CLATHRIN DYNAMICS AND ENDOCYTIC ORGANIZATION IN *Aspergillus nidulans*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Clathrin dynamics and endocytic organization in *Aspergillus nidulans*. (May 2014)

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Polarized growth at the apex of filamentous fungi produces a unique cell type called a hypha. This form of growth found only in fungi, relies on numerous proteins to direct new growth to the cell apex. Endocytic recycling has been hypothesized to be crucial to the maintenance of the polarized localization of these protein complexes. Amongst these important complexes is clathrin, which arrives at the endocytic assembly sites and acts as a coat protein for invaginating vesicles. Clathrin was observed at three different locations in the cell: along and near the sub-apical collar, surrounding late Golgi equivalents just behind the sub-apical collar, and coating vesicles that moved throughout the cell via microtubules. The localization of clathrin will prove important in furthering the knowledge of how endocytosis contributes to the growth of filamentous fungi.

ACKNOWLEDGMENTS

I would like to thank Dr. Brian D Shaw and graduate student, Zach Schultzhaus for their invaluable help and guidance with my research and the creation of this thesis. I would also like to thank Dr. Miguel A. Peñalva for providing me with the *PhosBP*:RFP strain.

CHAPTER I

INTRODUCTION

Importance of filamentous fungi

Currently, the world is plagued by a vast array of plant and human pathogenic filamentous fungi. These pathogens, with ever-increasing virulence and resistance to traditional control methods, will soon prove to be very troublesome for nearly all cereal crops, fruits, and even immune-compromised humans. Some of the most significant of these pathogens include: *Magnaporthe oryzae, Botrytis cinerea,* and many species within the genera Aspergillus and Fusarium. *M. grisea* has proven to be a significant pathogen of rice and other cereal grains while *B. cinerea* is a major pathogen of vineyards and similar fruit crops. Species within the genera *Fusarium* and *Aspergillus* are some of the most severe fungal-human pathogens because of their opportunistic pathogenicity of humans and production of deadly mycotoxins (Hudler 1998). While treatments and prevention practices do exist for many of the most deadly fungal pathogens, these often fall short of any long-term control solutions. Fungal biologists are currently working towards developing a more complete understanding of the growth of filamentous fungi in order to provide insightful responses to these mounting global issues.

Endocytosis is widely accepted to occur in filamentous fungi. It has, however, never been observed in this important group of organisms. In filamentous fungi, the cell grows only at the polarized tip. An intricate balance between exo- and endocytosis within the cell maintains this polarized hyphal elongation. The polar molecules that direct such growth are thought to move away from the cell's apex, and thus must be maintained by endocytic recycling. Endocytic

recycling of these polar molecules occurs at a site termed the sub-apical collar (Shaw et al. 2011), which is considered the most important site of endocytosis in filamentous fungi. This process is depicted in Figure 1. Researchers have proposed several models by which filamentous fungi are thought to achieve their characteristic hyphal growth (Bartnicki-Garcia et al. 1995; Upadhyay & Shaw, 2008; Higuchi et al., 2009; Peñalva, 2010). Recent data points to the crucial role of endocytic recycling in maintaining polarized growth at the apex of growing hyphae. It has been documented that when endocytosis is down regulated or completely prevented, growth of the hypha becomes aberrant and disrupted (Shaw et al., 2011). The objective now is to further the understanding of growth in filamentous fungi, developing a more complete model of growth with all its intricacies, and perhaps imaging the endocytic process for the first time in filamentous fungi.

The role of clathrin in endocytic recycling

In the organism *Saccharomyces cerevisiae* clathrin/actin-mediated endocytosis is well understood. Clathrin, although not required for endocytosis, plays a crucial role in stimulating early endocytic-site assembly (Kaksonen et al., 2005). It is also thought that clathrin might be involved in endocytic sorting and transport within the cell (Baggett and Wendland, 2001). Both of these conclusions affirm the important role of clathrin during endocytosis within yeast, and nearly all eukaryotic cells.

Growth and endocytosis in filamentous fungi (*A. nidulans*)

Clathrin will likely play similar roles in the growth and development of filamentous fungi as in yeast. Therefore, it is hypothesized that GFP-tagged clathrin could serve as a visual marker for

endocytic processes in *A. nidulans*, one of the few model filamentous fungi. Considering the role of clathrin in the early events of endocytosis and as a coat protein of endocytic vesicles, it is expected that localization will occur at the sub-apical endocytic collar as well as around certain invaginated vesicles. *A. nidulans* transformants with GFP-tagged clathrin heavy chain (*chcA*:GFP) have been constructed to help with the visualization of clathrin within the cell. The use of several drug treatments allowed for enriched conditions by which clathrin may be characterized during intracellular function. It is suspected that clathrin-coated vesicles are transported via microtubules and/or actin cables. The use of benomyl and cytochalasin A would in turn help in characterizing the movement of clathrin throughout the cell. Brefeldin A, a drug that disrupts traffic between Golgi equivalents and the endoplasmic reticulum (ER), was also utilized.

