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The thesis research presented here focused on studies of a novel myosin II heavy chain kinase D (MHCK D) expressed in *Dictyostelium discoideum* cells. MHCK D is made up of four distinct domains: a short coiled-coil region (Coil), a region rich in serine, asparagine, proline, & glutamine residues (SNPQ), a kinase catalytic domain (Cat), and a WD-repeat segment (WD). A major component of this project was to amplify the Catalytic and WD repeat domains (Cat-WD) from genomic DNA. The Cat-WD truncation of MHCK D was cloned into pTX-GFP & pTX-Flag plasmids for expression in *Dictyostelium* cells. The recombinant plasmids were electroporated into D*ictyostelium* cells to over-express the fusion protein (GFP, or flag-tagged). To determine if MHCK D does phosphorylate the Myosin heavy chain, which in turn drives bi-polar filament disassembly, the phenotype of cells over-expressing the Cat-WD domain were compared to the phenotype of Myosin II-null cells and non-recombinant wild type cells. Cells over-expressing the Cat-WD domain from MHCK D, showed a phenotype similar to Myosin II-null cells, indicating that the Cat-WD domain plays a role in Myosin II bi-polar filament disassembly resulting from phosphorylation of the myosin heavy chain. Another aim of the thesis research focused on expression of the *mhkd* gene during the multi-cellular development cycle of *Dictyostelium*, using Reverse Transcriptase-PCR (RT-PCR). The results indicate that *mhkd* is expressed throughout the multi-cellular development cycle, as well as during the vegetative growth phase of *Dictyostelium*. In summary, the results reveal that the Cat-WD domain of MHCK D drives Myosin II bi-

polar filament disassembly leading to defects in cytokinesis, and is expressed in both vegetative cells as well as cells undergoing the multi-cellular development cycle. These studies provide a basis for future research focused on activation, localization, and substrate targeting of MHCK D.

CLONING & CELLULAR CHARACTERIZATION OF MYOSIN II HEAVY CHAIN KINASE D FROM *DICTYOSTELIUM DISCOIDEUM*

by

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CHAPTER I

INTRODUCTION

Dictyostelium discoideum is a social amoeba that feeds on bacteria and is found in the soil. The *Dictyostelium* genome has been sequenced and is made up of 6 chromosomes totaling 34Mbp . Under normal conditions with sufficient food, the cells grow and divide by binary fission. When the nutrient levels decrease below a certain level, the cells are signaled to undergo multicellular development (Figure 1). This is initiated by a single cell, the founder, releasing cyclic adenosine monophosphate (cAMP) into the surrounding area . When the cAMP comes into contact with cAMP receptors on the surface of other *Dictyostelium* cells it activates a G-protein coupled pathway. This leads to the activation of the enzyme adenlyate cyclase which catalyzes the conversion of ATP into cAMP, which is then secreted into the surrounding area. Thus, the cAMP signal initiated by the founder cells is effectively amplified and relayed to cells in the surrounding area. Despite having cAMP receptors uniformly distributed on their surfaces, *Dictyostelium* cells respond to a gradient of cAMP by taking on a highly polarized shape and migrating (chemotaxing) toward the source of cAMP . While the early intracellular signaling events leading to chemotaxis toward cAMP are still unclear, the changes in the actin-myosin II cytoskeleton that actually drive cell shape change and locomotion have been well described .

Within 4 hours after the release of cAMP, approximately 100,000 cells will aggregate together and form a mound at the site of the initial release of cAMP. The aggregate of cells can then begin to move toward a stimulus to try and find a more nutrient rich area to increase their chance of survival. If they do not find nutrients, the cells will begin to differentiate into either stalk cells, or pre-spore cells. The stalk cells will lift the pre-spore cells within the fruiting body into the air. Both the stalk cells and the pre-spore cells will release cellulose, although the cells will react differently to this release. After the stalk cells release the cellulose, they will desiccate and die. The cellulose released from the pre-spore cells will form a barrier which will protect the spores when they are released into the air (Kessin, 2001).

Using *Dictyostelium* as a model organism has many advantages over using other organisms. The *Dictyostelium* genome contains many genes that are similar to those in higher eukaryotic organisms, but are not present in other model systems. This makes *Dictyostelium* very useful in functional analysis of sequenced genes. The ability of *Dictyostelium* to undergo certain cellular processes allows for research which can be applied to human health and disease. These cellular processes include cell motility, cytokinesis, signal transduction, and phagocytosis to name a few. Cancer cells undergoing metastasis as well as angiogenesis by endothelial cells rely on cell motility. Cytokinesis is essential in cellular maintenance and proliferation, and thus plays an important role in cells of the immune system. In higher eukaryotic organisms, signal transduction is essential to regulate the behavior of cells, as well as to sense both external and internal environments. Defects in signal transduction can lead to a variety of

diseases including cancer, diabetes, and autoimmunity. Many organisms require phagocytosis to efficiently remove pathogens through antigen presentation of the immune cells. Phagocytosis is also used as a method for some cells to internalize essential nutrients. What we know thus far about these fundamental cellular processes can be attributed, in part, to the use of model organisms like *Dictyostelium*.

Dictyostelium expresses twelve different types of myosin proteins, seven of which are myosin I (unconventional myosin), a single headed protein, and one double headed myosin protein known as myosin II (a conventional myosin). Myosin II is a hexamer consisting of two regulatory light chains, two essential light chains, and two heavy chains. Under appropriate conditions, myosin II hexamers aggregate to form bipolar filaments capable of contracting apposing actin filaments (Figure 2). The contraction of actin filaments is driven by the ATP-dependent movement of the globular "head" region of the myosin II heavy chain, and is activated upon phosphorylation of the regulatory light chain subunits of the myosin II molecule. In contrast, the ability of myosin II to contract actin filaments is inhibited by the phosphorylation of three threonine residues on the myosin heavy chain (MHC), which leads to disassembly of myosin II bipolar filaments into contraction-incompetent monomers (Figure 2) (De la Roche and Cote, 2001). Cellular, genetic, and biochemical studies have revealed that *Dictyostelium* cell division, efficient chemotaxis, and multicellular development rely on the ability of myosin II to contract actin filaments, and that MHC phosphorylation plays a central role in regulating myosin II activity and localization in the cell (Bosgraaf et al. 2006).

Myosin heavy chain kinase D (MHCK D) is one of four structurally related kinases, three of which (MHCK A, B, & C) have been shown to phosphorylate *Dictyostelium* myosin II heavy chain and drive bipolar filament disassembly *in vivo* and *in vitro*. Based on its predicted structural similarity to the MHCK A, B, and C enzymes (Figure 3), it is presumed that MHCK D catalyzes MHC phosphorylation in a manner that leads to myosin II bipolar filament disassembly (Yumura et al., 2005). All four members of the MHC kinase family share similar domain structure, with each having a carboxyl-terminal WD repeat domain, and an alpha kinase catalytic domain. The gene for MHCK D encodes a 96kDa protein that contains four distinct domains: a short coiled-coil region, a region rich in serine, asparagine, proline, & glutamine residues (SNPQ domain), an alpha-kinase catalytic domain, and a WD-repeat segment (Figure 3). Protein-protein interaction studies have demonstrated that the WD repeat domains of MHCK A and B target these kinases to myosin II by binding directly to the myosin filaments (Steimle et al., 2001). The catalytic domain then phosphorylates three threonine residues at positions 1823, 1833, & 2029 on the heavy chain of myosin II. In wild type (WT) *Dictyostelium* cells the phosphorylation of the three threonine residues results in the disassembly of the myosin II within the cell (Figure 2).

Cellular and genetic studies have shown that *Dictyostelium* cells that contain a mutant myosin II heavy chain gene, coding for the myosin II protein in which these three threonine residues were each changed to alanine (an amino acid unable to be phosphorylated), had severe over assembly of myosin II (Egelhoff et al., 1993). Studies have also shown that when MHCK A was over-expressed in these *Dictyostelium* cells

with the mutant myosin II protein, the myosin II was resistant to disassembly (Kolman & Egelhoff, 1997).

The catalytic domain of MHCK D (as well as MHCK A, B, & C) is known as an alpha-kinase catalytic domain. Alpha-kinases are proteins that contain multiple domains within the protein in addition to a catalytic domain. The domains include WD-repeats, TPR-repeats, ion-channels, integrin-like domains, or calmodulin binding domains to name a few (Drennan et al., 2004) The term alpha-kinase was suggested for this family of kinases due to their ability to phosphorylate amino acid residues in an alpha-helical structure (Ryazanov et al., 1999), as compared to conventional protein kinases (CPK's) that phosphorylate amino acids in a loop or an irregular structure. Alpha-kinases are also structurally different than CPK's (Manning et al., 2002a, b). There are a variety of alpha-kinases found in other organisms, for example fungi, protozans, and vertebrates. The completion of the human genome project reveled that human cells express 6 unique alpha-kinases. They include eEF-2 kinase, TRPM7, kidney alpha-kinase, lymphocyte alpha-kinase, muscle alpha-kinase, and heart alpha-kinase (Ryazanov et al., 1999; Ryazanova et al., 2001; Ryazanov, 2002). However, there are no known alpha-kinase proteins that phosphorylate the myosin II heavy chain within human cells. These alpha-kinases have also been shown to be absent in all prokaryotic organisms, yeasts, insects, and higher plants whose genomes have been sequenced thus far (Drennen et al., 2004).

The MHCK A, B, and C enzymes have been studied in considerable detail, with particular focus on their roles in myosin II disassembly during cytokinesis, and cellular migration (Egelhoff, 1999; Kolman et al., 1996; Liang et al., 2002; Nagasaki et al., 2002;

Rico and Egelhoff, 2003; Yumura et al., 2005). Recent studies using Fluorescence Recovery After Photobleaching (FRAP) analysis revealed that there is a significant increase in fluorescence recovery time of GFP-myosin II in the contractile ring during cytokinesis in cells lacking MHCK A, B, and C enzymes, compared to that of wild type Ax2 cells (Yumura et al., 2005). Ax2 *Dictyostelium* cells are a strain of cells selected for their ability to grow in HL5 media, rather than on bacterial lawns. This cell line was chosen as the wild type control group in all studies conducted during the thesis research. Yumura et al. also showed that MHCK A, B, and C null cells exhibit a significant increase in GFP-myosin II FRAP in the actin-myosin II cortex of interphase cells. This shows that these three kinases are required for proper turnover of myosin II filaments in the cell. In contrast, when Yumura et al. tested MHCK D null cells during cytokinesis and interphase, they saw no significant increase in recovery time, compared to the WT cells. The researchers concluded that if in fact MHCK D has a role in myosin II assembly control, that it would be in a setting other than that of the basal control of myosin levels (Yumura et al., 2005). This indicates the need for further research on MHCK D to determine what role, if any, this kinase plays in regulating myosin II function in the cell.

Thesis Research Aims

The broad aim of my thesis research was to determine if MHCK D indeed functions as a myosin II heavy chain kinase in *Dictyostelium* cells and to identify when in the multicellular developmental cycle this kinase is expressed & functional. To this end, I focused on performing the following sets of studies:

1) **Cloning of MHCK D catalytic and WD-repeat domains (Cat-WD) into vectors for over-expression of the Cat-WD protein with a Flag-epitope tag & Green Fluorescent Protein (GFP) tag in *Dictyostelium* cells.** These cells were then analyzed for the changes in phenotype resulting from increased myosin II heavy chain kinase D activity in the cell (see #2, below).

