (50mM Tris-HCl, pH=7.5, 300mM NaCl, 5mM MgCl2, 20mM imidazole, 4mM β-mercaptoethanol, 1mM PMSF) and basic lysis buffer mix, 1 mL 50% v/v resin was added to each sample of supernatant, or soluble fraction. This was thoroughly mixed together by rotation for 60 minutes at 4°C. This mixture was the gently centrifugated at 500 x g at 4°C, a 50 µL sample of the supernatant was taken (and labelled as the unbound fraction), and the rest of the supernatant was discarded. The pelleted beads were then washed three times using wash buffer, after which five half-column volumes of elution buffer (50mM Tris-HCl, pH 7.5, 300mM NaCl, 5mM MgCl2, 300mM imidazole, 4mM β-mercaptoethanol, 1mM PMSF) were used to elute off the protein. 20 µL samples of each elution were collected for imaging. The resin was then resuspended in 1 mL of wash buffer, and a 50 µL sample of this was taken as the bound sample. The elutions were flash-frozen in liquid nitrogen and frozen at -80°C, and the samples taken for imaging were stored at -20°C. These samples were analyzed using SDS-PAGE.

2.3.4 Atg11 *in vitro* binding test

To preload the Ypt1, two 2 µL samples of protein were incubated at 30°C for 30 minutes in 250 µL preloading buffer (20 mM Hepes pH 7.2, 5 mM EDTA, 1 mM DTT) with 7.25 µM GTP-γ-s. After incubation, 2.5 µL 1 M $MgCl_2$ was added and the samples were incubated for another 30 minutes at 30°C. During this time, two 1 µg samples same question as above of Atg11 were incubated with 15 µL Glutathione-Sepharose beads in 500 µL BY buffer (40 mM Hepes-KOH pH 7.0, 0.1 M KOAc, 5% glycerol, 1 mM DTT) for 30 minutes at 4°C. The beads were pelleted at 600 g for 1 minute, the supernatant sampled (Post-Bead Incubation) and the rest discarded. The pellets were the resuspended in 500 µL BY buffer supplemented with 2 µM MgOAc. To each of these mixtures, 0.2 µg Ypt1-GTP and Ypt1-GTP-γ-s were added, respectively. These mixtures were incubated for three hours at 4°C with rotation. Immediately upon completion of incubation, samples were taken from both mixtures and labelled Post-Ypt1 Incubation Supernatant. Both mixtures were then washed once with BY buffer, and samples taken from both for imaging (labelled Post Wash Supernatant). The proteins were eluted with 20µL 2X SDS-PAGE sample buffer, and all samples were imaged and analyzed using SDS-PAGE and Western Blot. The primary antibody for Ypt1 was a rabbit anti-histidine (rαHis; His-probe antibody G-18 (rabbit polyclonal) from Santa Cruz biotech), the primary antibody for Atg11CC2-3 was rabbit anti-GST (rαGST; GST antibody Z-5 (rabbit polyclonal)), and the secondary antibody used for both was goat anti-rabbit (gαr; Millipore AP312P).

2.3.5 Yeast 2-hybrid screen

BDu-Ypt1 was transformed into the multiple-knockout yeast-2-hybrid strain YCY149. The genes knocked out from this strain are detailed below (Table 2)[11]:

Table 2: Genotype of yeast strains.[11] YCY149 was used in the yeast 2-hybrid screens of this study.

Strain	Genotype
SEY6210	*MATα ura3-52 leu2-3, 112 his3-200 gal4Δ gal80Δ LYS2 ::GAL1-HIS GAL2-ADE2*
YCY123	SEY6210 atg1Δ, *2Δ, 3Δ, 4Δ, 5Δ, 6Δ, 7Δ, 8Δ, 9Δ, 10Δ, 11Δ, 12Δ, 13Δ, 14Δ, 16Δ, 17Δ, 18Δ, 19Δ, 20Δ, 21Δ, 23Δ, 24Δ, 27Δ, 29Δ*
YCY147	YCY123 *gal4Δ gal80Δ GAL1-HIS3 GAL2-ADE2*
YCY148	YCY147 *met2::GAL7-lacZ*
YCY149	YCY148 *atg31Δ::KanMX*

