SMALL GTPASE ARL3 REGULATES FLAGELLAR MOTILITY IN *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

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Movement of proteins and cargo from the cell body to the flagellar tip is carried out by the intraflagellar transport system (IFT). IFT is composed of multiple protein subunits that are responsible for the building and maintenance of the flagella. ADP- Ribosylaition Factor Like-3(ARL3) is an evolutionarily conserved protein found in all ciliated organisms that is hypothesized to play a role in IFT. ARL3 is a small GTPase that interacts with ciliary proteins as an allosteric regulator, effector, and localizer of proteins associated with retinal photoreceptor issues, retinisis pigmentosa, and poly cystic kidney disease respectively. Mutants with the ARL3 gene disrupted in Chlamydomonas reinhardtii display impaired swimming ability and quick deflagelleation. In an attempt to rescue the mutants, three independent point mutations gave rise to the ARL3 protein actively GTP bound, inactively GDP bound, and in a wildtype interchanging form. Swim speed tests and flagellation rate of wild type insertional mutants showed statistically insignificant difference from naturally occurring wild type in their ability to swim and flagellate. While the active and inactive forms show a significant difference in flagellation rate and swimming ability. Results support our hypothesis of Arl3's localization along the flagellar membrane and within the basal body of *Chlamydomonas reinhardtii.* The goal of this project is

to reestablish proper ciliary function in these ARL3 disputed mutants via transformation of a wildtype plasmid. If successful, similar methods could be implemented to resolve impaired ARL3 found in polycystic kidney disease and photoreceptor malformations.

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NOMENCLATURE

ARL3: ADP Ribosylation factor-Like 3 (Gene)

Arl3: ADP Ribosylation factor-Like 3 (protein)

WT: Wild Type

Chlamy: Chlamydomonas Reinhardtii

TAP: Tris-Acetate-Phosphate

GDP- Guanosine-5’ diphosphate

GTP- Guanosine-5'-triphosphate

IFT: Intraflagellar Transport System

CHAPTER I

INTRODUCTION

Objectives

Mutants with a disrupted ARL3 gene have been created by artificial insertion of a plasmid carrying hygromycin resistance gene (hyg3) with the aminoglycoside phosphotransferase gene (Aph7”) as well. These ARL3 disrupted mutants in *Chlamydomonas Reinhardtii* display abnormal phenotypes when compared to wildtype. Although they can grow phenotypical normal length flagella, Arl3 mutants seem to have an unstable flagella. This is evidenced by their uncoordinated swimming pattern and quick deflagellation rate. Our aim is to rescue these phenotypic issues found in Arl3 mutants by introducing the plasmid of WT properly function form of the ARL3 gene that will translate the correct form of the GDP-GTP interswitching protein. In addition constitutively inactive GDP bound (T30N) and constitutively active GTP bound (Q70L) form of Arl3 will also be analyzed as controls. Introduction of the WT form is hypothesized to restore a greater degree of stability of the cilia and fix these phenotypic issues.

Background:

Flagella

Flagella are extensions of the cell body important for locomotion and cell to cell communication. At the base of flagellum is the basal body, which anchors the flagellar axoneme to the cell membrane. There are two types of cilia, motile and primary. The motile ciliary axomeme contains a radial array of 9 outer doublet microtubules with two central singlet microtubules. The dynein and other force generating components allow motile cilia to beat. On the other hand, the

primary cilia are immotile. They lack the central pair microtubules and all the appendages for motility. The primary cilia are responsible for sensing signals of the extracellular environment while motile cilia, such as the ones in the trachea and oviduct, beat in a synchronized rhythm to generate the movement of fluids (Ishikawa 2011). Motile cilia are also referred to as flagella. These two names can be used interchangeably.

Ciliopathies

Ciliopathies are diseases that result from issues in the flagella include renal disease, retinal disease, situs inverus, and infertility. Many ciliopathies like retinitis pigmentosa (RP2) are caused by an X-linked mutation, however, when properly functioning RP2 serves a regulator of Arl3. Situs inversus is the phenomenon of mirrored location of internal organs in the body. Lack of dynein arms on the microtubules was originally found in patients with primary ciliary dyskinesia that caused infertility in males (Afzelius 1976). Ciliopathies tend to manifest as syndromes as well. Syndromes include Bardet-Biedl, Senior-Loken, Joubert's, Meckel-Gruber, and Leber's congenital Amaurosis, and Nephrononphthisis. Phenotypes associated with these syndromes vary but can include, retinitis pigmentosa, renal disease, polydactyl, hypoplasia of corpus callosum, hepatic disease (Badano, 2006). Recent studies (Gotthardt 2015) have revealed role of Arl13b, a protein when mutated results in Joubert's syndrome, as the guanine exchange factor (GEF) of Arl3. Symptoms of Joubert's syndrome include congenital brain malformation of the cerebral vermis and severity of the disorder is correlated with issues in kidneys and liver. Ciliopathies begin with a small mutation in the genome that goes on to alter protein synthesis and the subsequent interactions necessary for normal flagellar assembly and function. This study

focuses on the phenotypic manifestations of a mutations in the ARL3 gene in model organism Chlamydomonas Reinhardtii.