2) **Analysis of the ability of the MHCK D Cat-WD domain to function *in vivo* as a myosin II heavy chain kinase in *Dictyostelium* cells.** If the Cat-WD domain of MHCK-D does act similar to the other MHC kinases (Betapudi et al., 2005; Kolman et al., 1996; Liang et al., 2002; Nagasaki et al., 2002; Rico and Egelhoff, 2003), then the over-expression of the Cat-WD domain should show a similar phenotype to that of Myosin II-null cells, because the Cat-WD domain should keep the myosin II phosphorylated and in a disassembled state. Over-expression of MHCK A, B, & C in *Dictyostelium* cells causes an increase in phosphorylation of the three threonine residues at positions 1823, 1833, & 2029 of the myosin II heavy chain, which lead to a higher disassembled state of the myosin II filaments. This leads to a decreased

ability of the cells to undergo cytokinesis, and cellular migration, as is exhibited by Myosin II null cells. Myosin II null cells show a significant decrease in cell mobility due to the inability of the cells to contract F-actin, mainly at the rear of the cell. In addition, myosin II null cells do not divide normally in suspension culture and show multinuclearity because they cannot complete cytokinesis (De la Roche and Cote, 2001).

3) **Analysis of MHCK-D expression levels at different times in *Dictyostelium* multicellular development.** Using RT-PCR, I examined the relative levels of MHCK D expression during the multicellular development cycle. This indicates what phase during development that MHCK D is at its highest concentration, which would indicate when MHCK D has its greatest effect on a specific event that occurs during that phase of development. This is an important point since initial examination of MHCK D null cells indicated this enzyme does not appear to play a role in regulating myosin II bipolar filament assembly in vegetative *Dictyostelium* cells (Yumura et al., 2005).

CHAPTER II

MATERIALS & METHODS

PCR (Polymerase Chain Reaction)

PCR is a method used by researchers to amplify known fragments of DNA for use in further experiments. To amplify the Cat-WD truncation of the *mhkd* gene, primers were designed to anneal to the 5' ends of the 1557 base pair truncation (GenBank Accession Number AAFI02000047; dictyBase Gene DDB0220109) . The primers were also designed to have an overhang (CACC) on the 5' ends of the primer to allow for directional ligation into the pENTR/D-TOPO plasmid. The following primers were used to amplify the Cat-WD truncation:

GTWYMKDCAT-WDFWD – 5'-CACCCCAGATTCAAATTGTTAATTTG-3'

GTWYMKDFL-REV – 5'-CACCTTTATTTTCCAATTCCAAAC-3'

To amplify the Cat-WD truncation of the *mhkd* gene, genomic DNA was extracted from *Dictyostelium discoideum* (strain Ax2) cells. The *mhkd* gene does not contain any introns, which allows for amplification of the gene from genomic DNA (GenBank Accession Number AAFI02000047). The Cat-WD truncation was amplified using the iProof™ High-Fidelity PCR Kit (Bio-Rad, Hercules, CA). The high-fidelity PCR kit uses a *Pyrococcus*-like enzyme with a proof reading domain that allows amplification of longer DNA templates. The *Pyrococcus*-like DNA polymerase makes fewer errors, at a

rate of 50x less than *Taq* polymerase. The 50µl PCR reaction was set up by adding the following into a PCR tube: 5x High-Fidelity Buffer, dNTP mix (10mM each of dATP, dTTP, dGTP, & dCTP), MKDFWD primer (10µM), MKDREV (10µM), iProof DNA polymerase (1 unit), dH_20, 1µl (100µg/µl) total extracted genomic DNA ($5x10^6$ cells) from Ax2 *Dictyostelium* cells.

The reactions were placed in a thermocycler with the following PCR cycle settings:

1.	95°C X 30sec.		DNA Denature
2.	95°C X 30sec.	} 35x	DNA Denature
3.	60°C X 1min.		Primer Annealing
4.	72°C X 90sec.		DNA Extension
5.	72°C X 10min.		Final Extension

Steps 2-4 were repeated 35 times in order to amplify the DNA fragment. The PCR reaction (entire 50µl) was then loaded into an 0.8% agarose gel with ethidium bromide (EtBr) and run at 120 constant Volts for 30 mins. The gel was visualized using an ultraviolet light source. The DNA band corresponding to the PCR amplified Cat-WD truncation was excised from the gel and the DNA was extracted from the gel sample using a DNA Gel Extraction Kit (Qiagen, Valencia, CA).

TOPO Cloning Reaction

The PCR amplified Cat-WD truncation was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) to enable sub-cloning into the expression plasmid later. The cloning reaction was set up by adding the following to a micro-centrifuge tube: 2µl Cat-WD PCR amplified product, 1µl Salt Solution (1.2M NaCl, 0.06M $MgCl_2$), 2µl Sterile H20, 1µl pENTR/D-TOPO (20ng/ml linear plasmid in: 50% glycerol, 50mM

Tris-HCl, 1mM EDTA, 2mM DTT, 0.1% Triton X-100, 100μg/ml BSA, 30μM bromophenol blue). The reaction was mixed gently and incubated at 23^{o} C for 5 minutes.

Transformation of One Shot TOP10 Chemically Competent Cells and Selection of Positive Clones.

The TOPO cloning reaction was used in the transformation of *E. coli* chemically competent cells to allow for replication of the plasmid. Two micro-liters of the Cat-WD TOPO cloning reaction was added to a 50μl sample of One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA). The cells were incubated on ice for 30 minutes, then the cells were heat-shocked at 42^{o}C for 90 seconds. The cells were immediately transferred to ice for 2 minutes. S.O.C. medium (250μl) at room temperature was added to each tube of cells. The cells were incubated at 37^{o}C while shaking (220 RPM) for 1 hour. The cells were then spread onto LB/Kan agar plates in two volumes (50μl & 200μl) which were incubated at 37^{o}C overnight. Two colonies from each plate were added to 3ml LB/Kan liquid media and incubated at 37^{o}C overnight. To purify the plasmid from the liquid cultures, the QIAprep Miniprep Kit (Qiagen, Valencia, CA) was used. The purified plasmid was then used in a PCR reaction along with the Cat-fwd & MHCKD-rev primers to ensure that the Cat-WD truncation was present. To check for directionality of the insert in the plasmid, a PCR reaction was set up with an internal Cat-WD forward primer and the M13 reverse primer. The PCR samples were subjected to gel electrophoresis and the size of the band indicated that the insert was present and in the correct orientation.

Performing The LR Recombination Reaction

Recombination was used to transfer the Cat-WD truncation from the entry clone (pENTR/D-TOPO) to the destination vector (pTX-GFP, or pTX-Flag) (Levi et. al., 2000). Figure 4 shows a diagram of the pTX-GFP expression plasmid. Figure 5 is a diagram of the pTX-Flag expression plasmid. The following components were added to a microcentrifuge tube: one micro-liters pENTR/D-TOPO with Cat-WD insert, one micro-liter Destination vector (pTX-GFP, or pTX-Flag), five micro-liters TE Buffer, two micro-liters LR Clonase II Enzyme Mix. A recombination positive control was performed using the pENTR-gus (β-glucuronidase gene) entry clone along with the pDEST15 destination vector. A recombination negative control was performed by including a second positive control which did not receive the LR Clonase II Enzyme Mix. The reactions were incubated at 25^{o}C for 1 hour. One micro-liter Proteinase K solution (2μg/μl in: 10mM Tis-HCl, 20mM $CaCl_2$, & 50% glycerol) was added to each sample and incubated for 10 minutes at 37^{o}C.

Transformation of DH5α Cells and Selection of Expression Clones

The recombination reaction was used in a transformation reaction using DH5α cells to allow for replication of the expression plasmid. One micro-liter of recombination reaction was added to a 50μl sample of Library Efficiency DH5α *E. coli* cells (Invitrogen, Carlsbad, CA). The samples were then incubated on ice for 30 minutes, then they were heat-shocked at 42^{o}C for 30 seconds. The samples were then transferred to ice for 2 minutes. S.O.C. medium (450μl) was added to the cells, and they were incubated at 37^{o}C while shaking at 200 RPM for 1 hour. Twenty micro-liter and 100μl samples were

then plated on LB/Amp agar plates, and incubated at 37^{o}C overnight. Bacterial colonies that grew on the LB/Amp plates were then added to a 3ml LB/Amp liquid culture and incubated at 37^{o}C while shaking at 200 RPM overnight. To purify the plasmid from the liquid cultures, the QIAprep Miniprep Kit (Qiagen, Valencia, CA) was used. The purified plasmid was then used in a PCR reaction along with the Cat-fwd & MHCKD-rev primers to ensure that the Cat-WD truncation was present. The PCR samples were subjected to gel electrophoresis and the size of the band indicated that the insert was present.

Transformation and Selection of *Dictyostelium* Cells with Recombinant pTX-Cat-WD-Flag & pTX-Cat-WD-GFP Expression Plasmids

The pTX-GFP & pTX-Flag expression vectors containing the Cat-WD truncation of MHCK D gene were transformed into *Dictyostelium* cells using electroporation. *Dictyostelium discoideum* strain:Ax2 cells were used in the transformation. The cells were electroporated at 0.85 kV/25mF two times. Then the cells were allowed to recover in HL5 medium. After a one day recovery, the antibiotic G418 (10μg/ml) was added for selection of those cells containing the expression vector. Cells containing the expression vector over express the Cat-WD truncation of MHCK D. The level of over-expression will depend, in part on the number of plasmids per cell. The expression vector contains a G418 resistance coding region, which allows for selection of cells that over express the Cat-WD domain, and increased over expression was achieved by increasing the concentration of G418 (max 50μg/ml) in the culture medium. Those cells that contain more copies of the expression vector will produce more G418 resistance gene product

(neomycin phospho-transferase) which will allow them to survive and replicate in higher levels of G418 (Pang et. al., 1999).

Analysis of Cells for MyoII-null Phenotype

When *Dictyostelium* cells lacking the myosin II protein, or are over-expressing an MHCK, are grown in suspension culture, they are unable to divide and develop into large multi-nucleated cells. This indicates that myosin II plays a key role in cytokinesis. To determine if MHCK D can function as a myosin II heavy chain kinase *in vivo,* *Dictyostelium* cells selected for over-expression of the Cat-WD truncation of MHCK D were collected after 72 hours in suspension culture. The cells were seeded on glass microscope slides, and the growth medium was removed after allowing the cells to settle on the bottom of the slide. The cells were then fixed using ice cold methanol and incubated for 5 minutes. The methanol was removed and the cells were washed with 1x Tris Buffered Saline (1x TBS: 10ml 1M Tris-HCl, 8g NaCl, 0.2g KCl, & dH_20). To visualize nuclei, the fixed cells were stained with 4,6-diamidino-2-phenylindole (DAPI), after 5 minutes the DAPI solution was removed and the cells were washed with distilled water (Betapudi et al., 2005). The cells were then visualized using an inverted fluorescence microscope. The number of nuclei per cell were counted and recorded.

To examine the ability of *Dictyostelium* cells to grow and divide while grown in suspension culture, cells over-expressing the MHCK D Cat-WD truncation (30μg/ml & 50μg/ml) were grown in a shaking (190 RPM) suspension culture for 7 days. Cells were then collected and counted on days 1, 2, 3, 4, & 7. Ax2 cells not over-expressing the recombinant protein were included as a control. The test was performed in triplicate.