CHAPTER II

METHODS

Culture and pharmacological techniques

All strains were cultured on minimal and complete media according to standard procedures. These procedures have been previously outlined (Kaminskyj, 2001). Cells were imaged either as 18-24 hour old cultures on agar blocks or in aluminum chambers attached to coverslips (Kuo and Hoch, 1996). Cytochalasin A was added to leading hyphae on agar blocks at a concentration of 200μg/mL from a stock solution in dimethyl sulfoxide to block the polymerization and elongation of actin within the cell (Upadhyay and Shaw, 2008). Benomyl was added in a similar manner at a concentration of 5 μg/mL to bind to the microtubules within the cell and prevent intracellular transportation (Markina-Iñarrairaegui et al. 2013). FM4-64 was added to cells using a Hamilton syringe directly on the stage of the microscope to a final concentration of 20μM, staining membrane within the cell. This lipophilic dye is taken up through endocytosis and produced red-fluorescence within the cell when bound to membrane (Peñalva, 2005; Fischer-Parton et al. 2000).

Genetic techniques

Several strains of *A. nidulans* were created *de novo* or by crossing with existing laboratory stock to investigate the role and structure of clathrin within the cell. A complete list of strains used in this study is contained in Table 1. Three techniques were used to produce the strains: Fusion Polymerase Chain Reactions (PCR), transformation, and sexual mating between strains. Clathrin

light- and heavy-chain strains (*clcA* & *chcA*, respectively) with each protein tagged with C-terminal GFP were the first to be generated.

PCR

Fusion PCR, a process that uses DNA polymerase and constructed overhangs to stitch together DNA sequences, was used to create the desired construct. The GFP coding sequence was inserted via C-terminal fusions into the native locus by targeted transformation. This process uses Novozyme (NovoNordisk) to digest the cell wall to produce protoplasts. Then polyethylene glycol was used to make the cell membrane permeable and allow for the integration of the DNA construct within the genome of the target strain. The complete methodology has been previously published (Oakley and Edgerton, 2012). Transformants such as: *PhosBP*:RFP, Tubulin:GFP, and LifeAct were also created using similar methodologies, Fusion PCR protocol to transformation procedures, however these were already available in the laboratory (Pantazopoulou and Penalva, 2009).

Transformation

Selection during transformation in *A. nidulans* is facilitated by the use of auxotrophic strains, which are mutated in a gene that synthesizes essential nutrients required for growth. Selection for insertion of the targeted gene is mediated by inserting a good copy of the nutritional gene in order to restore prototrophy. Each strain used in this project is auxotrophic (Table 1).

Sexual crosses

Sexual mating was the last procedure in the overall process, and allowed for the transfer/sharing of phenotypes following selection of the offspring. A complete methodology has been previously published and involves plating strains with the desired phenotypes and complementary auxotrophies on media without any supplements, which promotes heterokaryon formation between two strains (Todd et al. 2007). The mating of most mutants took approximately 2 weeks. These mating mutants were kept sealed with parafilm tape to limit oxygen exchange, kept in the dark to limit asexual reproduction, and incubated at room temperature (approximately 22 °C). After two weeks, cleistothecia, the sexual fruiting structure containing progeny, were fully developed near the surface of the colonies. These cleistothecia were picked and cleaned of vegetative tissue. Ascospore progeny were plated on nutrient supplemented media in order to select for desired characteristics. *PhosBP*:RFP was crossed with *chcA*:GFP in order to aid in the characterization of clathrin within the fungal cell.

Microscopy

An Olympus BX51 equipped with a Hamamatsu ORCA ER camera, a mercury florescent light component, and a 100x objective lens (NA=1.4) was used for visualization. A complete list of specifications for this microscope was previously published (Upadhyay and Shaw, 2005). Green (GFP) and red (mCherry, FM4-64, mRFP) compatible florescence filter sets were utilized. Most of the images were collected via time-lapse with a variety of time-delay(s): dark periods of 1, 2, 5, 10, 30 seconds, and several minutes, which helped to minimize phototoxicity. In addition to these time-lapse images, Z-stack images through multiple focal planes were rendered in order to elucidate clathrin structure throughout the cell. Characteristics were noted and some quantified

such as prevalence of clathrin, average velocity of clathrin-coated vesicles, and rate of growth. These variables were then compared between different laboratory strains/crosses. All images were acquired using a computer connected microscope and camera that utilized Slidebook (version 5.0) imaging software. Images were prepared for publication using Photoshop CS5.

CHAPTER III

RESULTS

Clathrin localization

ChcA:GFP was observed in three locations within the hyphae: at the sub-apical collar, behind the sub-apical collar labeling late Golgi equivalents, and moving throughout the cell on microtubules and/or actin cables (Figure 2 & 3). ChcA:GFP localized at or near the sub-apical collar as a cytoplasmic 'haze'. This localization pattern likely represents ChcA:GFP molecules that are moving too fast and/or are too small to image with a light microscope. ChcA:GFP also localized to punctate spots at the cortex long the cell membrane, and thus labeled the sub-apical collar. ChcA:GFP coated late Golgi equivalents were found throughout the cytoplasm, and were generally excluded from the tip region and the collar region in the sub-apex. Co-localization with PhosBP:RFP revealed that ChcA:GFP ring structures encircled each late Golgi equivalent. Small, moving vesicles were also a common manifestation of clathrin within the cell, these vesicles typically tracked in a straight line for several microns. This indicates a likely association with a cytoskeletal element. The cytoplasmic localization and clathrin-coated collar are relatively stable, persisting in structure and position relative to the growing hypha. The small, moving vesicles showed the most movement throughout the cell, and were observed moving both towards and away from the cell apex at velocities ranging from 1-2 um/sec (n=30). It is suspected that these vesicles use both microtubules and actin cables for long- and short-distance travel throughout the cell, respectively. The last dynamic pattern of ChcA:GFP within the cell are the apparent ring structures formed around the late Golgi. The movement of ChcA:GFP on these structures was restricted to the late Golgi. ChcA:GFP movement off of these ring structures

did occur, but the majority of clathrin vesicles remained on the late Golgi for extended periods of time. Over 90% of the observed late Golgi equivalents were associated with clathrin (n=40).