Chlamydomonas Reinhardtii

Chlamydomonas Reinhardtii (Chlamy) is a photosynthesis, unicellular, green algae used to study flagella due to its evolutionarily conserved intraflagellar transport system. The ARL3 gene has been found in many ciliated organisms including Chlamy (Pazour 2005). This project studies chlamy strains have an artificial insertion that disrupts the whole ARL3 gene. Therefor these cells are not capable of producing the ARL3 protein products, thus we suspect issues in the flagellum where Arl3 protein is hypothesized to localize. When observing ARL3 disrupted chlamy cells, unusual phenotypes including uncoordinated movement and increased frequency of deflagellation. This project aims to determine if the insertion of naturally cycling form of the GTPase Arl3 can fix these issues and restore the flagellar integrity.

ADP- Ribosylation Factor-Like 3

The ADP- Ribosylation factor (ARF) family contains guanine tri-phosphate (GTP) binding protein class which includes ARF and ARF-Like (ARL) classes of proteins, collectively known as GTPases of the super family of Ras proteins. ARL3 is classified as a small GTPase that functions by switching between an inactive GDP bound form and an active GTP bound form. A GTPase Activating Protein (GAP) inactivate GTPases by switching the bound GTP to a GDP. Retinitis Pigmentosa 2 (RP2), mutated in X-linked retinitis pigmentosa, serves as the GAP for Arl3 (Veltel 2008). A Guanine Exchange Factor (GEF) serves to reactive Arl3 by exchanging the bound GDP for a GTP, Arl13b has recently been found to serve this role for Arl3 (Gotthardt

2015). Arl3's exact role in vivo is a topic of current investigation. Arl3 is associated with the microtubule and necessary for cytokinesis (Zhou 2006). Majority of the findings show defects in Arl3 lead to phenotypic issues in the formation and stability of the ciliary structures in internal organs as well as the development of photoreceptor outer segment.

A study of Arl3 (-/-) mice has shown distension of the liver, pancreas and kidney (Schrick 2006). These findings indicate issues in the development of epithelial tubule structures. This is evidenced by renal parenchyma covered with cysts along the cortex, medullary regions, and throughout the nephron. Arl3 has also been found to localize within the human retina (Grayson 2002). The connecting cilium of the rod and con photoreceptor serves as a conduit for proteins and lipids between the outer and inner segments. Schrick's study found the connecting cilium of the photoreceptors present in a rudimentary form. These mice seem to have an inner segment structure like those of wild type, however, only a minimal number of developed outer segments could be found. This finding indicates that Arl3 is needed to in order to develop an outer segments of the photoreceptor (Schrick 2006). Recent studies in mice photoreceptors have shown Arl3 as a regulator of protein trafficking within the photoreceptors (Hanke-Gogokhia 2016).

A study of the flagellated protozoans *Leishmania donovani* (Ld) and *Leishmania amazonensis* analyzed the expression level of LdARL-3A in these Trypanosomatids in order to link level of ARL-3A present with degree of ciliogenesis. Fluorescently labeled anti- LdArl-3A was used to visualize localization. During the non-flagellated life stage of amastigote there was no labeling of LdArl-3A, indicating little to no expression level. However, during the promastigote life stage or flagellated stage localization visible around the cell body, flagella, and flagellar pocket.

Overexpression of the protein did not affect the morphology of the cell. Alternations to the proteome of LdARL-3A where the GTP binding site was point mutated, in the same way our experiments were performed in *chlamydomas reinhardtii* was used. Variables include a constitutively active form, Q70L-GTP, a constitutively inactive form T30N-GDP and unique myristoylation target site of glycine 2 to alanine, G2A. Results showed that the G2A form and the T30N-GDP form had the same defects in viability and poorly centralized labeling of LdARL-3A. A glycine 2 mutation probably results in a form that is similar to that of the T30N-GDP inactive form. The Q70L-GTP form had tiny immotile flagella that were not labeled and poor localization of LdARL-3A throughout the cell. These findings suggest the mutations in ARL3 alter the localization of ciliary proteins needed for proper ciliogenesis (Cuvillier, 2000).

A study in *Caenorhabditis elegans* shows the role of Arl3 to be indirectly connected with the intraflagellar transport system. Arl3 mutants observed under transition electron microscopy (TEM) shows misplacement of the microtubule doublet structures. Arl3 seems to regulate the association of IFTB and the OSM-3 motor as they move along the axonemal surface in a manner similar to that of histone deacteylase 6 (HDAC6). Results also showed that Arl3 was able to rescue mutated Arl-13 through a HDAC6 dependent mechanism. However, over expression of dominant active Arl3 (Q72L) shows cilia missing from the transition zone. This is possibly due to ineffective transport of proteins between the Golgi apparatus and transition zone (Li, 2010). Phenotypically the Arl3 mutants observed here were similar to that of wildtype. This is consistent with our observation that Arl3 are able to produce a phenotypically full length flagella, however, issues on what happened to the flagella during environmental changes were probably not assessed during their experiment.