This transformation was plated on SMD -ura growth media. These plates were incubated at 30°C overnight. Two colonies from this plate were used to inoculate SMD-ura cultures, which were incubated overnight in a 30°C shaker. The ODs of both cultures were read at a wavelength of 600 nm, and split into 3 separate samples. Into one sample, AD-Atg11 was transformed, AD-Atg11CC2-3 was transformed into another, and AD-empty was transformed into the third. These were plated on separate SMD -ura -leu plates, which were incubated at 30°C. After incubation, one colony from each transformation was restruck onto a new SMD -ura -leu plate. This plate was replica plated onto a clean SMD -ura -leu plate, and a SMD -ura -leu -ade plate, all of which were incubated at 30°C.

3.0 Results

3.1 DNA selection and PCR

To purify Ypt1, we needed to create a plasmid containing the Ypt1 gene and its six-histidine tag (His_6). We began by creating a large amount of Ypt1 DNA. Because we had plasmid and genomic His_6-Ypt1 DNA, we had to decide which to use as a source. After amplifying both samples using PCR, they were imaged using gel electrophoresis (Figure 1).

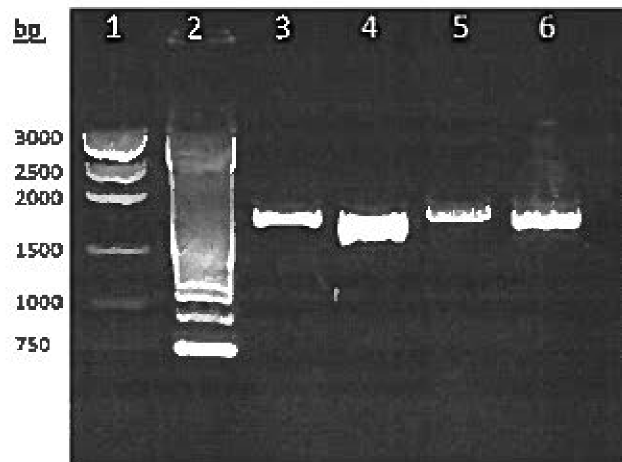

Figure 2: Ypt1 PCR. Lane 1: Ladder. Lane 2: Unknown marker. Lane 3: genomic DNA amplified with Q5 polymerase. Lane 6: plasmid DNA prepared using Q5 polymerase. Lane 7: genomic Ypt1 prepared with Platinum Hot Start 2X Master Mix Polymerase from Invitrogen. Lane 8: plasmid DNA prepared using Platinum Hot Start 2X Master Mix Polymerase from Invitrogen. The DNA in lanes 7 and 8 were prepared by Dr. Steven Backues.

Because both genomic samples showed brighter bands than their plasmid counterparts, we mixed the genomic samples together and used the mixture for the rest of the cloning. The concentration of this mixture was read using the Nanodrop and measured to be 111.1 ng/µL.

3.2 Results of Ypt1 transformation

After the concentration was determined, these samples were successfully treated with T4. During the ligation independent cloning process, these were then annealed into the pMCSG7 vector, which was then transformed into *E. coli*. This annealing process resulted in our desired plasmid pMCSG7-Ypt1, which would be used to create pure His_6-Ypt1. From the plate containing 10% of the transformation, four separate colonies were used to start cultures. These cultures were grown overnight, and the plasmid was extracted. The concentrations of plasmid were measured as follows (Table 2):

Table 3: Concentration of purified pMCSG7-Ypt1 plasmid DNA.

Sample Name	pMCSG7-Ypt1 concentration (ng/µL)
#1	32.5
#2	27.6
#3	27.9
#4	26.0

These concentrations were significantly lower than expected, so a PCR was run to see if we had indeed generated pMCSG7-Ypt1. The four samples, along with Ypt1 plasmid as a positive control and empty pMCSG7 as a negative control, were tested by PCR using primers 3 and 4 (Table 1) and then imaged using gel electrophoresis (Figure 3).