Clathrin and the cytoskeleton

Three pharmacological agents were used: brefeldin A, benomyl, and cytochalasin A were utilized. Benomyl disrupts microtubule dynamics and resulted in cessation of all long-distance intracellular ChcA:GFP traffic, indicating that the fast moving ChcA:GFP coated vesicles move on microtubules. Polarity maintenance was reduced after benomyl treatment, which resulted in a significant decrease in the cell's rate of growth as well as abnormal cell shape. As expected, many of the cells treated with benomyl grew abnormally. A similar effect was observed when the cell was treated with cytochalasin A, which affects actin microfilament assembly. Cytochalasin A treatment resulted in abnormal cell morphology, reduced growth, but ChcA:GFP traffic was not affected (Figure 4). The effects of these drug treatments on long-distance (> 5μm) vesicle traffic are outlined in Figure 5. The last treatment utilized brefeldin A, a drug that inhibits traffic between the Golgi and endoplasmic reticulum, which did not significantly reduce the fitness of the cell. This treatment did, however, produce an abnormal disappearance of a late Golgi equivalent (Figure 6). The dissolution of the late Golgi equivalent was followed by the disassembly of the corresponding clathrin ring structure. LifeAct:RFP and Tubulin:GFP were treated with cytochalasin A and benomyl as controls in order to show the effects of the drugs on the cytoskeleton, respectively (Figure 7).

Additional results

An additional experiment was designed to determine if there was a correlation between rate of growth and apical volume. Apical volume was essentially the three-dimensional volume of the hyphal tip; the measured area began at the cell's tip and ended at the beginning of the clathrin-coated sub-apical collar (see Figure 1). A linear relationship between rate of growth and apical volume was observed (Figure 8). As apical volume of the cell increased, so did the cell's rate of growth.

CHAPTER IV

CONCLUSION

Clathrin in *A. nidulans*

ChcA:GFP was observed in three distinct locations within the hyphae: throughout the cell as a cytoplasmic 'haze', free-moving punctuate vesicles, and larger ring structures. The cytoplasmic localization was visible throughout the cell, but growing cells exhibited a brighter, more concentrated, 'haze' within the sub-apical collar. This diffuse cytoplasmic localization is likely free-floating clathrin proteins and/or subunits, which are directly related to the sub-apical collar via endocytosis. The clathrin coat along the sub-apical collar is persistent because of the large volume of endocytic vesicles that are continuously being absorbed into the cell. Furthermore, this could explain the residual cytoplasmic localization within the sub-apical collar, as it is likely expended and/or readily available clathrin molecules. In addition to this localization, the volume of clathrin was greatest and most densely concentrated just behind the sub-apical collar. The free-moving clathrin-coated vesicles are expected to act as intracellular transportation of cellular cargo as they travel about the cytoskeleton. These vesicles were seen going both towards and away from the tip in equal numbers. The pharmacological treatments revealed that most of these ChcA:GFP vesicles travel along microtubules, but some short-distance movement may be reserved for actin cables. The last manifestation of ChcA:GFP was as ring structures closely associated with late Golgi equivalents. The late Golgi equivalents are important in packaging proteins for transportation throughout the cell. A continual production of vesicles to be transported from the late Golgi equivalents would require a constant supply of clathrin. As expected, over 90% of observed late Golgi equivalents were associated with ChcA:GFP. In

addition, the brefeldin A treatment produced a unique response by clathrin: when the late Golgi equivalent(s) disappeared, the corresponding clathrin ring structure dispersed shortly afterwards (Figure 5). This affirms the fundamental relationship between the late Golgi and corresponding clathrin structures, which were bright, concentrated, and reserved around the late Golgi.

Additional conclusions

ChcA:GFP also allowed for an assessment of the correlation between rate of growth and cell apical volume. The rate of growth of a cell was linearly correlated to the volume of the cell's apical area. Generally speaking, larger hypha will have a faster rate of growth. Ultimately, the characterization of clathrin within the cell of *A. nidulans* was successful. In the future this information can be applied towards further elucidation of inter- and intracellular processes such as endocytosis. GFP-tagged clathrin can be used as a visual aid in the observation of these cellular processes.