The function of Arl3 in the cilium has also been investigated. Arl3 was found to bind specifically to the GTP found on the membrane. Binding of Arl3 to uncoordinated C. elegan mutant 119 (UNC119) causes specific release of myristoylated cargo (Ismail 2012). This is a process needed for proper localization of proteins to the ciliary membrane (Wright 2011). ARL-GDP domain doesn't fold the same way of other ARF-GDP forms, this could indicate a specialized function of Arl3 to target a membrane specific compartments (Hillig 2000). This points us closer to the role of Arl3 in the formation of cilium.

One study focused on the role of Arl3 in localizing nephronophthisis 3 (NPHP3) protein to the primary cilium (Writght 2011). NPHP3 is responsible for the proper development of renal tubules. The study showed that the ARL2, ARL3, UNC119a, UNC119b, and RP2 present in retinal pigment epithelial (RPE) cells could serve a function in the localization of NPHP3 to the primary cilium. Subsequent experimentation with siRNAs specific for targeted degradation of the mRNA of these binding proteins showed that in the absence of Arl3 there was only 12% localization of LAP tagged NPHP3 to the primary cilium, RP2 showed 25% localization but UNC119b showed 0% and is therefore critical for NPHP3 localization to the primary cilium. In the absence of ARL2, 75% localization of the LAP tagged NPHP3 was seen in the primary cilium. In addition UNC119a did not seem to play a large role since there was still 65% localization (Wright 2011). This study shows that Arl3 serves as a traffic controller in localizing proper proteins to the cilium. In addition Arl3 and RP2 have been shown to mediate the traffic of membrane associated ciliary proteins (Schwarz 2012).

Intraflagellar transport system

The flagellum is a cellular organelle but not an autonomous structure. It is formed continuously with the plasma membrane and stabilized by the microtubule based axoneme. Moreover, the structure of flagella is not stationary. Therefore, to assemble and maintain the structure, the flagellum must obtain all of its building blocks from proteins synthesized within the cell body. Intraflagellar transport system (IFT) is the multi-subunit protein complex responsible for the assembly of the flagella, IFT-A and IFT-B are the two large subunits (Marshall 2001). They are conserved from Chlamydomonas to humans. This is why it is possible to study issues in *Chlamydomonas reinhardtti* flagella function and relate the findings to human ciliopathies. IFT-A works retrograde to move these recyclable materials back to the basal body where they will be refined before being sent out again. IFT-B is used to move necessary proteins from the basal body to the tip of the flagellum in an anterograde motion. For this to occur kinesin and dynein motors are used to catalyze the anterograde and retrograde movement, respectively (Pedersen 2008). IFT can be thought of as an ecofriendly postal service. Where necessary materials are packaged at the basal body, sent out using an anterograde motor that drops off the cargo along the flagella. Effectors like Arl3 are necessary to bind to cargo carriers like UNC119 to release their cargo within the flagella. After the cargo has been dropped off they take the empty boxes back to headquarters where they can be used again to send out more cargo to different sites along the axoneme. This dynamic cycle needs an incredible amount stability and coordination, however, dysfunction in any protein within the system can lead to a plethora of diseases and impairments.

CHAPTER II

METHODS

Establishing the mutants

Samples that were grown and transformed previously in the lab needed to be checked via western blot for the presence of the transformed gene. After performing a western blot the T30N samples A12 and B12 both did not have the transformed gene. This is concurrent with the hypothesis because the T30N is the GDP form, which is much more unstable then the Q70L GTP form of ARL3. A fresh round of transformation was performed with the ARL3-T30N-Paramycine resistance-GFP plasmid. Fresh transformants were then cultured and confirmed with another western blot.

Transformation via Electroporation

Arl3 (+ mating type form was used) backcross progenies were streaked on hygromycin TAP plates. Arl3+ contains the hygromycin resistance gene from the original disruption used to create the mutants. Samples were inoculated in 23mL of TAP solution until densely green growth was observed. Then transferred to 120mL TAP solution until the culture again was densely green. Finally the sample was transferred to a 900mL TAP solution for 20-24 hours. Transformation was carried out during this critical period when the cells were in the mid log growth phase. 1mL of concentrated cells, 60mM of sorbitol and 10μL of desired linearized plasmid were chilled in an electroporation cuvette. Electroporation with the parameters was carried out at 825Ω, 750V,

and 650µC to shock cell and create temporary holes in the plasma membrane. The plasmid was linearized at the BGLII restriction site. Figure 1 below shows details of the transformation.

Figure 1.