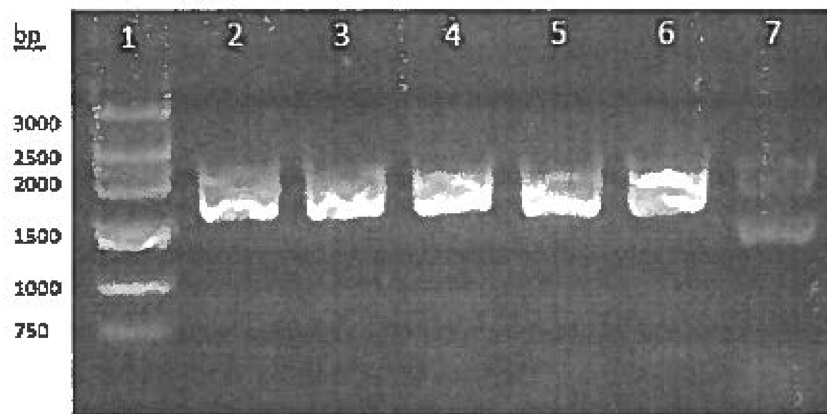

Figure 3: Gel electrophoresis of pMCSG7-Ypt1 Lane 1: Ladder. Lanes 2-5: pMCSG7-Ypt1. Lane 6: Ypt1 plasmid. Lane 7: empty pMCSG7.

The bands in lanes 2-5 appear to be the correct size to be pMCSG7-Ypt1, and are at the same height as the positive control of Ypt1 plasmid. Therefore, this gel (Figure 3) showed that the samples did indeed contain Ypt1, suggesting that we had in fact generated pMCSG7-Ypt1. After confirming this with sequencing performed at the University of Michigan Sequencing Core, we were then able to begin small scale protein expression.

3.3 Small scale protein expression

To yield an abundance of our protein of interest, pMCSG7-Ypt1 (the samples verified by sequencing) was transformed into the *E. coli* expression strain Rosetta. To find a clone with the best expression, small scale cultures of six independent clones of pMCSG7-Ypt1 were induced via autoinduction[12], along with a control autoinduction culture of mTangerine. mTangerine is a fluorescent protein that shows the conditions

for autoinduction were optimal for protein production. After completing the small-scale protein expression protocol, the samples were analyzed using SDS-PAGE.

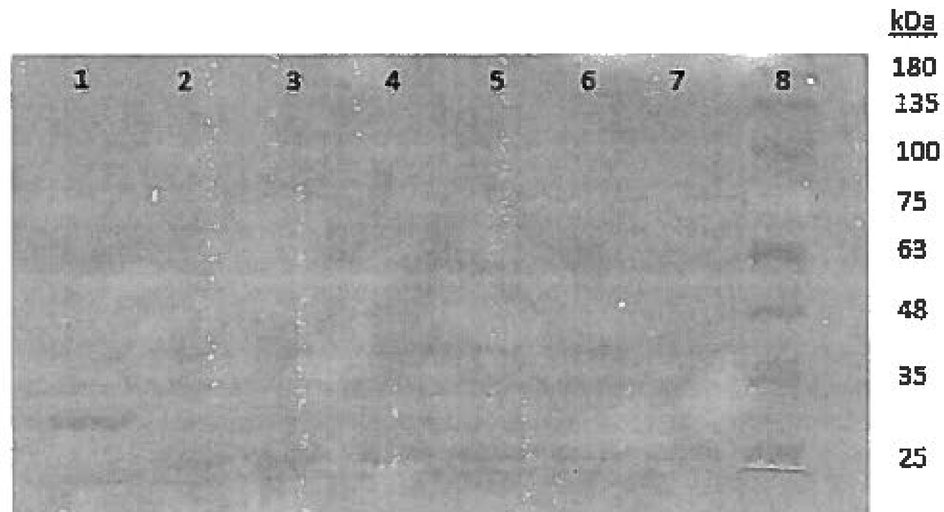

Figure 4: Small scale protein expression test results. Total lysates of autoinduced cultures expressing mTangerine (lane 1) or 6 different clones of pMCSG7-Ypt1. Induced Ypt1 can be seen running at approximately 26 kDa in all six of the clones. Lane 7: Marker.

While the resulting image is very faint, there are apparent bands for each Ypt1 autoinduction culture in the proper spot (26.2 kDa) on the gel. The positive control, mTangerine, worked as expected, and a large band appears at approximately 30 kDa. These images showed that our protein of interest was being expressed by Rosetta, and we could proceed with the large-scale protein expression protocol. Because the samples in lanes 3 and 4 appear to be the darkest, these were used further in the purification studies.