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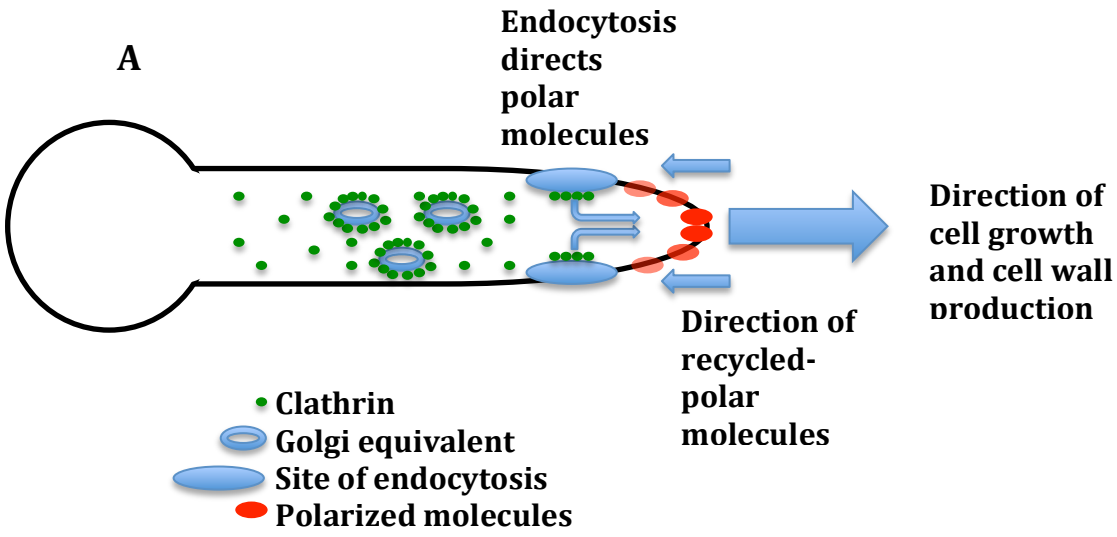
APPENDIX

Table 1. Strains. The following table outlines the strains that were used in this study.

Strains	Genotype	Description	Source
TN02A7	*pyrG89, pyroA4, riboB2, nkuA::argB*	Control strain	Oakley, 2006
MAD2243	*wA4, argB2, pyroA4::[pyro*-gpdamini::mrfp:HOSBP]; niiA4*	Late Golgi Marker-RFP (mRFP-PHOSBP)	Pantazopoulou and Penalva, 2009
ZS1	*pabaB22, ChcA::GFP::PyrG::ChcA*	*chcA*:GFP	This study
MZ1	*pyroA4::[pyro*-gpdamini::mrfp:HOSBP]; ChcA::GFP::PyrG::ChcA*	*chcA*:GFPxLate Golgi Marker-RFP	This study
L01028	*pyrG89, metG1, choA1, chaA1, GFP::tubA*	Tubulin-GFP	Horio and Oakley, 2004
LQ2	*wA4, pyroA4, pyrG89; pyrG::pniiA::LifeAct::RFP*	LifeAct-RFP	This study


A
Endocytosis directs polar molecules
Direction of cell growth and cell wall production
Direction of recycled-polar molecules
Clathrin
Golgi equivalent
Site of endocytosis
Polarized molecules

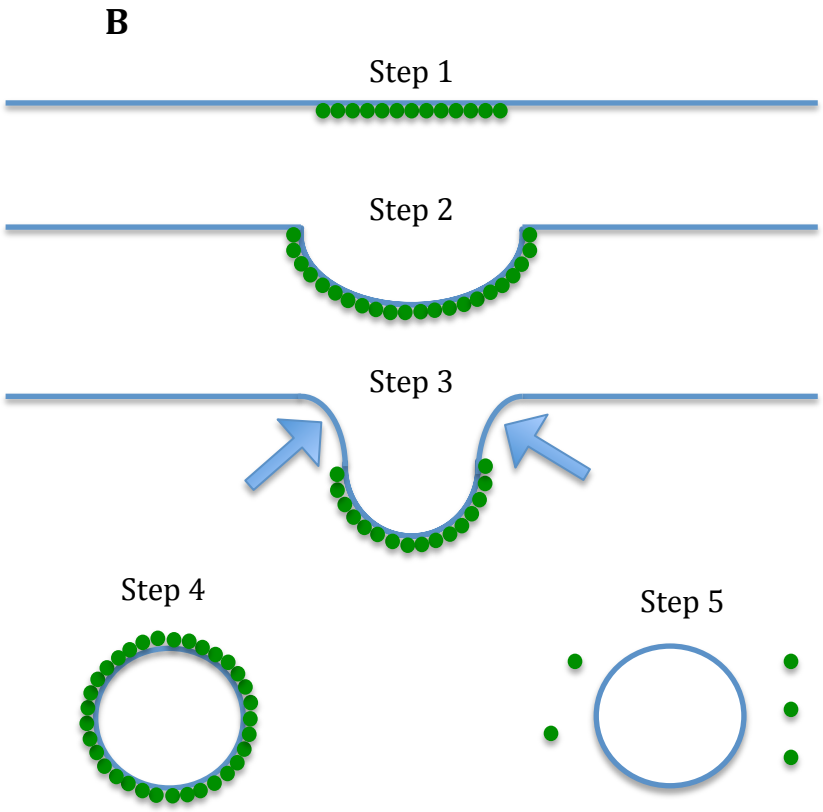

B
Step 1
Step 2
Step 3
Step 4
Step 5

Figure 1. Model depiction of apical-recycling and clathrin budding. A) The localization of clathrin and Golgi equivalents is shown. The cell is thought to grow at the tip via polarized extension; the polar molecules will be forced back away from the site of active growth. These polar molecules are maintained at the tip by endocytosis. B) Clathrin first coats the surface of the membrane. A membrane invagination begins to form with the aid of other cellular proteins. Clathrin continues to aid in shaping the membrane invagination while other proteins apply pressure at the membrane, behind the invagination. The invagination is sliced from the membrane and becomes an intracellular vesicle, and then the clathrin dissipates.