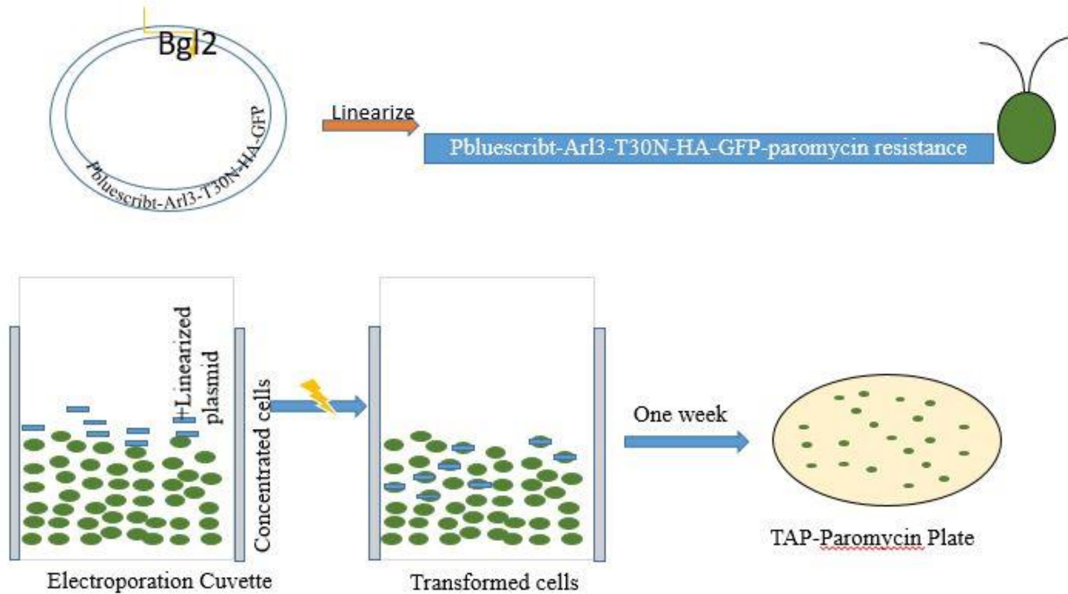


Figure 1. Arl3+ cells were shocked with electric current in the presence of the linearized form of *E.coli* plasmid "pbluescript-Arl3-T30N-paromycine resistance-HA-GFP". Only cells that took up the plasmid will be able to grow on the plate after 1 week. Isolated colonies were collected and cultured for further testing.

Phenotypic classification

Before each test freshly samples were cultured by inoculating cells from paromycin TAP plates into 22mL of TAP medium in 50mL flasks. Mutants that presented dark bands indicating high expression levels in the previous western blot (8 samples) along with controls (CC125, ARL3+) were used for further analysis. In an attempt to analyze all tested samples A8T30N was still used as a negative control.

Figure 2.

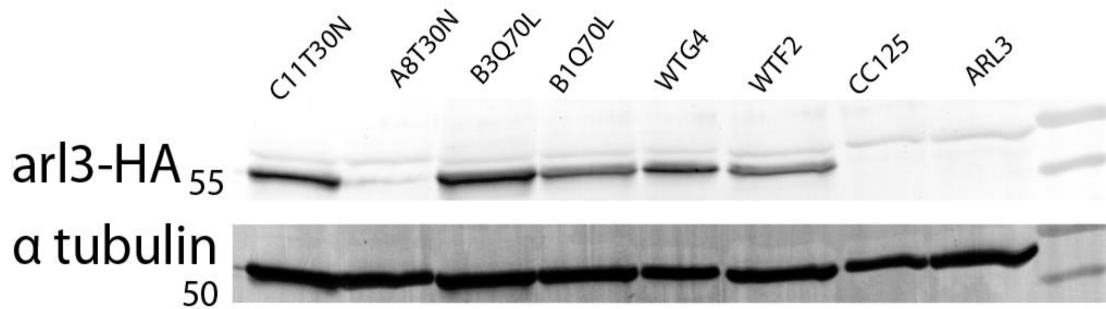


Figure 2. Results from second immunoblot. A8T30N, originally shown to have the highest expression level of the transformed gene, now shows little expression and will no longer be considered an inactive mutant. CC125 is the WT control and does not have the Arl3-HA tag we have inserted**.** HA tag and anti-rat secondary antibody was used to visualize the expression level of the transformed gene. Alpha tubulin was probed for using anti alpha tubulin and anti-mouse secondary antibody. Stained protein ladder was used as a marker.

Immunofluorescence localization:

We would like to see the localization of the transformed protein products within the cell. For this experiment formaldehyde fixation was used to visualize Arl3 since it is a membrane related protein. Total internal reflection fluorescence (TIRF) was used to see the labeling of flagellar and membrane proteins. Fla10 is a known IFT motor protein that is localized along the length of the flagella. It was visualized with red alexa flouro 594. We probed for our HA-Arl3 tag and with green “alexa flouro 488”. 100x objective was used to visual the cells. Image J software was used to analyze this data.

Measurement of flagellar length:

Cells are fixed with 2% glutaraldehyde for clear visualization. 200μL of cells cultured in TAP medium and 200μL of 4% glutaraldehyde are mixed in a 1.5 mL Eppendorf tube. 12μL of this solution is placed on a microscope slide and allowed to dry for three minutes before placing a

cover slip. Flagella were measured using light microscope at 63x objective with emersion oil. Measurements were calculated using "Image J" software.

Swim speed measurement:

Cell cultures were placed in a swimming chamber and recorded with a high speed camera using Köhler illumination. Photos were taken at 2 day culture time as well as 7 day culture time. Samples were serially diluted before being evaluated. For the 2 day sample 10µL of cultured cells + 90µL of fresh TAP were mixed for a 10x dilution. Then 8µL of the first dilution was mixed with 72µL of fresh TAP for a final 100x dilution and a final volume of 80µL in the swim chamber. Infinity capture software was used to take the photos of the cells. Exposure time was set to two seconds, the gain was set to 1.75 and the 4x objective lens was used.