Analysis of *mhkd* gene expression during multi-cellular development by Reverse Transcriptase-PCR

A total of $1.0x10^7$ wild type Ax2 *Dictyostelium* cells were applied to a nitrocellulose membrane which was soaked in 1x starvation buffer (20mM M.E.S., 0.2mM $CaCl_2$, 2.0mM $MgSO_4$, pH 6.8). Under these conditions, *Dictyostelium* cells enter into the multi-cellular development cycle, allowing them to aggregate together on the membrane. The cells were collected ($5.0x10^6$ cells) at the following time points following the initiation of starvation: 0h, 4h, 8h, 12h, 18h, 21h, 24h, & 48h. The cells were washed off of the membrane, counted, and 5.0×10^6 cells were pelleted and stored. The samples were stored at -20°C immediately following collection. The total RNA was isolated from each sample using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA samples were then subjected to a RQ1 DNase digestion to ensure there was no genomic DNA contamination. RNA (1.2μg) was added to a micro-centrifuge tube along with 1μl RQ1 10x Buffer, and 2 units RQ1 RNase-Free DNase, and Nuclease-free water up to a final volume of 10μl. The reaction was then incubated at 37°C for 30 minutes. One micro-liter RQ1 DNase Stop solution was then added to each sample and incubated at 65°C for 10 minutes. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed on the RNA samples (1.2μg) collected previously, using the Access RT-PCR System (Promega, Madison, WI). The primers used in the reaction specifically amplified the catalytic (Cat) domain of the *mhkd* gene, and the amount of amplified Cat domain reflected the relative amount of MHCK D mRNA that is present in that sample. A positive control was performed along with the RT-PCR reaction, as well as IG7 which is

a constitutively expressed gene in *Dictyostelium* (Nagasaki et al., 2002) which acted as a loading control. The primers for the RT-PCR reaction are as follows:

GTWYMKDCAT-WDFWD – 5'-CACCCCAGATTCAAATTGTTAATTTG-3'

GTWYMKDCATREV – 5'-CACCTTGAAGACAGATTGGTGAACATTTATG-3'

IG7-S 5'-TTACATTTATTAGACCCGAAACCAAGCG-3'

IG7-AS 5'-TTCCCTTTAGACCTATGGACCTTAGCG-3'

A negative control was also performed by replacing the reverse transcriptase with dH_20 in the reaction. The negative control ensured there was no contamination of the RNA samples with genomic DNA. All RT-PCR reactions were placed in a thermocycler and cycled as follows:

1. 45°C X 45min.		Reverse Transcription
2. 94°C X 2min.		RT Inactivation
3. 94°C X 30sec.	28x	Denaturation
4. 60°C X 1min.		Annealing
5. 68°C X 3min.		Extension
6. 68°C X 10min.		Final Extension

After the RT-PCR cycle was completed, the samples were loaded into a 0.8% agarose gel with Ethidium Bromide and electrophoresis was performed by running the gel at 120 constant volts for 50 minutes. The gel was visualized using the Bio-Rad Gel Documentation System (Bio-Rad, Hercules, CA). The difference in band intensity throughout the time points corresponded to the amount of MHCK D gene expression at that time during the *Dictyostelium* developmental cycle in wild-type cells.

CHAPTER III

RESULTS

PCR (Polymerase Chain Reaction)

Using the GTWYMKDCAT-WDFWD and GTWYMKDFL-REV primers the Cat-WD domain was successfully amplified using the iProof™ High-Fidelity PCR Kit (Bio-Rad, Hercules, CA). The Cat-WD truncation of MHCK D is made up of a 519 amino acid chain (Figure 3), therefore the amplified gateway prepared Cat-WD gene will be 1565 base pairs (1557bp + 4 base addition at each 5' end) in length. Following gel electrophoresis, the size of the Cat-WD amplified DNA migrated slightly above the 1500 base pair (bp) band in the 1kb DNA marker (Figure 6). This result indicates that the amplified band is of the correct size. Following DNA gel extraction, a sample of the extracted DNA was subjected to gel electrophoresis to ensure the extraction worked properly (data not shown).

Analysis of TOPO Cloning Reaction

To test for proper insert and orientation after the TOPO cloning reaction, a double restriction enzyme digest was performed. The pENTR/D-TOPO plasmid with the Cat-WD insert was digested with *NotI* and *NdeI* restriction enzymes. The pENTR/D-TOPO plasmid contains a *NotI* cleavage site at position 673, and the Cat-WD domain contains an *NdeI* cleavage site at position 638. When the pENTR/D-TOPO plasmid contains the Cat-WD truncation in the correct orientation and is double digested with *NotI* and *NdeI,*

the resulting bands will be 650bp & 3487bp long. Figure 7 shows the electrophoresis gel with the double digested pENTR-Cat-WD/D-TOPO plasmid in lane 2; lane 1 contains 1kb DNA marker. Lane 2 contains 2 DNA bands, 1 band above the 3000bp marker, and 1 band above the 1000bp marker. It is unclear why the lower band did not migrate to the correct position (650bp), so a PCR reaction was also performed using a forward primer which binds to the pENTR/D-TOPO plasmid at bases 537-552 (M13 forward) and a reverse primer that binds within the MHCK D catalytic domain at bases 699-726. If the Cat-WD truncation is present and in the correct orientation there will be an amplified DNA band with a length of 870bp. Figure 8 shows the electrophoresis gel with the PCR sample using the pENTR-Cat-WD/D-TOPO plasmid as the DNA template. Lane 2 contains the PCR reaction with an amplified DNA band slightly larger than the 750bp marker.

Analysis of Expression Plasmids Following Recombination Reaction

To ensure the pTX-Flag, and pTX-GFP expression plasmids contain the correct MHCK D Cat-WD truncation, a restriction enzyme digest was performed. *EcoRI* was used to digest the plasmids following the recombination reactions. Both expression plasmids contain two *EcoRI* cleavage sites, and the Cat-WD truncation contains one *EcoRI* cleavage site. If the Cat-WD truncation is present in the plasmids the following bands should appear following the restriction digest:

pTX-Cat-WD-Flag: 1600bp, 4000bp, & 6500bp.

pTX-Cat-WD-GFP: 1600bp, 3500bp, & 7500bp.

Figure 9 shows the electrophoresis gel with the recombinant plasmids after restriction enzyme digest. Lane 1 contains the 1kb DNA marker, lanes 2 and 3 contain the pTX-Cat-WD-Flag plasmid, and lanes 4 and 5 contain the pTX-Cat-WD-GFP plasmid. The pTX-Cat-WD-Flag plasmid has bands at ~1800bp, ~3500bp, and 6500bp, while the pTX-Cat-WD-GFP plasmid has bands at ~1800bp, ~3500bp, and 7500bp. The banding profiles indicate that the recombinant plasmids contain the MHCK D Cat-WD truncation, and that the insert is also in the correct orientation.

Analysis of Protein Expression in *Dictyostelium* Cells with pTX-Cat-WD-Flag & pTX-Cat-WD-GFP Expression Plasmids

Following electroporation of *Dictyostelium* (Ax2 cell line) cells with either the pTX-Cat-WD-GFP or pTX-Cat-WD-Flag recombinant expression plasmid, the selection antibiotic G418 (10μg/ml) was added to the culture media. Cell lysates were made and a western blot was performed. Figure 10 shows the western blot probed with an anti-Flag antibody (Invitrogen, Carlsbad, CA). Lanes 1 and 2 show bands at ~62.5kD and ~70kD (Cat-WD-Flag peptide). Lane 3 contains a cell lysate from Ax2 *Dictyostelium* cells not expressing the Flag-tagged fusion protein. The Flag-tagged Cat-WD fusion protein has a calculated size of ~58.6kD, although the western blot shows a band at ~70kD. It is unclear at this point as to why the Cat-WD-Flag fusion protein migrates at a slower rate than predicted. The band at ~62.5kD is due to non-specific binding of the anti-Flag antibody to another *Dictyostelium* protein. This is shown by the anti-Flag binding in the Ax2 cell line not expressing a Flag-tagged fusion protein (lane 3) at the same position of ~62.5kD.

Figure 11 shows a western blot probed with anti-GFP antibody (Invitrogen, Carlsbad, CA). Lanes 1 and 2 contain cell lysates from *Dictyostelium* cells containing the pTX-Cat-WD-GFP recombinant plasmid. Lanes 1 and 2 show a band at ~98kD which corresponds to the Cat-WD-GFP fusion protein predicted size.

Analysis of Recombinant Protein Over-expression Level in *Dictyostelium* Cells

A western blot was performed on *Dictyostelium* cells cultured in HL5 media containing either 10μg/ml or 50μg/ml G418 selection antibiotic. A rabbit polyclonal anti-GFP antibody was used to probe the blot. G418 is used to select for cells containing multiple copies of the recombinant expression plasmid. *Dictyostelium* cells grown in medium containing higher concentrations of G418 should express the recombinant protein at higher level than those cells grown in medium containing lower concentrations of G418. Figure 12 shows the developed western blot of cell lysates from *Dictyostelium* cells containing the pTX-Cat-WD-GFP recombinant plasmid grown at G418 concentrations of 10μg/ml and 50μg/ml. Lane 1 contains cells grown at 10μg/ml G418, lane 2 contain cells grown at 50μg/ml G418.

Analysis of Cat-WD Over-expressing Cells for Cytokinesis Defects

Dictyostelium cells containing the pTX-Cat-WD-GFP recombinant plasmid grown in HL5 with either 30μg/ml or 50μg/ml G418 were stained with DAPI to visualize the number of nuclei per cell. Wild type Ax2 cells were included as a control group. The cells were visualized using an inverted fluorescent microscope. The number of nuclei per cell were counted and recorded. Figure 13 is a graph indicating the results of the nuclei count test. Over 80% of Ax2 cells lacking the recombinant expression plasmid contained

only one nucleus per cell, while more than 75% of cells over-expressing the Cat-WD truncation contained 2 or more nuclei per cell (Figure 13). Figure 14 shows images of Ax2 cells expressing recombinant protein from the pTX-Cat-WD-GFP plasmid. The images show that cells expressing recombinant protein at 30μg/ml and 50μg/ml G418 contained multiple nuclei per cell. Ax2 cells not overexpressing the protein were included as a control.

Figure 15 shows the growth curve of the shaking suspension culture. *Dictyostelium* cells over-expressing the recombinant MHCK D Cat-WD protein grew and divided at a much slower rate than Ax2 cells not over-expressing the protein. The results also indicate that the level of over-expression at 30μg/ml G418 was sufficient to cause a defect in growth rate. Figure 15 shows one of the three sets of results recorded during the test. All three tests showed similar results to that of figure 15.

Analysis of *mhkd* gene expression during multi-cellular development by Reverse Transcriptase-PCR

To examine gene expression levels during *Dictyostelium* multi-cellular development, reverse transcriptase-PCR was performed on RNA samples from cells collected at specific time points during the multi-cellular development cycle. Figure 16 show the RT-PCR samples from the following time points: 0h, 4h, 8h, 12h, 18h, 21h, 24h, & 48h. The results of the catalytic domain amplification indicate that MHCK D is expressed throughout the multi-cellular development cycle in *Dictyostelium* cells, however a more accurate assessment of differences in MHCK D expression level will require real-time PCR, or Northern Blot analysis. The results of the IG7 RT-PCR

indicate that similar levels of RNA sample was loaded for each time point, while the negative control (no reverse transcriptase included in the reaction) indicates that there is no genomic DNA contamination in the RNA samples.