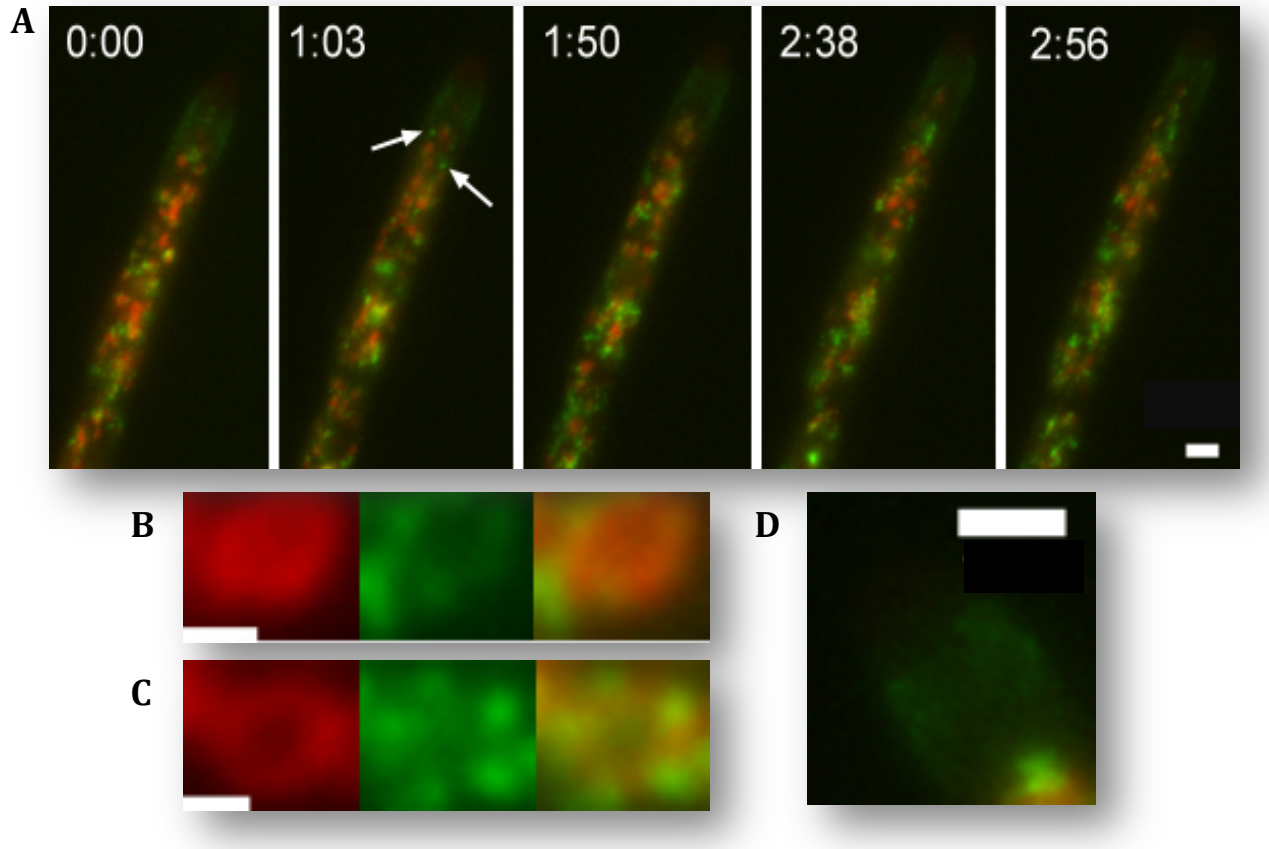


Figure 2. **Localization of clathrin within the cell**. A) Time-lapse imaging of a growing hypha. Clathrin can be seen as green fluorescent structures, and Golgi equivalents are the red fluorescent circles/rings. In the frame marked 1:03, the two white arrows are pointing at two small clathrin-coated vesicles. Scale bar = 2µm B, C) Colocalization of clathrin and the cell's Golgi equivalent(s) is depicted in B&C. The left and middle frames (of B&C) show close-ups of the Golgi equivalents and clathrin, respectively. The ring structure of both clathrin and the Golgi equivalents are clearly visible. Scale bar = 0.5µm D) The cytoplasmic-collar 'haze' is shown. This is considered the site of endocytosis and green fluorescence, suggesting the presence of clathrin, is evident. Scale bar = 2µm