CHAPTER III

RESULTS

Figure 3.

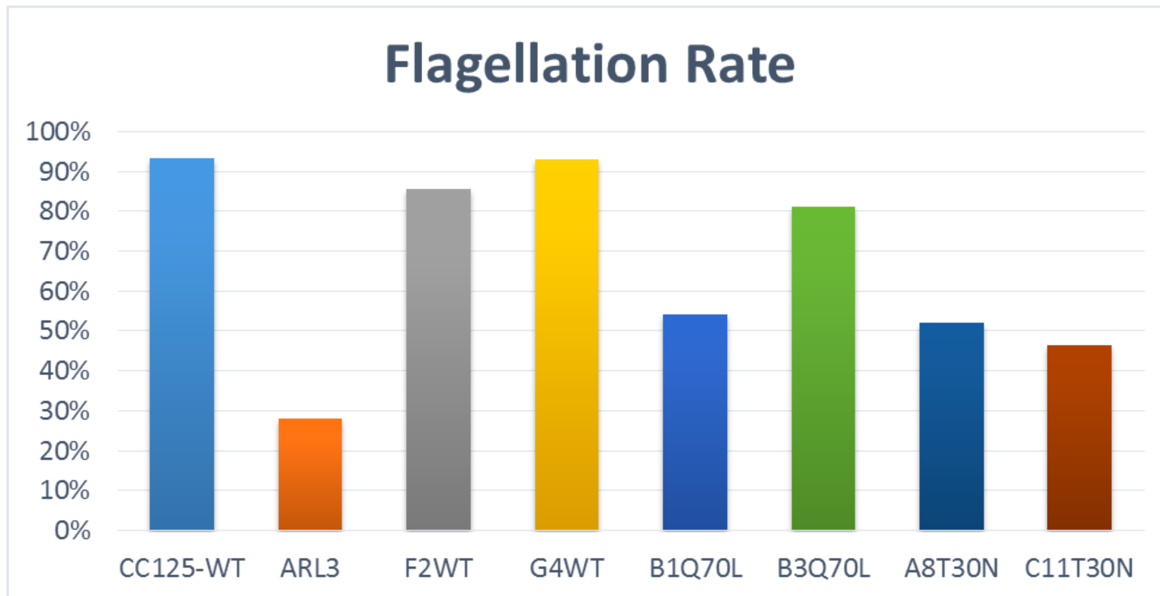


Figure 3.Flagellation Rate: 300 cells for each sample were counted for the presence of flagella with a light microscope, percentage values are displayed above. Arl3 tends to deflagellated rapidly. The trend is analogs to the expression level of the Ha-arl3 tag in wild type and Q70L insertional mutant. However for the T30N form there is no apparent trend since A8 actually has lower level of expression than C11. Majority of the mutant strains are lacking flagella which is supported the observation of immotile cells during the swim test.

Figure 4.