3.4 Large scale protein purification

Because we intend to test the interaction of Ypt1 and Atg11CC2-3, we need a large amount of purified protein, which is not possible to obtain using the small-scale expression test. We used the His_6 tag on the Ypt1 to purify the protein using affinity chromatography. Cultures containing 50 mL of autoinduction media were inoculated with our His_6-Ypt1 pMCSG7 expression strain. After overnight incubation at 30°C, these were spun down and the pellets saved. These pellets were broken apart using lysis and sonication, after which they were washed, incubated with the affinity resin, and eluted off the column. A sample of each step in the purification process was analyzed using SDS-PAGE.

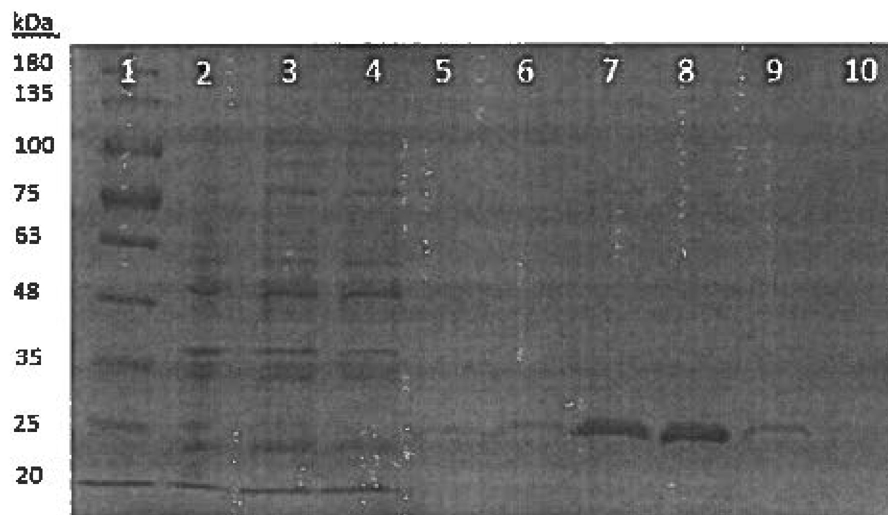

Figure 5: Large scale protein expression results. Lane 1: Marker. Lane 2: Total lysate. Lane 3: Soluble fraction. Lane 4: Unbound fraction. Lane 5: Bound after elution fraction. Lane 6-10: Elutions 1-5, respectively.

The dark bands in Elutions 7 and 8 indicate that a large amount of His_6-Ypt1 was produced. In other words, the large-scale purification was a success. Using a Bradford

protein assay with the microplate reader and BSA standards, the concentration of Elution 2 was found to be 0.242 mg/mL.

3.5 Western blot of Atg11 *in vitro* binding test

Now that we had our purified Ypt1, it was time to test its interaction with Atg11CC2-3 *in vitro*. Because Ypt1 is a GTPase and is activated when bound to GTP, it was incubated with with GTP-γ-S, a GTP analogue that does not hydrolyze. GST tagged Atg11CC2-3 was incubated with the Glutathione-Sepharose beads, then mixed with His_6 tagged Ypt1 and the complex was reisolated. Upon completion of the test, the three samples were run on an SDS-PAGE gel, which was then transferred to a Western blot to detect both proteins.