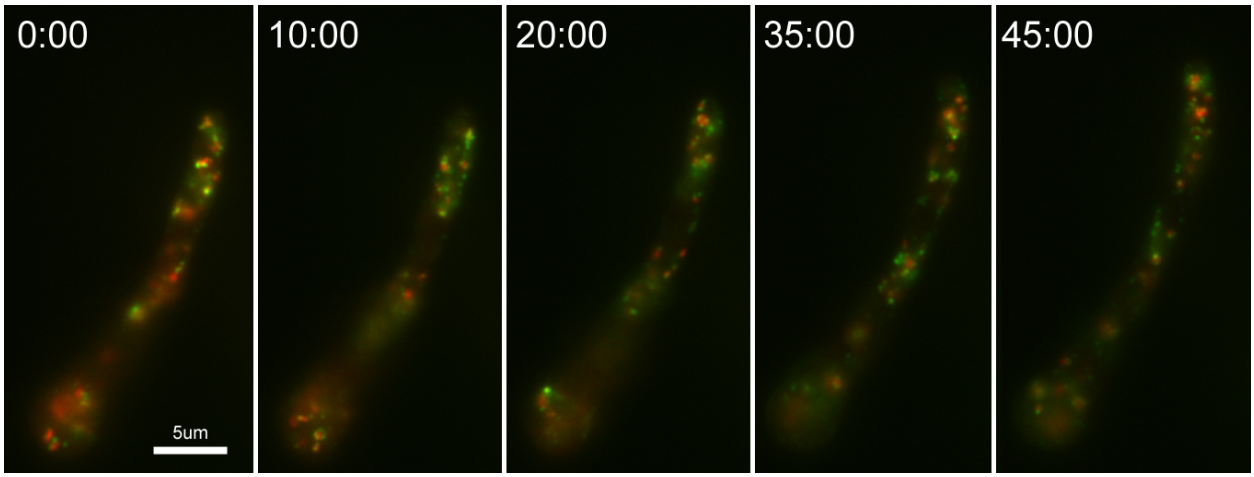


Figure 3. Clathrin localization continued (germination). Germination of hyphae is a slow process. The apical volume (area between the tip and sub-apical collar) of germinating hyphae is generally smaller than that of mature hyphae (see Figure 8). The localization of clathrin and late Golgi equivalents were similar whether the hypha was young or old. Scale bar = 5µm

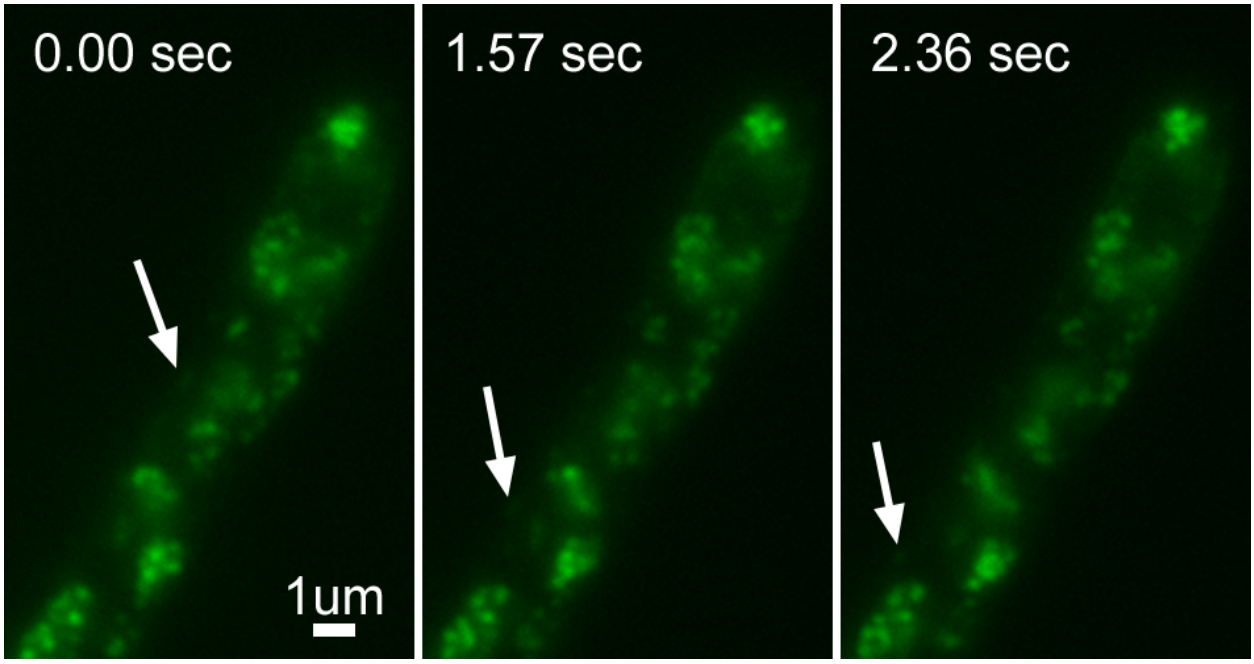


Figure 4. Cytochalasin A drug treatment. Cytochalasin A disrupts actin assembly. Here there was no effect on ChcA:GFP trafficking (arrow), indicating that this trafficking is not occurring on the actin cytoskeleton. Scale bar = 1µm