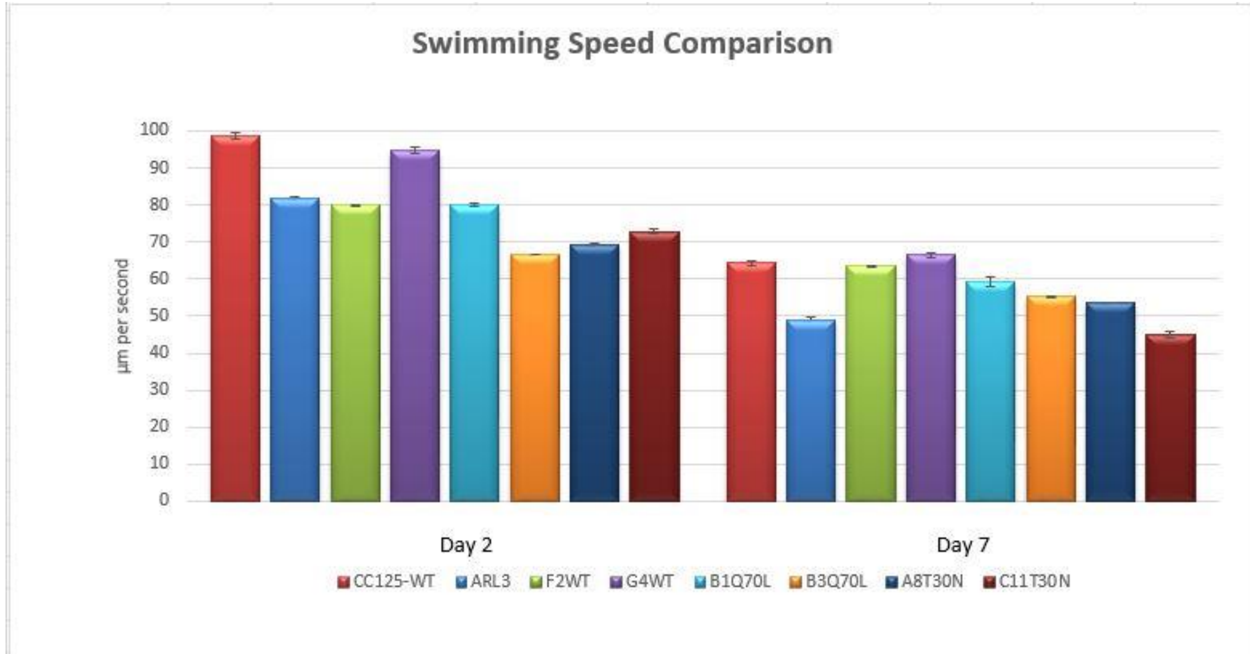


Figure 4. Swimming Rate**:** Three independent rounds of measurements showed similar trends among the swim speeds of the swimming cells in each sample. Data from a single trial is reported above. This data only takes paths of motile cells for averages. A t-test for significance at α=.05 revealed differences in the mean swim speed at 2 days for all samples, of these values G4WT was least significant in difference. However for the 7 day culture there is only a statistical significance between the Arl3 and cc125. G4WT shows marginally better swimming ability at this time point. Error bars are displayed for the different in means from the two experimental trails.

Figure 5. (Below) Protein localization: Immunofluorescence was used to visualize localization Arl3-HA in green and an IFT motor protein Fla-10 in red. Negative control shows the absence of green within the flagellar region. In the presence of Arl3 localization of both to the flagella is apparent in the active GTP bound mutation. In the presence of Arl3 shows localization in the flagella is apparent in the inactive GDP bound form. Wild type insertional mutation shows co-localization indicated by the yellow color found within the flagellar region.

Figure 5.