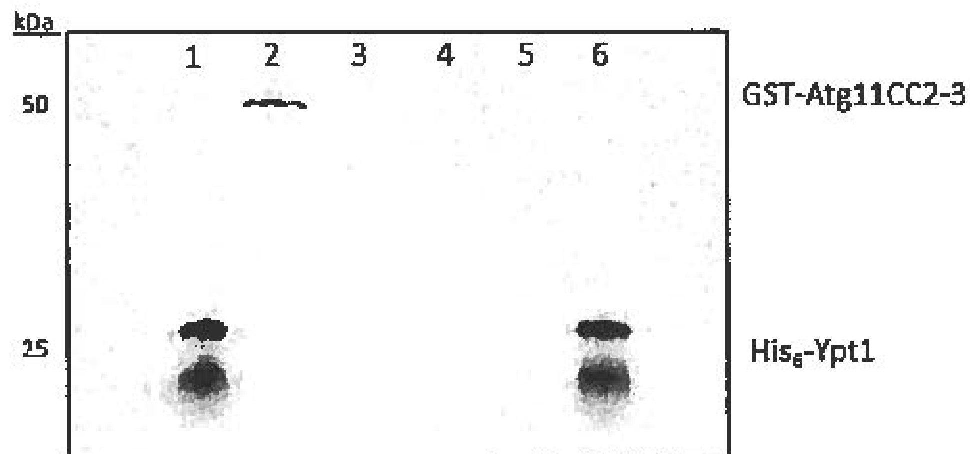

Figure 6: Western blot of His_6-Ypt1 and GST-Atg11CC2-3. Lane 1: protein ladder. Lane 2: post-GTP-γ-S incubation supernatant. Lane 3: Atg11CC2-3 + beads supernatant. Lane 4: post-Ypt1 incubation wash supernatant. Lane 5: Atg11CC2-3 Ypt1 bound Lane 6: protein ladder. The bottom half of the nitrocellulose was incubated in rαHis antibody to detect His_6-Ypt1, and the top half was incubated in rαGST.

Because GST-Atg11CC2-3 is approximately 50 kDa, it should appear in the top portion of the nitrocellulose, and His_6-Ypt1 (26 kDa) should appear in the bottom portion. The post-GTP-γ-S incubation supernatant in lane 2 should contain Ypt1 bound

to GTP-γ-S. Therefore, the band in lane 2 is GST-Atg11CC2-3, and the bands in lanes 1 and 6 are proteins in the ladder that the antibodies happened to bind to. We expected to see a His_6-Ypt1 band in lane 2, a faint GST-Atg11CC2-3 band or no band in lane 3 (indicating that all the protein had bound to the beads), possibly faint His_6-Ypt1 band in lane 4 (indicating that all of the protein had bound to GST-Atg11CC2-3), and both a GST-Atg11CC2-3 and a His_6-Ypt1 band in lane 5. There should not be any bands in lanes 1 and 6, because these are the markers. Figure 6 is highly unexpected, since no bands appear where they were predicted, and antibodies are apparently illuminating proteins in the marker. This unusual gel could have been flipped during the Western blot preparation, causing mislabeling of the bands. However, this does not explain the apparent antibodies attached to proteins in the marker. Because of this unusual blot, the binding assay was repeated by Dr. Steven Backues to verify these results.

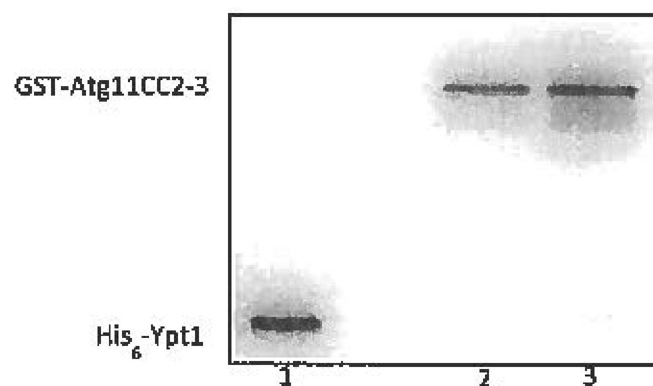

Figure 7: Western blot of GST-Atg11CC2-3 and His_6-Ypt1. Top portion: GST-Atg11CC2-3. Bottom portion: His6-Ypt1. Lane 1: Ypt1 only. Lanes 2 and 3: Atg11-CC2-3 and Ypt1 pulldown. Courtesy of Dr. Steven Backues.

The band in Lane 1 shows how much Ypt1 should appear in the pulldown assay. However, no Ypt1 appears in the pulldown assay, although both Atg11CC2-3 bands appear. From this blot, we can conclude that under these conditions, Ypt1 and Atg11CC2-3 do not interact *in vitro*.