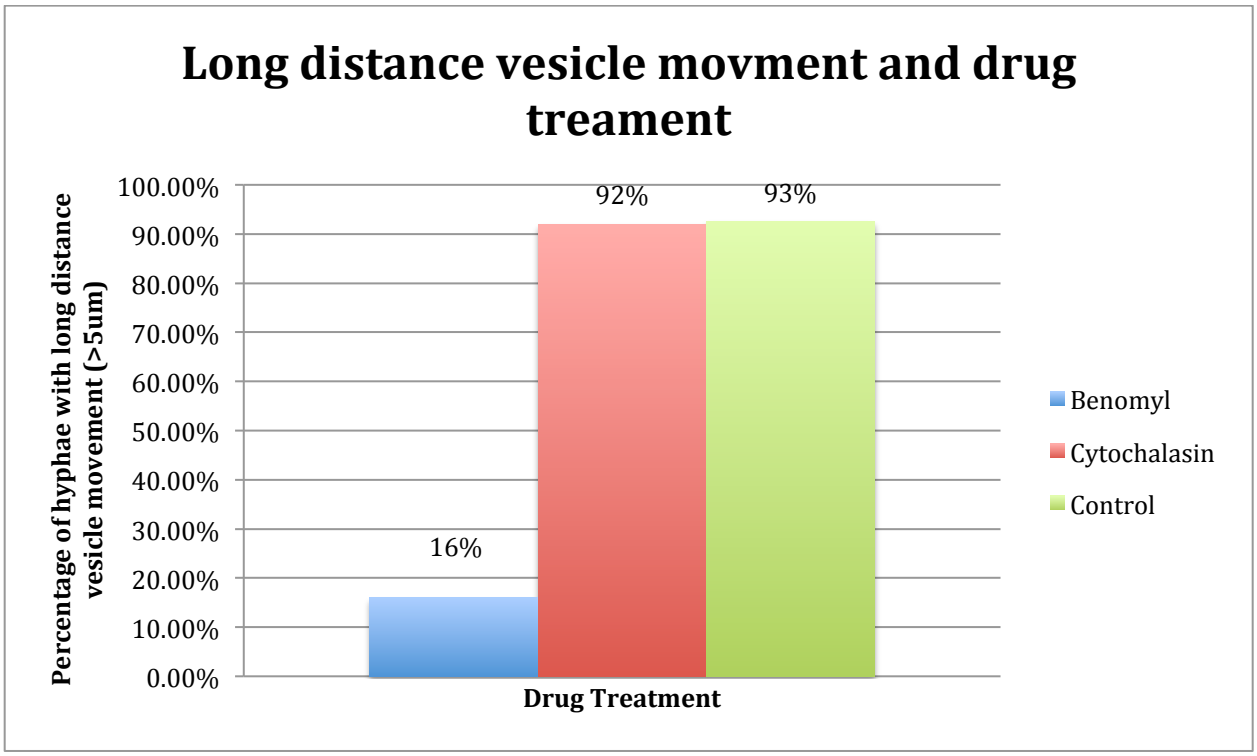


Figure 5. Graph: Long distance vesicle movement and drug treatment. Percentages pertaining to the observation of long-distance (>5um) vesicle traffic were created from a pool of hyphae (n=90). Of the observed hyphae treated with benomyl, only 16% exhibited long-distance vesicle traffic. Control hyphae and those treated with cytochalasin A exhibited greater than 90% occurrence of long-distance vesicle traffic. In other words, microtubules play a crucial in the long-distance traffic of vesicles within *A. nidulans*.

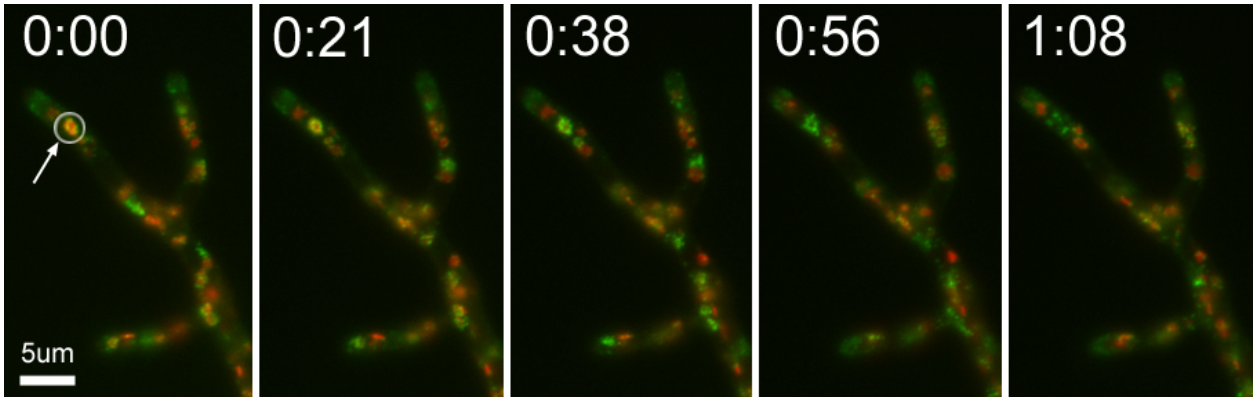


Figure 6. Brefeldin A drug treatment. Brefeldin A disrupts protein (i.e. clathrin) transport between the Golgi apparatus (pictured as red vesicles) and the endoplasmic reticulum (not pictured). The arrow points out a Golgi equivalent that dissipates, which in turn resulted in the dissociation of the corresponding clathrin ring structure. Scale bar = 5µm