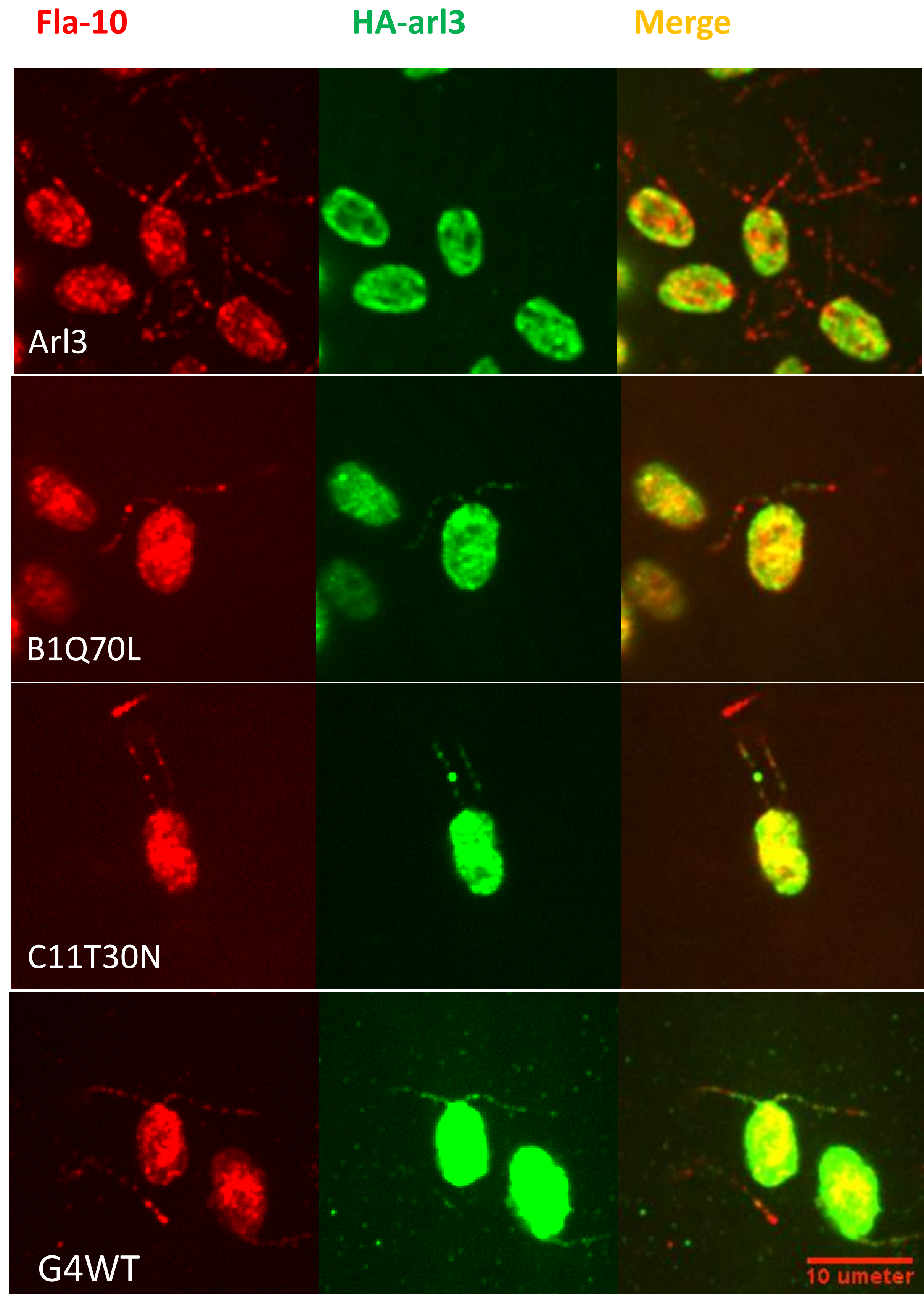
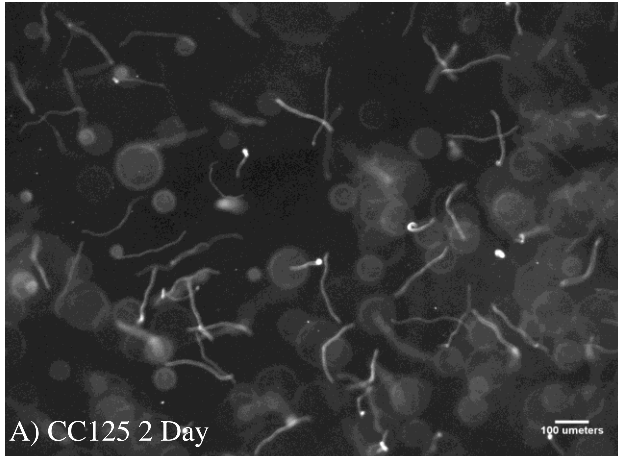
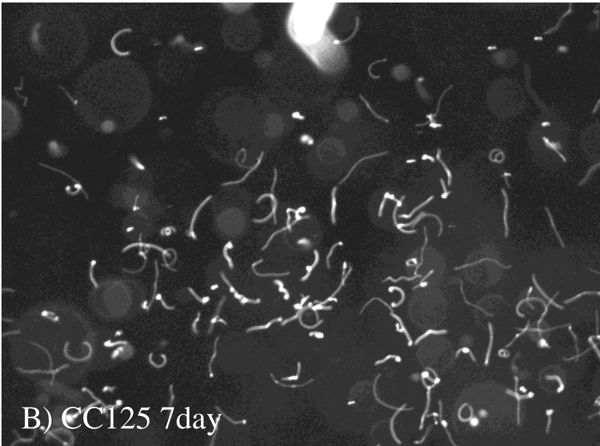
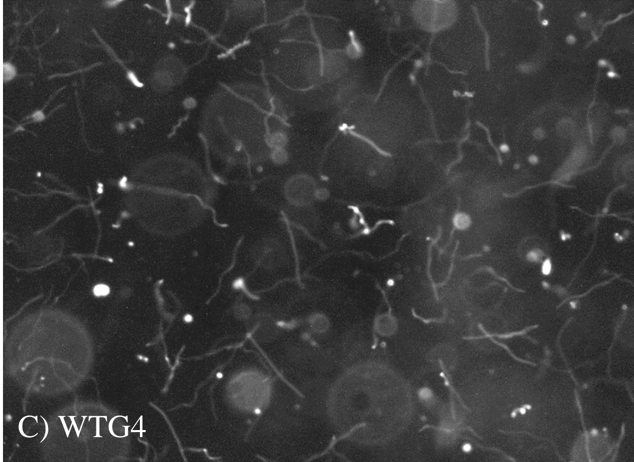
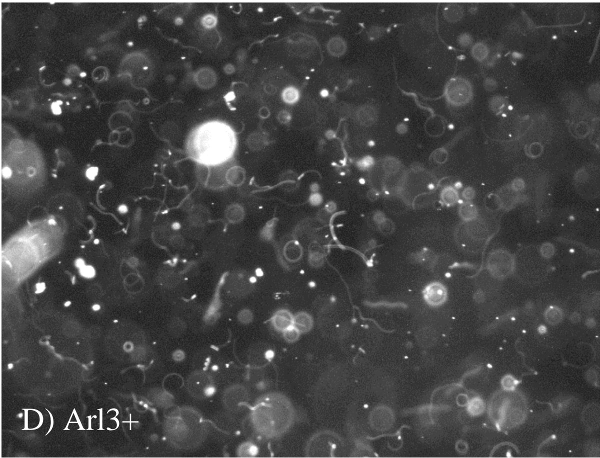


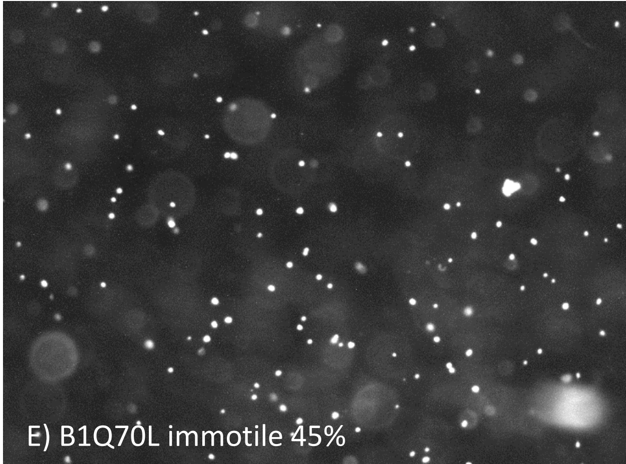
Figure 6.