3.6 Yeast 2-hybrid screen

After obtaining the negative results from the *in vitro* binding assay, we wanted to see whether or not the two proteins interact *in vivo*, as previously reported[2], and, if so, whether the Atg11CC2-3 region was sufficient for this interaction. For this we used a yeast-2-hybrid assay with BD-Ypt1 as the bait and either full length Atg11 (AD-Atg11) or just the CC2-3 region (AD-Atg11CC2-3) as the prey. BD-Ypt1 was transformed into the yeast strain YCY149, which is a multiple-knockout strain that has had the genes coding 25 selective autophagy proteins removed. Into three separate YCY149 BD-Ypt1 cultures, AD-Atg11, AD-Atg11CC2-3, and a negative control of AD-empty were transformed. Because the Atg11-Ypt1 interaction has been previously reported[2], AD-Atg11 was used as a pseudo-positive control. These transformations were plated onto SMD -ura -leu plates, which were then replica plated onto a new SMD-ura -leu plate and a SMD -ura -leu -ade plate. The results of these replica plating are below in Figure 7.

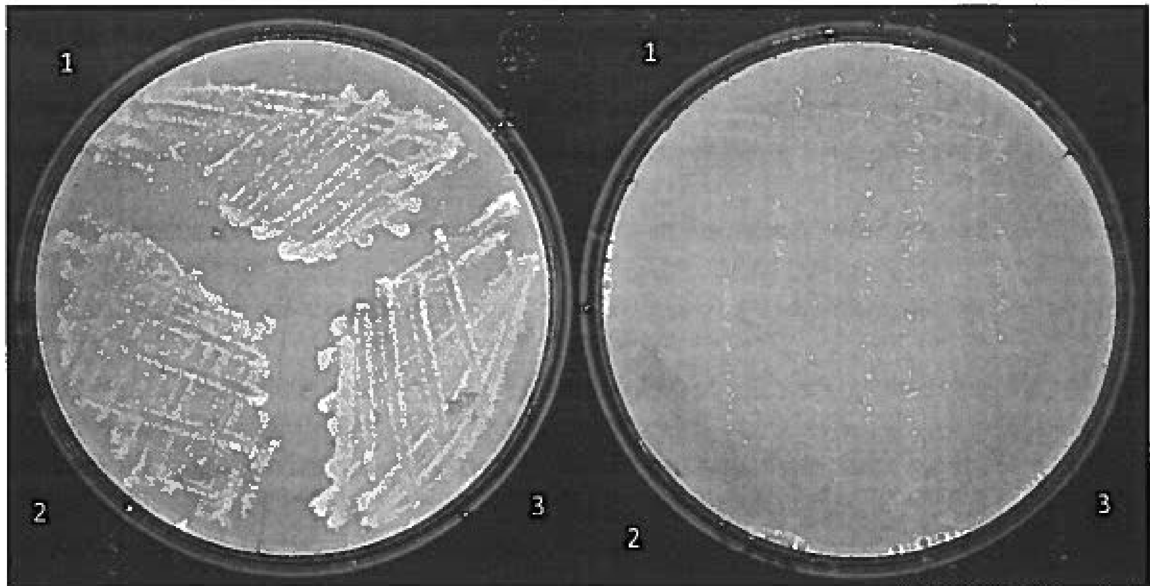

Figure 8: Yeast 2-hybrid screen. Left plate: SMD -ura -leu. Right plate: SMD -ura -leu -ade. Section 1: BD-Ypt1 AD-Atg11CC2-3. Section 2: BD-Ypt1 AD-Atg11. Section 3: BD-Ypt1 AD-empty.

The BD-Ypt1 AD-Atg11 interaction was meant to act as a positive control, while BD-Ypt1 AD-empty acted as a negative control. Surprisingly, no interaction was seen on the selective plate, not even the pseudo-positive control. This result seems inconsistent with the published literature,[2] but could be due to the type of selection used or the strength of the interaction between the proteins. To test whether this interaction occurs in a wild-type yeast strain, we repeated the screen using the pJGY-4a wilt-type yeast strain. The results are below in Figure 8:

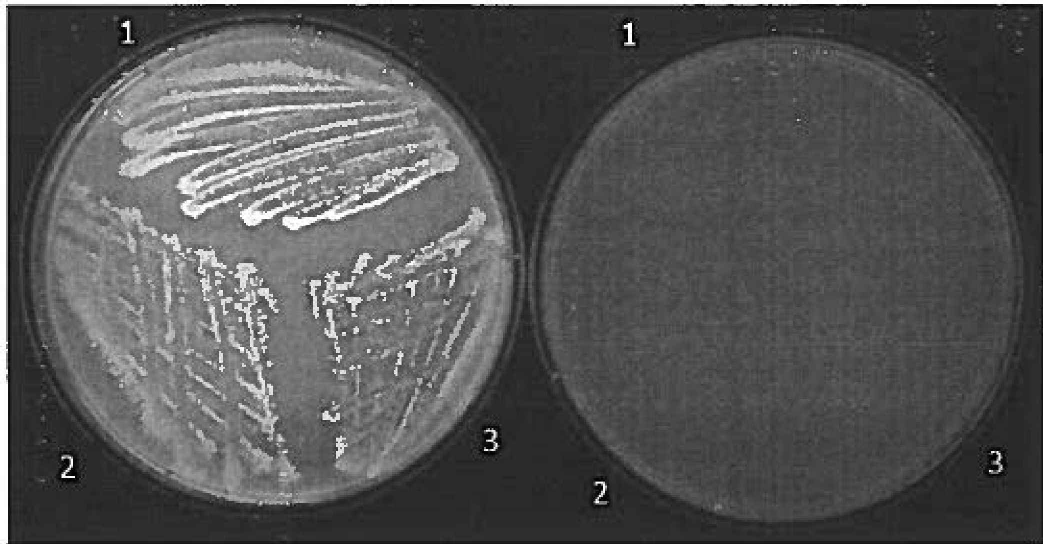

Figure 9: Yeast 2-hybrid screen. Left plate: SMD -ura -leu. Right plate: SMD -ura -leu -ade. Section 1: BD-Ypt1 AD-Atg11CC2-3. Section 2: BD-Ypt1 AD-Atg11. Section 3: BD-Ypt1 AD-empty.

The selective -ade plate in Figure 9 shows the same results as the selective plate in Figure 8. This indicates that the proteins are not interacting *in vivo*, or the interaction might be transient in nature and not strong enough to be detected by the -ade selection.

4.0 Discussion

It has been reported that Ypt1 interacts with the CC2-3 region of Atg11 both in vitro and in vivo.[2] Because we are interested in elucidating the mechanism of Atg11's interactions during selective autophagy, we wanted to verify that the Ypt1-Atg11 interaction does occur experimentally, to use as a control for future experiments. Ypt1 was purified before, but it had a GST tag rather than a histidine tag.[2] Because of this, the protocol used for large-scale purification was developed from existing literature and used for the first time during this research.

When dialyzing Ypt1 into BY buffer after purification, a dialysis cassette was used. The sample was injected and removed using a small needle, which kept coming loose from the syringe. Because of this, a large amount of the sample spilled or wasn't recovered from the cassette. Either more practice is needed with the dialysis cassette, or a different method of protein dialysis is needed.

The *in vitro* binding tests showed that there was no interaction between Atg11 and Ypt1. This is surprising, since the interaction has been reported in literature with His_6-tagged Atg11 and both GST- and HA-tagged Ypt1.[2] However, these reports relied primarily on *in vivo* methods of measuring the interaction such as Yeast-2-hybrid and Bimolecular Fluorescence Complementation. Our lack of pulldown could be because the interaction is transient *in vitro*, not enough of the Ypt1 had been activated by the GTP, or one or both purified proteins are not functional. Future *in vitro* tests could be done by using immunoprecipitation, and testing the pulldown of Ypt1 with a protein A (PA) tag and Atg11 with an HA tag. Because of this, we went forward with testing the interaction *in vivo*.

Previous literature has reported that Ypt1 requires just the CC2-3 region to successfully interact with Atg11 *in vivo* by yeast 2-hybrid screen.[2] To test this ourselves, we used full-length AD-Atg11 as a pseudo-positive control, and AD-empty as a negative control. Much to our surprise, BD-Ypt1 didn't interact at all, even with full-length Atg11. One possible explanation for this could be the type of selection we used. The selective plate was without adenine, which is more stringent than a histidine selection. In other words, an adenine selection is used to test strong protein interactions, while a histidine

selection is used to detect weak protein interactions.[13] A histidine selection was used in previous Ypt1-Atg11 interaction tests[2], but in our hands Atg11 shows autoactivation on –his plates, giving a false positive. Therefore, we decided to use an adenine selection. In the future, a histidine selection could be used to see if the interaction occurs, and 3-AT (3-Amino-1,2,4-triazole, a competitive inhibitor of histidine synthesis) could be used to control the Atg11 autoactivation.