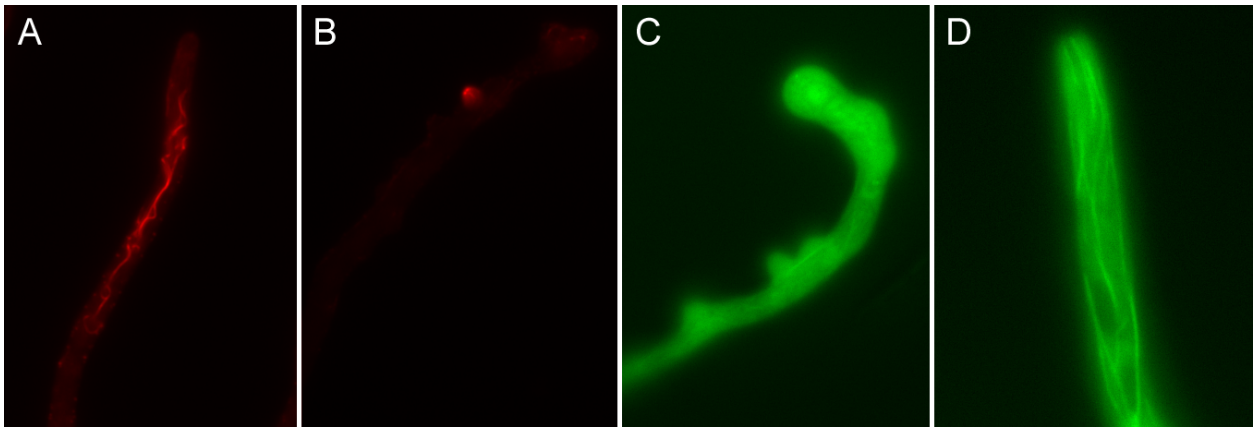


Figure 7. Cytochalasin A and benomyl intended effects. These images depict the effects that cytochalasin A and benomyl have on the cytoskeleton. Images A & D are both controls and were not given either drug; both of these images should be considered typical of *A. nidulans* cells. Image B shows the effect that cytochalasin A has on the polymerization of actin (RFP-LifeAct). Image C shows the effect that benomyl has on the formation of microtubules (GFP-Tubulin).

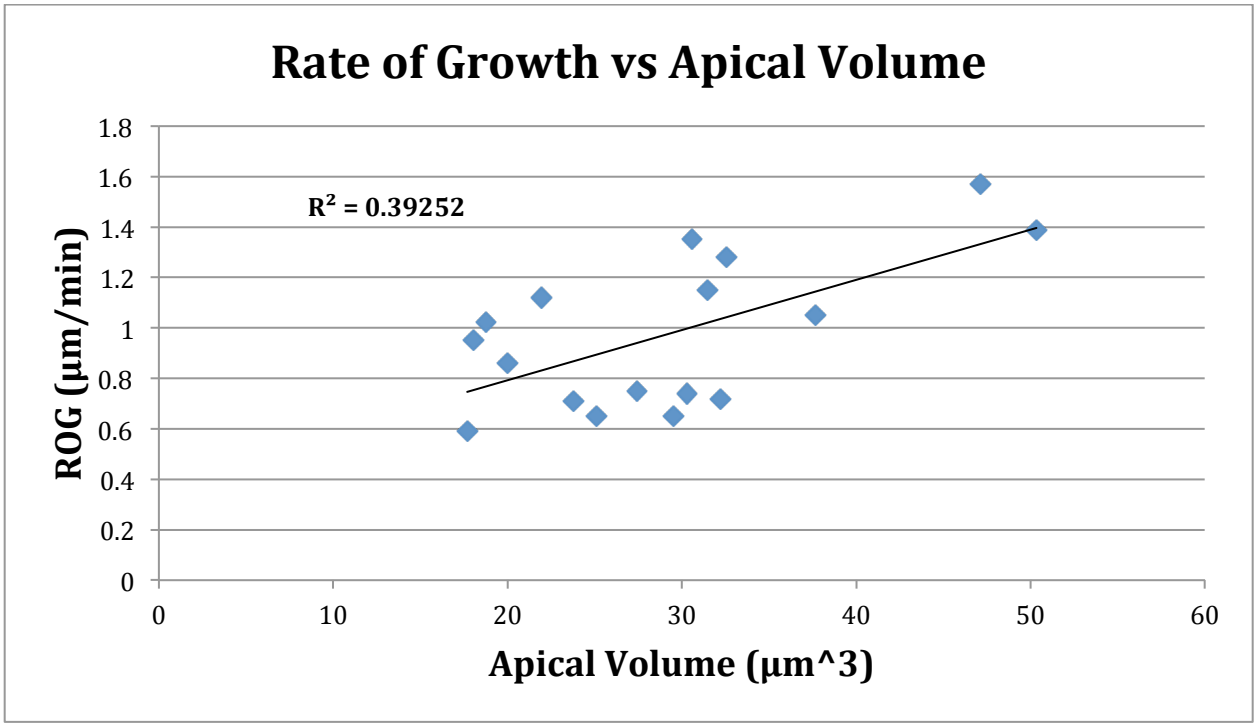


Figure 8. Graph: Rate of growth vs. cell apical volume. This graph tests the correlation between hyphal rate of growth and the apical volume of the cell. The apical volume was calculated by measurements of the cell's tip: length from tip to beginning of clathrin collar, width of hyphae at collar, and the concavity of the hyphal tip were considered. According to the data, the correlation was linear.