CHAPTER IV

DISCUSSION

The results presented here demonstrate that the Cat-WD domain of MHCK D, when over-expressed, induces a Myosin II-null phenotype in *Dictyostelium* cells. The results also indicate that MHCK D is expressed throughout the multi-cellular development cycle as well as in cells during their vegetative state. Previous studies (Yumura et. al., 2005) suggest that if in fact MHCK D is a myosin II heavy chain kinase, that it likely has a role in some setting other than that of the basal control of cortical myosin II levels. The fact that MHCK D is expressed up to 48 hours after initiation of the multi-cellular development cycle suggests that MHCK D does play some important role in regulating myosin II filament disassembly during this process. It should also be noted that this is the first time that any truncation of the *mhkd* gene has been cloned in an expression plasmid allowing over-expression studies to be conducted.

Phosphorylation of the three threonine residues (1823, 1833, 2029) on the myosin II heavy chain tail drives myosin II filament disassembly in *Dictyostelium* cells. Myosin II heavy chain kinases are responsible for the phosphorylation of these threonine residues. Due to the extensive research on MHCK A, B, & C, *in vitro* and *in vivo* studies have shown that they do phosphorylate the myosin II heavy chain and regulate bipolar filament disassembly (Egelhoff, 1999; Kolman et al., 1996; Liang et al., 2002; Nagasaki et al., 2002; Rico and Egelhoff, 2003; Yumura et al., 2005). The structural similarity of

MHCK D to the other known kinases indicates that it also may have a role in regulating myosin II bipolar filament disassembly via phosphorylation of the MHC. Over-expression of MHCK A, B, or C in *Dictyostelium* cells leads to a myosin II-null phenotype as seen by larger multi-nucleated cells resulting from defects in cytokinesis when grown in suspension culture. The studies presented here demonstrate that when the Cat-WD truncation of MHCK D is over-expressed in *Dictyostelium* cells, the cells are large and have multiple nuclei per cell (figures 13 and 14). When grown in suspension culture *Dictyostelium* cells over-expressing the Cat-WD truncation of MHCK D show defects in cytokinesis and do not divide as often as wild type Ax2 cells (figure 15). Taken together these results suggest that MHCK D may act like a myosin II heavy chain kinase and thus may play a role in regulating bipolar filament disassembly. These assays do not distinguish between MHCK D directly phosphorylating the myosin II heavy chain tail or being required for the activity of another kinase that directly phosphorylates the heavy chain. Other possible events that could be occurring to yield the same outcome, include MHCK D could possibly be phosphorylating another myosin II heavy chain kinase, causing it to hyper-phosphorylate the MHC. Another possibility is that MHCK D could be targeting another cytokinesis component other than phosphorylating the MHC, leading to a higher level of inactive myosin II monomers, indicated by the myosin II-null phenotype observed in these studies.

Further research will be needed to understand the role of MHCK D in regulating the myosin II bi-polar filament disassembly. To determine if MHCK D is phosphorylating another myosin II heavy chain kinase, MHCK D could be over-

expressed in triple knock-out *Dictyostelium* cells not expressing MHCK A, B, & C. If the same myosin II-null phenotype is observed in these cells it would indicate that MHCK D is not over-activating the other myosin II heavy chain kinases. To understand whether MHCK D is directly phosphorylating the MHC, or another mechanism regulating cytokinesis, MHCK D should be over-expressed in *Dictyostelium* cells expressing the 3x Ala myosin II. These cells express a mutated version of myosin II where the three theronine residues at positions 1823, 1833, & 2029 have been changed to alanine (an amino acid unable to be phosphorylated). If, upon being grown in a shaking cell culture, these cells show a normal WT phenotype, this would indicate that MHCK D directly affects those three theronine residues on the MHC that were replaced in the 3x Ala cell line. Another possible scenario is that MHCK D could be inhibiting a positive regulator of myosin II bipolar filaments, for example a MHC phosphatase. To test this possibility, *in vitro* studies would need to be performed using purified MHCK D and purified MHC phosphatase, and test the ability of the phosphatase to dephosphorylate a substrate in the presence and absence of MHCK D. If there is a decrease in the ability of the MHC phosphatase to dephosphorylate the substrate in the presence of MHCK D, that would indicate that MHCK D has some affect on the function of the MHC phosphatase.

Gene expression studies were performed to determine when MHCK D is expressed during the developmental cycle of *Dictyostelium* cells. The results show that MHCK D is expressed in the vegetative cell as well as up to 48 hours into the multi-cellular development cycle (figure 16). Previous studies show that MHCK D may play a small role in disassembly control but does not show a significant role when using FRAP

analysis (Yumura et. al., 2005). These studies used FRAP analysis to measure recovery times of GFP-myosin II at the cleavage furrow, and the cellular cortex. To further understand the role that MHCK D plays in the vegetative cell cycle, localization studies should be performed to show where in the cell MHCK D has its greatest effect. With the generation of a new cell line over-expressing the MHCK D Cat-WD-GFP fusion protein, localization tests would provide valuable new insight into the role MHCK D has within *Dictyostelium* cells.

Future studies, following the results of this paper indicating that MHCK D is in fact a myosin heavy chain kinase, should be performed focusing on activation, localization, and substrate targeting of MHCK D. Activation of MHCK A and MHCK C is initiated *in vitro* by autophosphorylation and MHCK A has also been shown to be activated by binding to F-actin (Liang et. al., 2002; Medley et. al., 1990; Egelhoff et. al., 2005). The WD repeat domains of MHCK A and MHCK B have been shown to target the kinases to myosin II by binding directly to myosin II filaments (Steimle et. al., 2001a). Since the WD repeat domains of MHCK A, B, C, and D are all structurally related, the role of the WD repeat domain of MHCK D may also be used to target the kinase to myosin II within the cell. Activation, localization, and substrate targeting studies of MHCK D would give further insight into the upstream regulation of MHCK D and provide a better picture of the role that MHCK D has in regulating myosin II heavy chain phosphorylation.

REFERENCES

Affolter, M., and C.J. Weijer. 2005. Signaling to cytoskeletal dynamics during chemotaxis. *Dev Cell*. 9:19-34.

Betapudi, V., C. Mason, L. Licate, and T.T. Egelhoff. 2005. Identification and characterization of a novel alpha-kinase with a von Willebrand factor A-like motif localized to the contractile vacuole and Golgi complex in *Dictyostelium discoideum*. *Mol Biol Cell*. 16:2248-62.

Chisholm, R.L., P. Gaudet, E.M. Just, K.E. Pilcher, P. Fey, S.N. Merchant, and W.A. Kibbe. 2006. dictyBase, the model organism database for *Dictyostelium discoideum*. *Nucleic Acids Res*. 34:D423-7.

De la Roche, M.A., and G.P. Cote. 2001. Regulation of *Dictyostelium* myosin I and II. *Biochim Biophys Acta*. 1525:245-61.

Drennan, D., and A. G. Ryazanov. 2004. Alpha-kinases: analysis of the family and comparison with conventional protein kinases. Biophysics & Molecular Biology 85:1-32.

Egelhoff, T. T., Croft, D., and Steimle, P. A. 2005. Actin activation of myosin heavy chain kinase A in *Dictyostelium*: a biochemical mechanism for the spatial regulation of myosin II filament disassembly. J. Biol. Chem. 280:, 2879-2887.

Egelhoff, T.T., and Cote, G.P. 1999. Guidebook t the Cytoskeletal and Motor Proteins. Oxford University Press, Oxford. 460 - 464 pp.

Egelhoff, T.T., R.J. Lee, and J.A. Spudich. 1993. *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo. *Cell*. 75:363-71.

Eichinger, L., J.A. Pachebat, G. Glockner, M.A. Rajandream, R. Sucgang, M. Berriman, J. Song, R. Olsen, K. Szafranski, Q. Xu, B. Tunggal, S. Kummerfeld, M. Madera, B.A. Konfortov, F. Rivero, A.T. Bankier, R. Lehmann, N. Hamlin, R. Davies, P. Gaudet, P. Fey, K. Pilcher, G. Chen, D. Saunders, E. Sodergren, P. Davis, A. Kerhornou, X. Nie, N. Hall, C. Anjard, L. Hemphill, N. Bason, P. Farbrother, B. Desany, E. Just, T. Morio, R. Rost, C. Churcher, J. Cooper, S. Haydock, N. van Driessche, A. Cronin, I. Goodhead, D. Muzny, T. Mourier, A. Pain, M. Lu, D. Harper, R. Lindsay, H. Hauser, K. James, M. Quiles, M. Madan Babu, T. Saito, C. Buchrieser, A. Wardroper, M. Felder, M. Thangavelu, D. Johnson, A. Knights, H. Loulseged, K. Mungall, K. Oliver, C. Price, M.A. Quail, H. Urushihara, J. Hernandez, E. Rabbinowitsch, D. Steffen, M. Sanders, J. Ma, Y. Kohara,

S. Sharp, M. Simmonds, S. Spiegler, A. Tivey, S. Sugano, B. White, D. Walker, J. Woodward, T. Winckler, Y. Tanaka, G. Shaulsky, M. Schleicher, G. Weinstock, A. Rosenthal, E.C. Cox, R.L. Chisholm, R. Gibbs, W.F. Loomis, M. Platzer, R.R. Kay, J. Williams, P.H. Dear, A.A. Noegel, B. Barrell, and A. Kuspa. 2005. The genome of the social amoeba *Dictyostelium discoideum*. *Nature*. 435:43-57.

Kessin, R.H. 2001. *Dictyostelium*: evolution, cell biology, and the development of multicellularity. Cambridge University Press, Cambridge. 294 pp.

Kolman, M.F., L.M. Futey, and T.T. Egelhoff. 1996. *Dictyostelium* myosin heavy chain kinase A regulates myosin localization during growth and development. *J Cell Biol*. 132:101-9.

Levi, S., M. Polyakov, and T. Egelhoff. 2000. Green Fluorescent Protein and Epitope Tag Fusion Vectors for *Dictyostelium discoideum. Plasmid.* 44: 231-238.

Liang, W., L. Licate, H. Warrick, J. Spudich, and T. Egelhoff. 2002. Differential localization in cells of myosin II heavy chain kinases during cytokinesis and polarized migration. *BMC Cell Biol*. 3:19.

Manning, G., Plowman, G.D., Hunter, T. and Sudarsanam, S., 2002. Evolution of protein kinase signaling from yeast to man. *Trends Biochem. Sci.* **27**, pp. 514–520.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T. and Sudarsanam, S., 2002. The protein kinase complement of the human genome. *Science* **298**, pp. 1912–1934.

Medley, Q. G., Gariepy, J., and Côté, G. P. (1990). *Dictyostelium* myosin II heavy-chain kinase A is activated by autophosphorylation: studies with *Dictyostelium* myosin II and synthetic peptides. Biochemistry 29:, 8992-8997.

Nagasaki, A., G. Itoh, S. Yumura, and T.Q. Uyeda. 2002. Novel Myosin Heavy Chain Kinase Involved in Disassembly of Myosin II Filaments and Efficient Cleavage in Mitotic *Dictyostelium* Cells. *Mol Biol Cell*. 13:4333-42.