5.0 Conclusion

A plasmid coding for His_6-Ypt1 was successfully constructed and transformed into Rosetta cells, with successful construction verified by sequencing. His_6-Ypt1 was then expressed and purified on a large scale, yielding a concentration of 0.242 mg/mL. This protein was then tested for interaction with GST-Atg11CC2-3 *in vitro*, and showed no interaction. The two proteins were then tested for interaction *in vivo* using a yeast 2-hybrid screen with adenine selection. Despite the Ypt1-Atg11CC2-3 interaction being reported in previous literature, no interaction was seen with either Atg11CC2-3 nor full length Atg11 in either the multiple-knockout strain or a wild-type yeast-2-hybrid strain. This could be due to the use of a more stringent selection. With these results, further yeast 2-hybrid assays could be performed to determine the reason that no interaction appeared and to further understand the mechanism of the Ypt1-Atg11 interaction.

6.0 References

1) Mao, K., Chew, L. H., Inoue-Aono, Y., Cheong, H., Nair, U., Popelka, H., Yip, C., & Klionsky, D. (2013). Atg29 phosphorylation regulates coordination of the Atg17 Atg31-Ag29 complex with the Atg11 scaffold during autophagy initiation. *PNAS 110 (31)*. doi:10.1073/pnas.1300064110.

2) Lipatova, Z., Belogortseva, N., Qi Zhang, X., Kim, J., Taussig, D., & Segev, N. (2012 May 10). Regulation of selective autophagy onset by a Ypt/Rab GTPase module. *PNAS 109 (18)*

3) Lipatova, Z., Shah, A. H., Kim, J. J., Mulholland, J. W., & Segev, N. (2013, July 30). Regulation of ER-phagy by a Ypt/Rab GTPase module. Retrieved November 28, 2016, from http://www.molbiolcell.org/content/24/19/3133.full

4) Mao, K., Wang, K., Liu, X., & Klionsky, D. (2013, July 15). The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy. *Developmental Cell 26 (1)*.

5) Popovic, D. & Dikic, I. TBC1D5 and the AP2 complex regulate Atg9 trafficking and initiation of autophagy. (2014, April). *PMS 15 (4)*. doi: 10.1002/embr.201337995

6) Suzuki, K. (2012, June 15). Selective autophagy in budding yeast. *Cell Death and Differentiation 20 (1)*. doi: 10.1038/cdd.2012.73

7) Cao, Y., & Klionsky, D. J. (2008). New insights into autophagy using a multiple knockout strain. *Autophagy, 4*(8), 1073–1075.

8) Behrends, C., Sowa, M. E., Gygi, S. P., & Harper, J. W. (2010). Network organization of the human autophagy system. *Nature*, *466*(7302), 68–76. http://doi.org/10.1038/nature09204

9) Yorimitsu, T., & Klionsky, D. J. (2005). Atg11 Links Cargo to the Vesicle-forming Machinery in the Cytoplasm to Vacuole Targeting Pathway. *Molecular Biology of the Cell*, *16*(4), 1593–1605. https://doi.org/10.1091/mbc.E04-11-1035

10) Wang, J., Menon, S., Yamasaki, A., Chou, H., Walz, T., Jiang, Y. Ferro-Novick, S. (2013, May 28). Ypt1 recruits the Atg1 kinase to the preautophagosomal structure. *PNAS 110 (24)*. doi: 10.1073/pnas.1302337110

11) Cao, Y., Nair, U., Yasumura-Yorimitsu, K., & Klionsky, D. J. (2009). A multiple *ATG* gene knockout strain for yeast two-hybrid analysis. *Autophagy*, *5*(5), 699–705.

12) Studier, F. W. (2005, January 7). Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification 41*.

13) Mordacq, J. and R. Ellington. 2008. The yeast two-hybrid assay. Pages 167-187, in Tested Studies for Laboratory Teaching, Volume 29 (K.L. Clase, Editor)