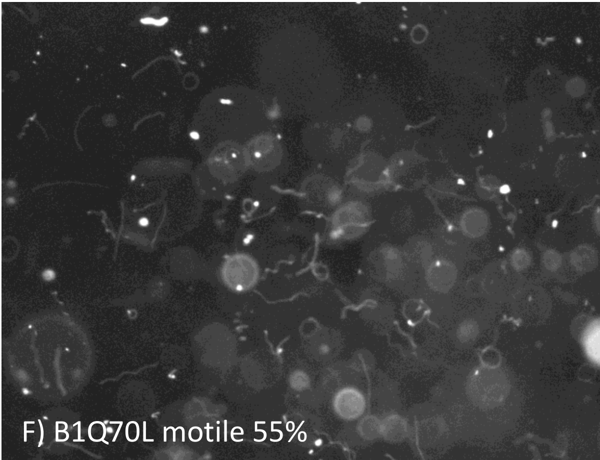












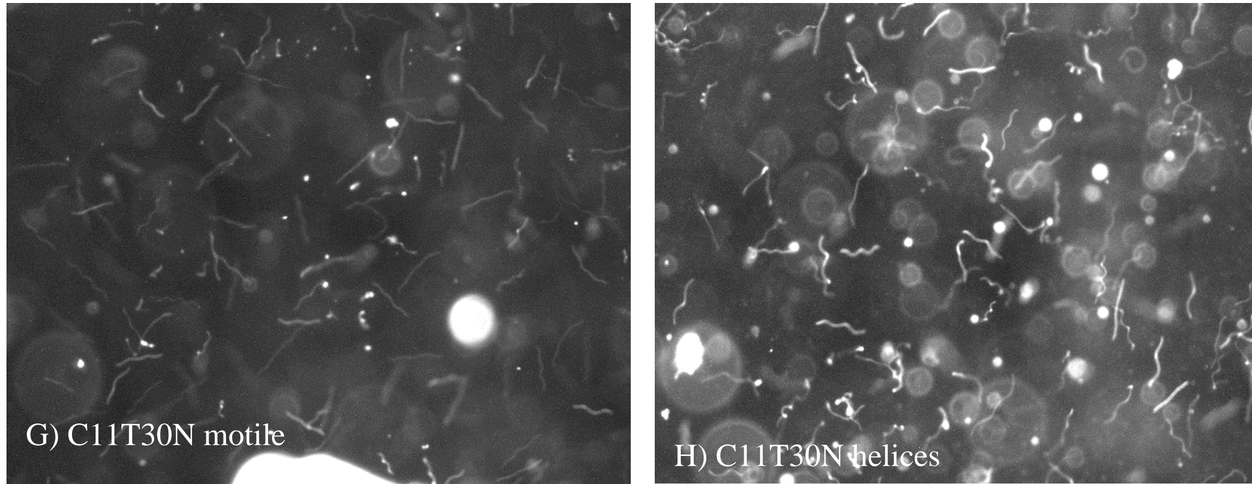


Figure 6. Swim test: Tracked produced by swimming cells over a two second period were visualized using Köhler illumination. Tracks visualized are those of moving cells only and analysis of swimming speeds only includes motile cells. CC125 at 7 days is shown in panel B, remaining images displayed are of day 2 of cell cultures. 7 day culture showed consistently slower swimming speed among all strains. Bright small circle depict immotile cells. CC125 WT (A) cells swim in a linear path. ARL3 (D) disrupted mutants swim in a curved path and make helical turns. WT insertional (C) mutants have restored linear swimming ability. Q70L active GTP form are also able to swim if they have flagella (F), however some cells did not have flagella (E) and remain immotile during the swim test. T30N inactive GDP form cells swim in varied forms, some cells (G) seem partially rescued while others (H) have reverted to the helical swimming turns found in Arl3. Images were taken 4x magnification and pictures were obtained through infinity capture software.

CHAPTER IV

CONCLUSION

Swim speed test shows that Arl3 is deficient in coordinated swimming ability. The rescued strains were able to restore linear swimming pattern. Although the rescue of G4WT is not complete it is very similar to wild type. Arl3*GDP bound showed 40% paired flagellation and was able to swim if flagella were present, however majority of the cells did not have flagella. Arl3*GTP bound had a significantly larger amount of bald cells, but those with flagella seemed to be moving around. Although both GTP and GDP bound forms of Arl3 can swim if they have flagella, they do not swim as far as wild type cells. This could be due to issues in flagellar coordination or strength of flagella. Both GTP and GDP forms show evidence of partial rescue. Immunofluorescence imaging shows minimal localization of Ha-Arl3*GDP and Ha-Arl3*GTP within the flagella. Although there are both green and red signals, co-localization shown in yellow with Fla-10 is not as apparent as that found in the WT rescued strain. This could mean the GDP form can enter the flagella, but it is not actually apart of the IFT train. The dot pattern seen in the flagella with HA-arl3 could indicate involvement of IFT cargo or could be an artifact of intense membrane lysis. Further imaging is needed for conclusive results. Comprehensive analysis of the results support that Arl3 mutants have been rescued by these transformations evidenced by restored linear swimming path and higher flagellation rates.

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