Pang, K., M. Lynes, and D. Knecht. 1999. Variables Controlling the Expression Level of Exogenous Genes in *Dictyostelium. Plasmid*. 41: 187-197.

Parent, C.A., and P.N. Devreotes. 1996. Molecular genetics of signal transduction in *Dictyostelium*. *Annu Rev Biochem*. 65:411-40.

Rico, M., and T.T. Egelhoff. 2003. Myosin heavy chain kinase B participates in the regulation of myosin assembly into the cytoskeleton. *J Cell Biochem*. 88:521-32.

Ryazanov, A.G., 2002. Elongation factor-2 kinase and its newly discovered relatives. *FEBS Letters* **514**, pp. 26–29.

Ryazanov, A.G., Pavur, K.S. and Dorovkov, M.V., 1999. Alpha-kinases: a new class of protein kinases with a novel catalytic domain. *Curr. Biol.* **9**, pp. R43–R45.

Ryazanova, L.V., Pavur, K.S., Petrov, A.N., Dorovkov, M.V. and Ryazanov, A.G 2001. Novel type of signaling molecules: protein kinases covalently linked with ion channels. *Mol. Biol.* **35**, pp. 271–283.

Steimle, P.A., T. Naismith, L. Licate, and T.T. Egelhoff. 2001. WD repeat domains target *Dictyostelium* myosin heavy chain kinases by binding directly to myosin filaments. *J Biol Chem*. 276:6853-60.

Yumura, S., M. Yoshida, V. Betapudi, L.S. Licate, Y. Iwadate, A. Nagasaki, T.Q. Uyeda, and T.T. Egelhoff. 2005. Multiple myosin II heavy chain kinases: roles in filament assembly control and proper cytokinesis in *Dictyostelium*. *Mol Biol Cell*. 16:4256-66.

APPENDIX

FIGURES

Figure 1

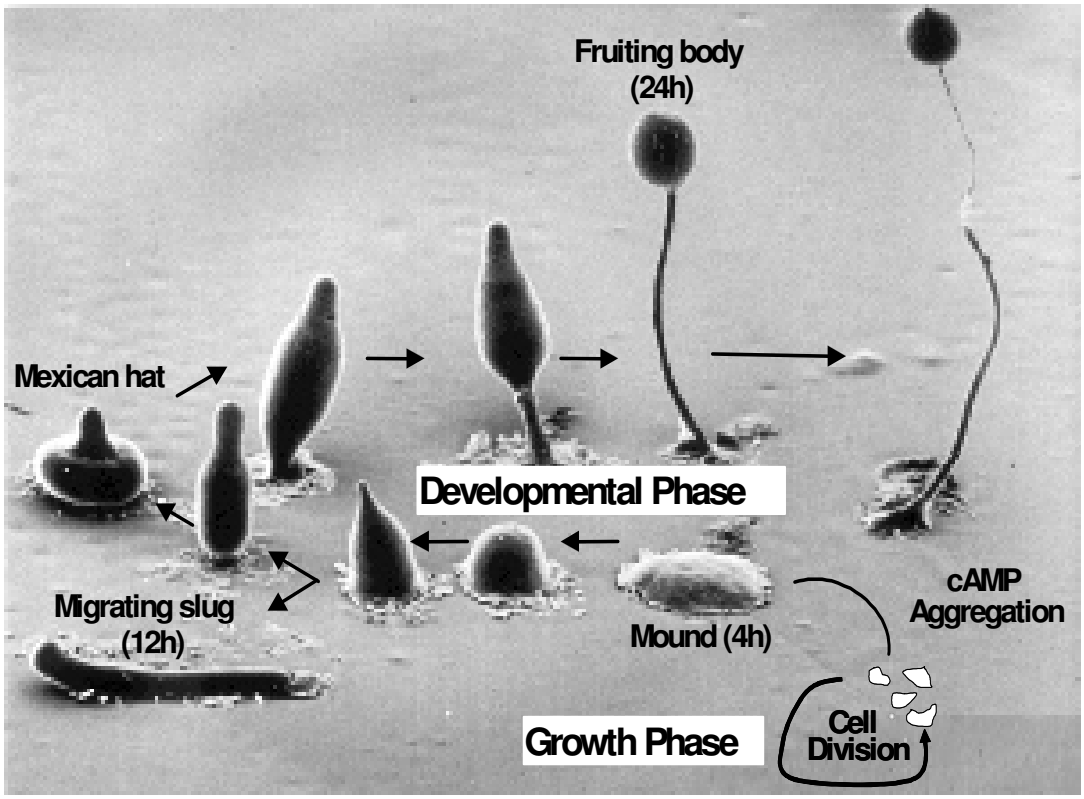


Figure 1: Scanning electron micrograph of the major life cycle stages of *Dictyostelium discoideum* Image courtesy of M.J. Grimson & R.L. Blanton - Biological Sciences Electron Microscopy Laboratory, TexasTech University

Figure 2

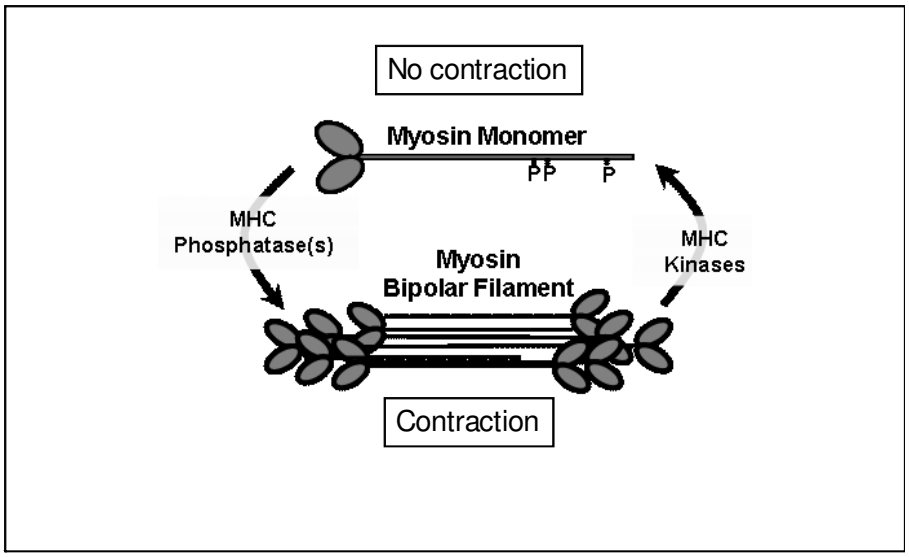
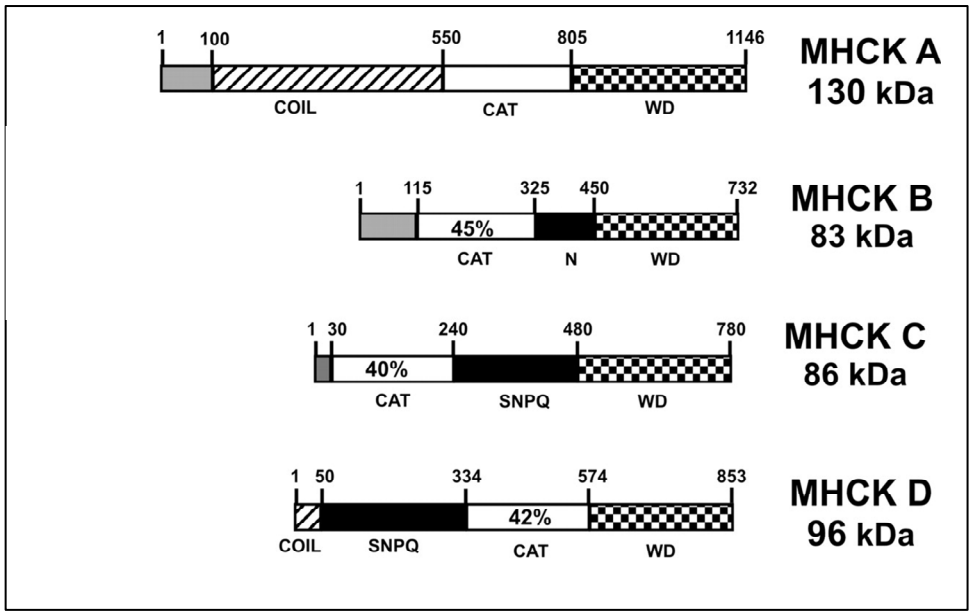


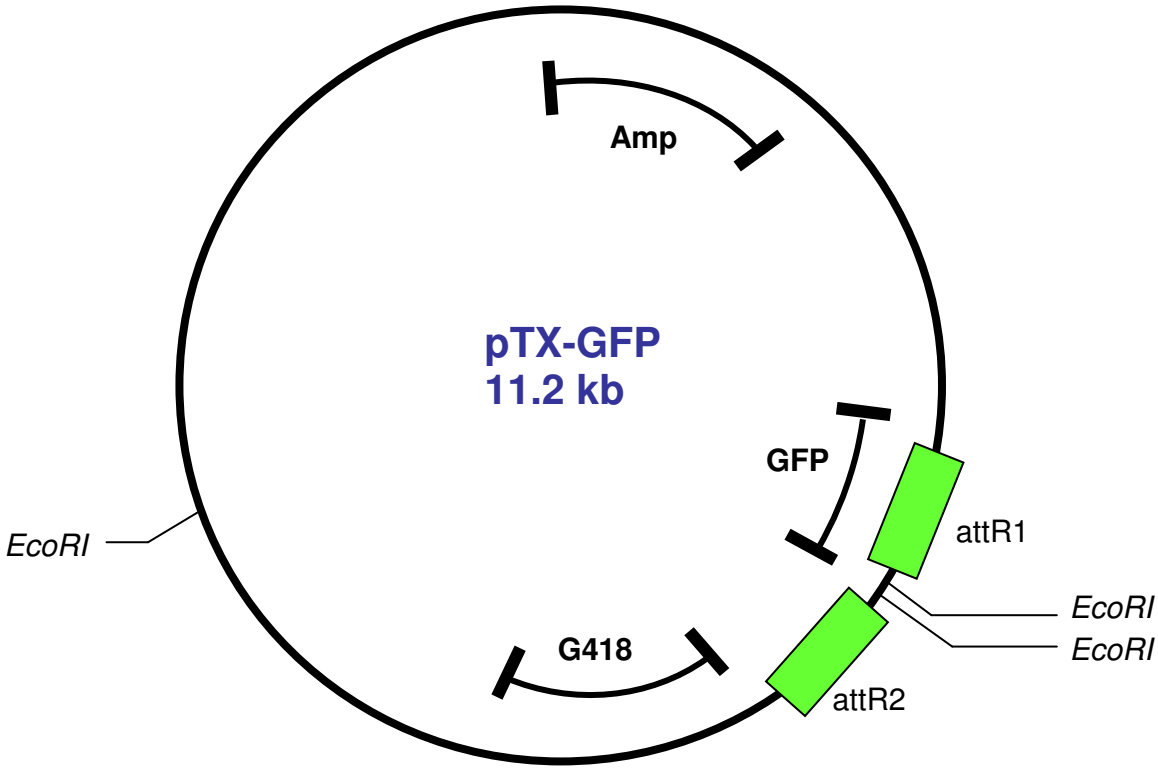
Figure 2: Regulation of Myosin II Filament Assembly in *Dictyostelium*. MHC kinases phosphorylate the myosin heavy chain leading to the inactive myosin monomer which cannot contract actin filaments. MHC phosphatases dephosphorylate the myosin heavy chain leading to myosin bipolar filamnet assembly which are able to contract actin filaments.

Figure 3



<u>Figure 3:</u> Schematic representation of the domain structure of the myosin II heavy chain kinase family members in *Dictyostelium*
The images above are based on GenBank sequences, mhka (U16856.1), mhkb (U90946.1), mhkc (AB079663.1), and mhkd (M93393.1) for MHCK A, MHCK B, MHCK C, and MHCK D, respectively.

Figure 4



<u>Figure 4:</u> Diagram of pTX-GFP Plasmid
Map of plasmid that will allow for over-expression of Cat-WD-GFP recombinant fusion protein in *Dictyostelium* cells. (attR= Gateway recombination sites)

Figure 5

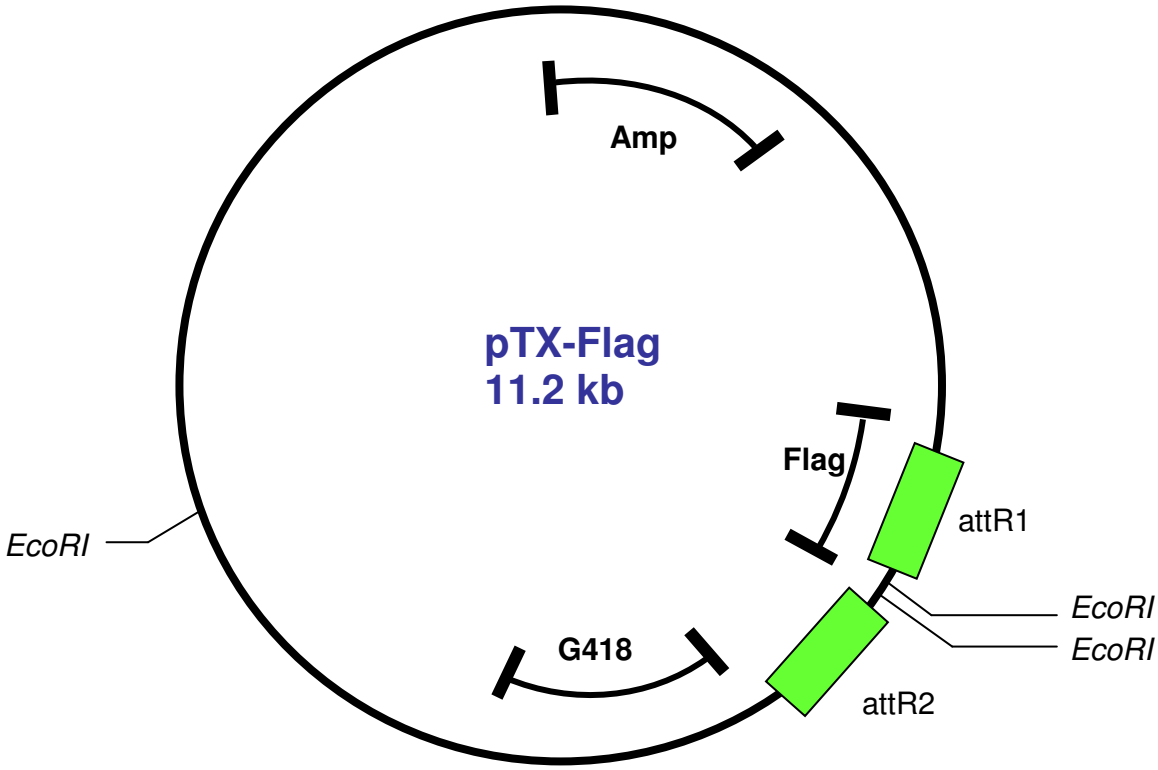


Figure 5: Diagram of pTX-Flag Plasmid
Map of plasmid used to allow for over-expression of Cat-WD-Flag recombinant fusion protein in *Dictyostelium* cells. (attR= Gateway recombination sites)

Figure 6

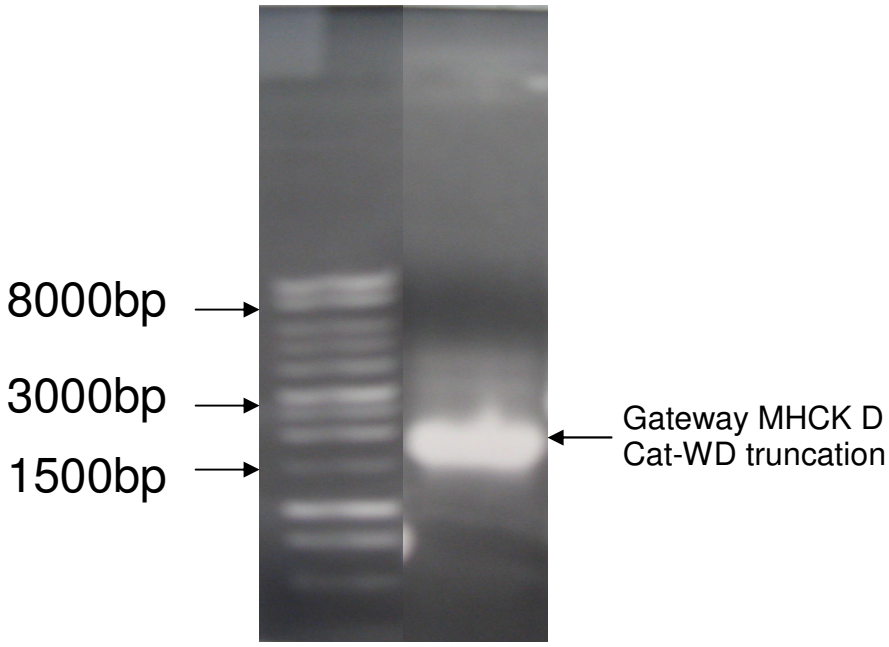


Figure 6: Gel Electrophoresis of Amplified Gateway Cat-WD Truncation
A sample of amplified Gateway Cat-WD truncation was separated electrophoretically to ensure proper size before subsequent cloning reactions.

Figure 7

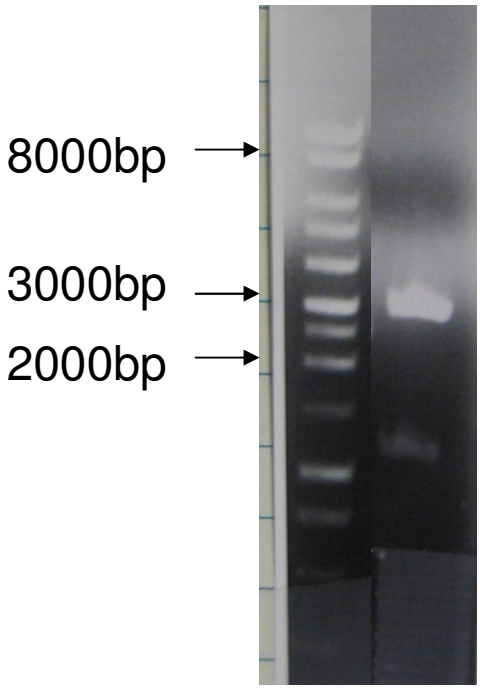
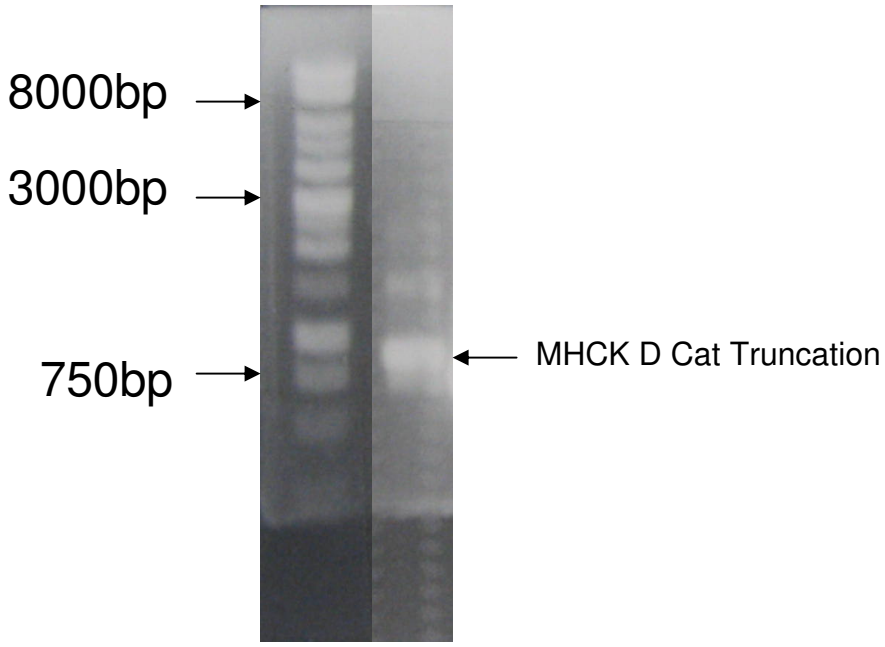


Figure 7: Double Digested pENTR-Cat-WD/D-TOPO Plasmid
An isolated plasmid sample was digested with *NotI* and *NdeI* restriction enzymes to check for correct MHCK D Cat-WD insert and proper orientation.

Figure 8



<u>Figure 8:</u> PCR amplification of Cat domain from pENTR-Cat-WD/D-TOPO Plasmid
A PCR reaction was performed using pENTR-Cat-WD/D-TOPO as the DNA template to amplify the Catalytic domain to ensure the presence of the MHCK D Cat-WD insert.

Figure 9

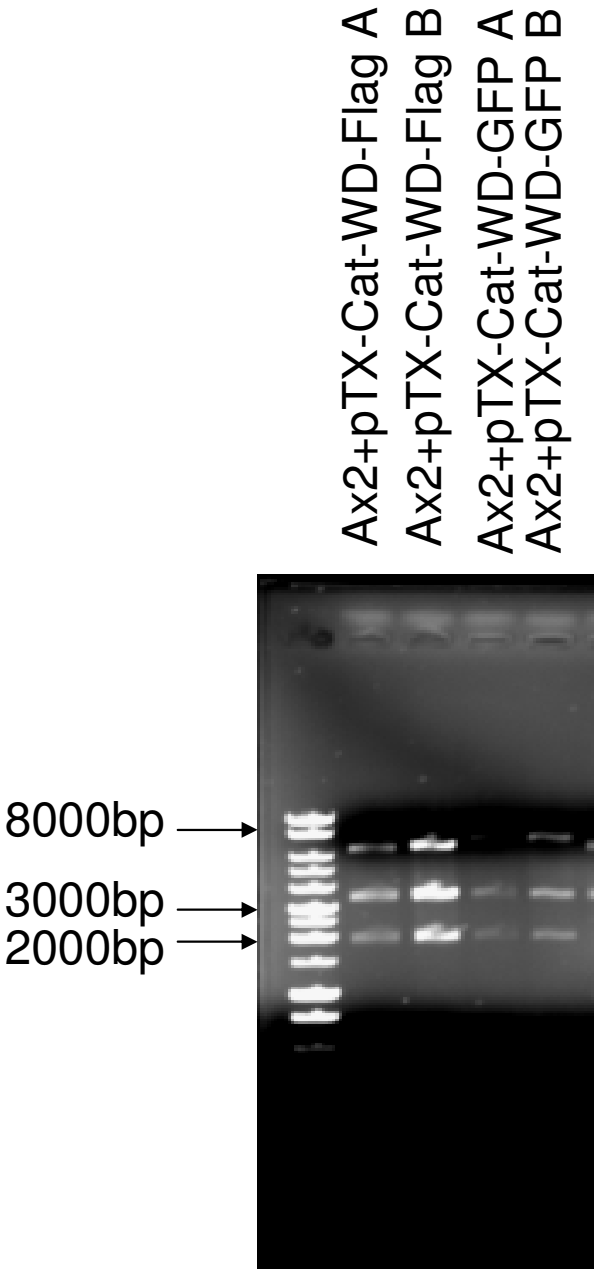


Figure 9: ***EcoRI*** **Restriction Enzyme Digest**
Recombinant plasmids were digested using *EcoRI* to check for correct MHCK D Cat-WD insert and proper orientation.

Figure 10

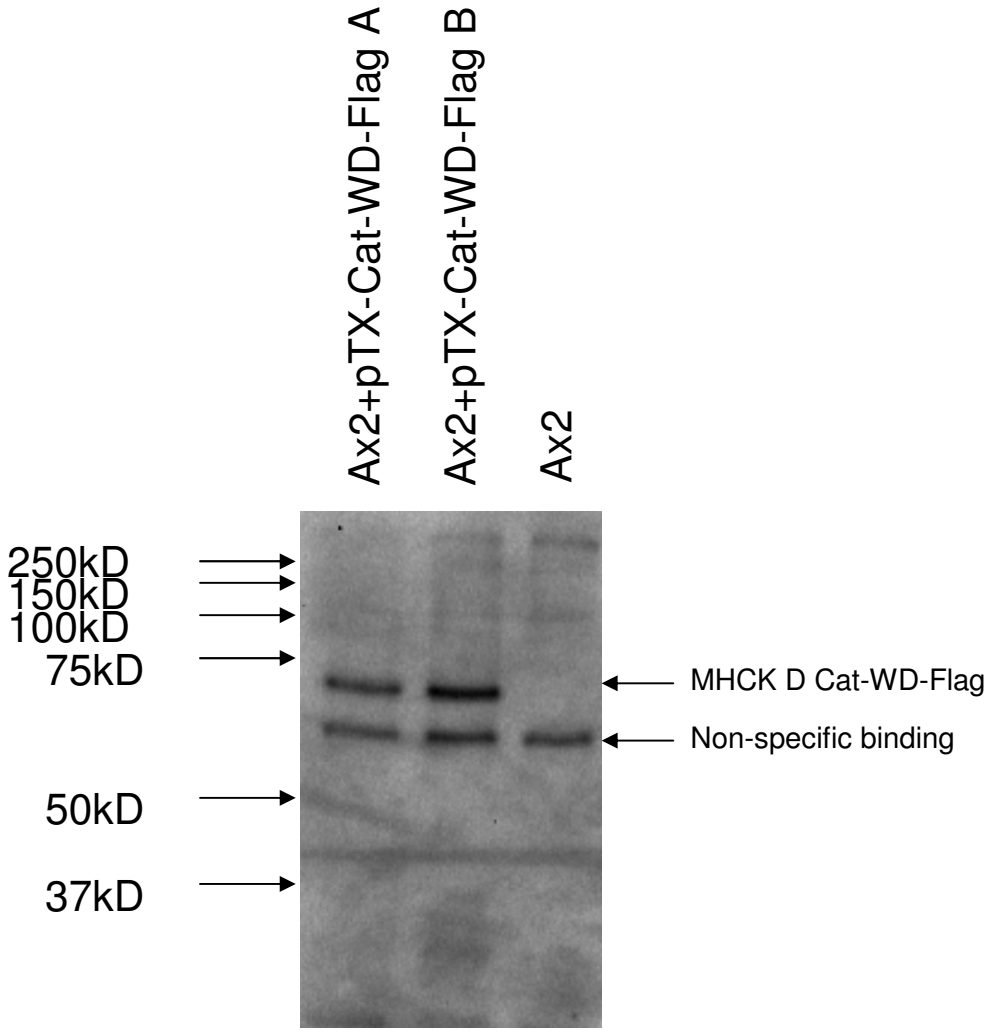


Figure 10: Anti-Flag Western Blot
Cell lysates from *Dictyostelium* cells containing the pTX-Cat-WD-Flag recombinant plasmid, as well as cells not expressing recombinant protein (lane 3) were probed using a anti-flag antibody.

Figure 11

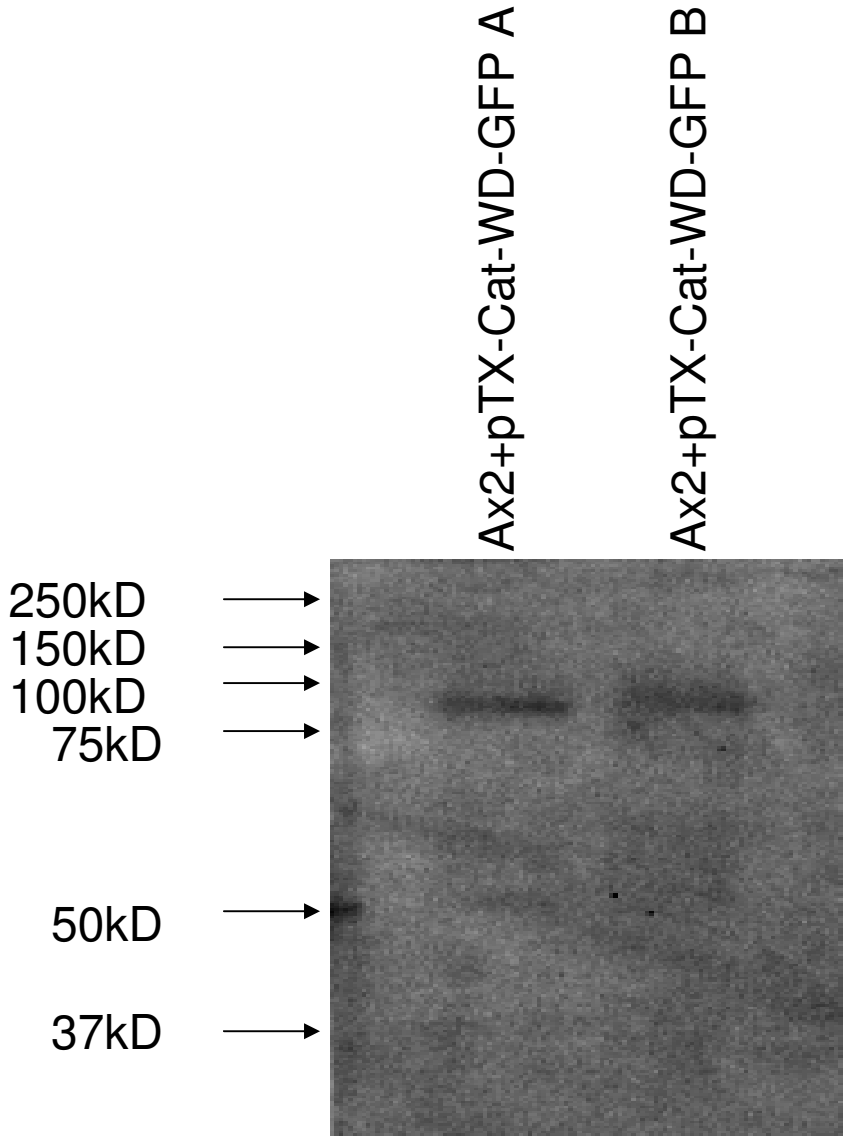


Figure 11: Anti-GFP Western Blot
Cell lysates from *Dictyostelium* containing the pTX-Cat-WD-GFP recombinant plasmid were probed with an anti-GFP antibody.

Figure 12

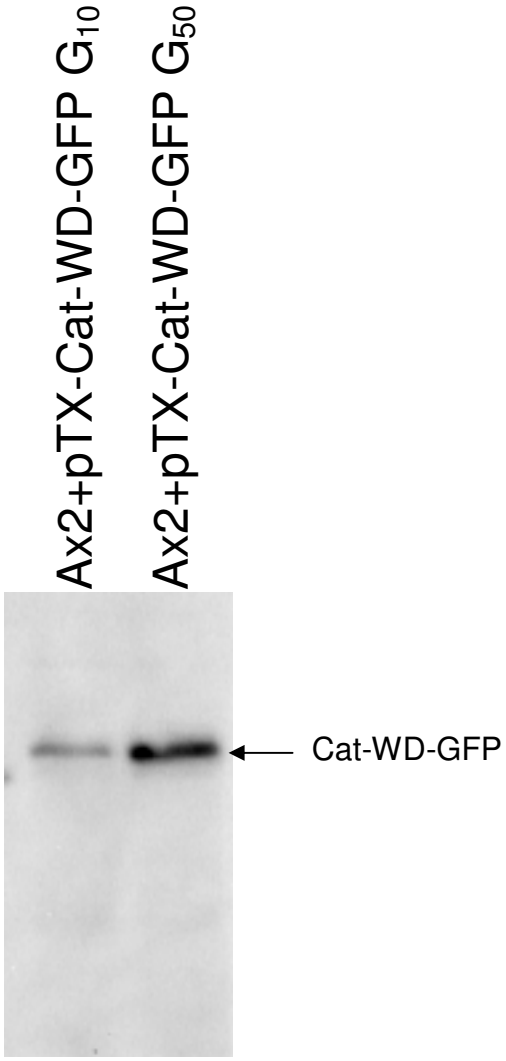


Figure 12: Analysis of Fusion Protein Over-expression
Anti-GFP western blot of cell lysates from *Dictyostelium* cells containing the pTX-Cat-WD-GFP recombinant plasmid grown at different levels of G418 were probed with an anti-GFP antibody. (G10 - Cells grown at [G418]=10μg/ml G50 - Cells grown at [G418]= 50μg/ml)

Figure 13

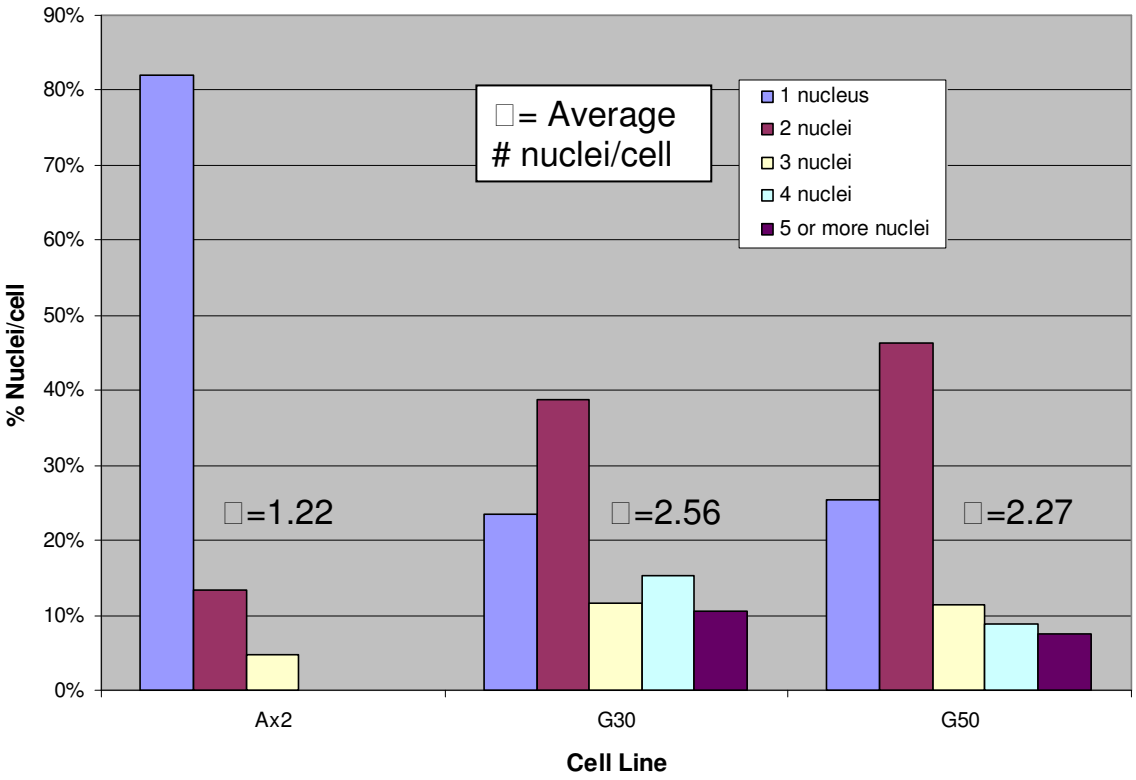
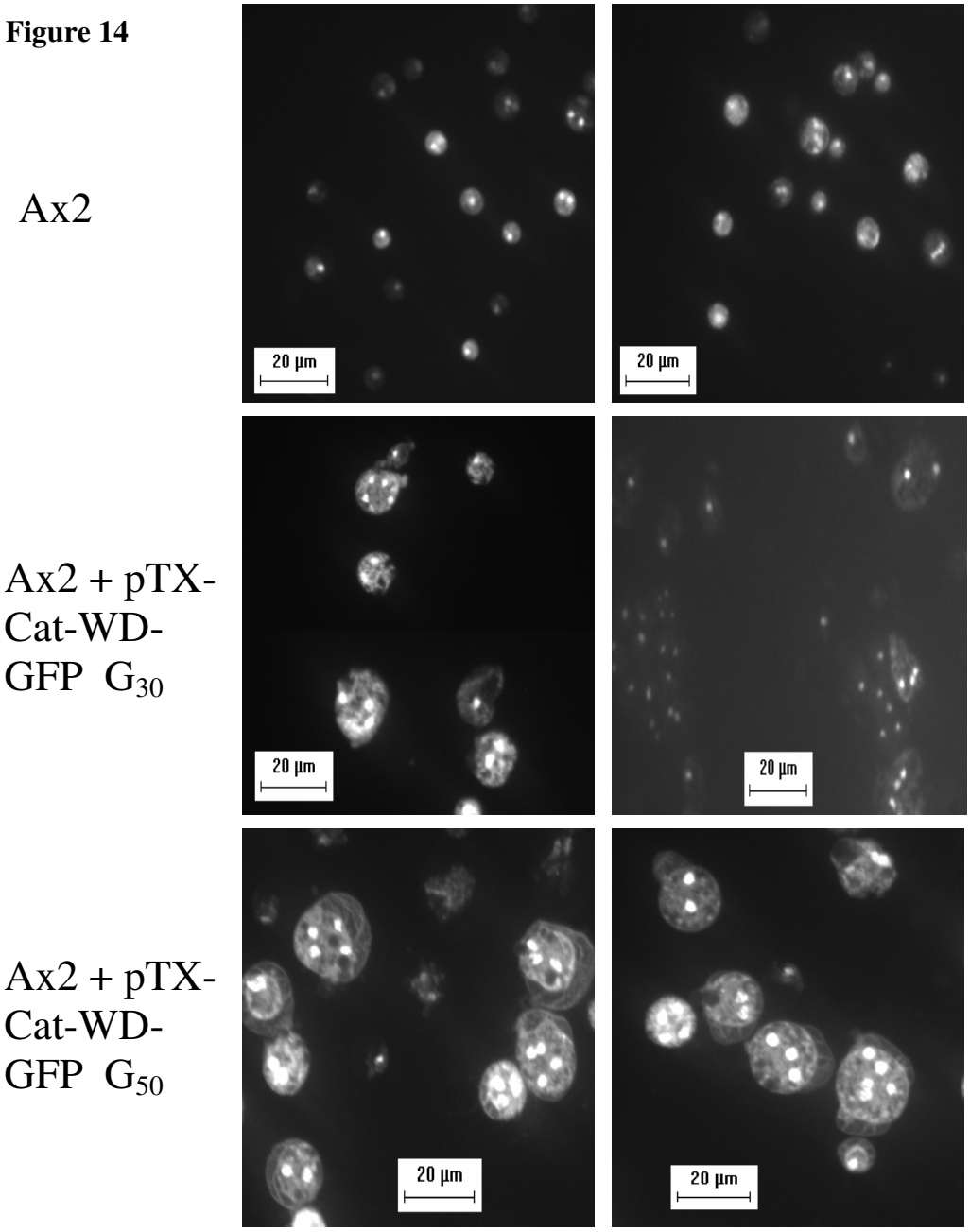


Figure 13: Analysis of Cytokinesis Defects by Nuclei Count
The number of nuclei in DAPI stained *Dictyostelium* cells were counted and recorded. (G30 - Cells grown at [G418]=30µg/ml G50 - Cells grown at [G418]= 50µg/ml)

Figure 14



<u>Figure 14:</u> DAPI Staining of *Dictyostelium* Cells. *Dictyostelium* cells were stained with DAPI to visualize nuclei. (G30 - Cells grown at [G418]=30mg/ml G50 - Cells grown at [G418]= 50mg/ml)

Figure 15

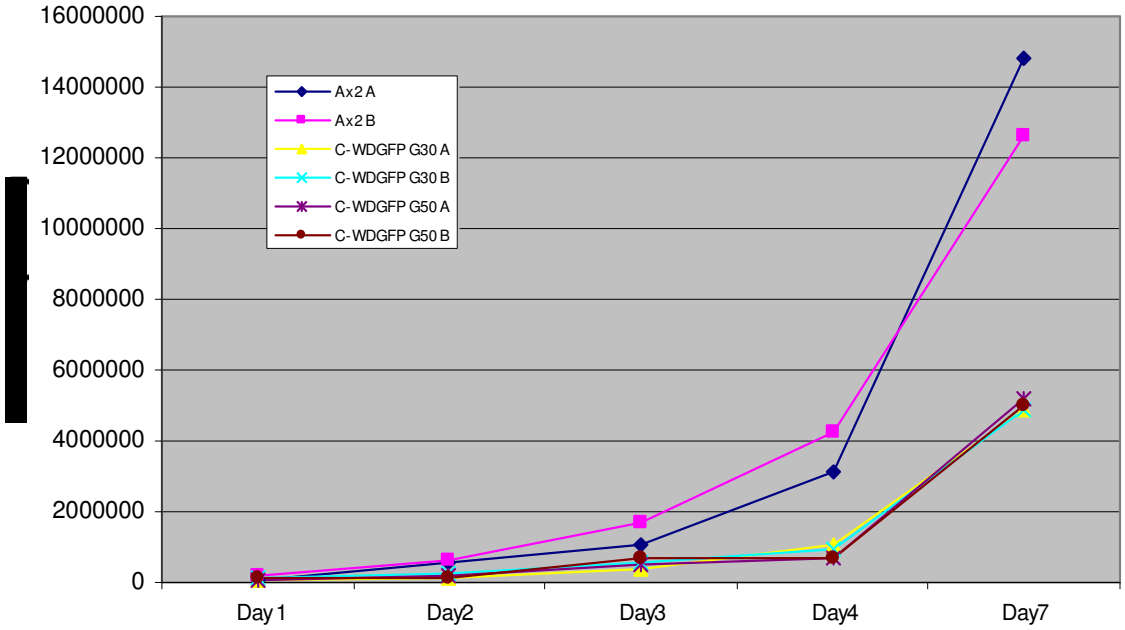


Figure 15: MHCK D Cat-WD-GFP Over Expression Growth Curve
Dictyostelium cell samples were counted at specified days following the start of shaking culture, to analyze for defects in cytokinesis. (G_{30} - Cells grown at [G418]=30µg/ml G_{50} - Cells grown at [G418]= 50µg/ml)

Figure 16

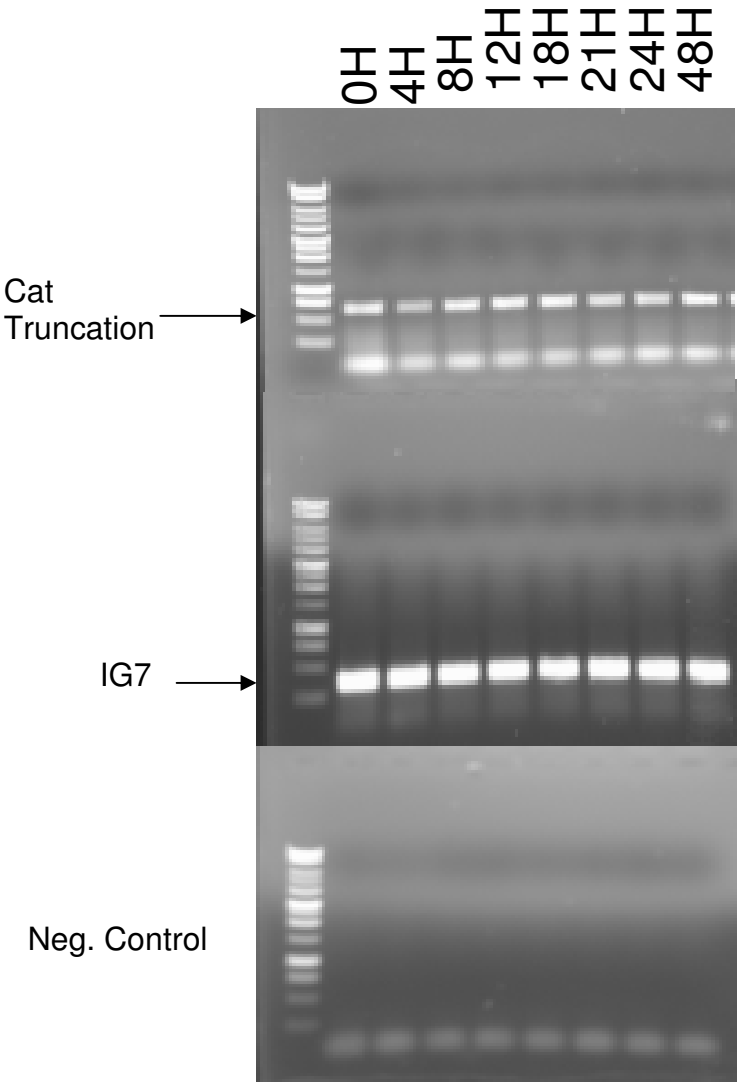


Figure 16: Reverse Transcriptase-PCR
RNA samples were collected at 0h, 4h, 8h, 12h, 18h, 21h, 24h, & 48h following initiation of multi-cellular development cycle. The RNA samples were subjected to RT-PCR using internal primers.