ABSTRACT

CLEVELAND PIGGOTT, JR.
The role of microtubules and myosin II in the formation of Hirano bodies
(Under the direction of Drs. MARCUS FECHHEIMER and RUTH FURUKAWA)

Hirano bodies are intracellular, paracrystalline, F-actin rich, structures that are most commonly found in the autopsied brains of humans suffering from neurodegenerative diseases. However, their biochemistry and physiology are not well understood despite their association with aging and several diseases. Recently, an in vitro and in vivo model to induce formation of Hirano bodies in living cells by expression of various mutants of a 34 kDa actin bundling protein mutant (Maselli et, al., 2002, 2003; Davis et, al., 2008) was developed. This allows further questions concerning Hirano bodies to be investigated. To examine what is necessary for the formation of Hirano bodies, we investigated the role of microtubules and an actin-dependent motor protein, myosin II. An inducible promoter for a protein that forms Hirano bodies fused to green fluorescent protein (pVEII-E60K-GFP) was introduced in *Dictyostelium discoideum* amoeba in the presence of pharmacological reagents. Cells were treated with nocodazole, a microtubule depolymerizing drug, or blebbistatin, a myosin II ATPase inhibitor. Cells were analyzed via fluorescence microscopy. After 24 hours of induction, no statistical difference in Hirano body size was seen between control cells and those treated with nocodazole, but blebbistatin-treated cells contained significantly smaller Hirano bodies, but still retained some large Hirano bodies. This suggests that myosin II plays a role in the formation of Hirano bodies but is not essential. Further experiments are needed to elucidate what components of the cytoskeleton are absolutely essential for the formation of Hirano bodies.

INDEX WORDS: Hirano bodies; neurodegenerative disease; microtubules; myosin II; actin

THE ROLE OF MICROTUBULES AND MYOSIN II IN THE FORMATION OF HIRANO BODIES

by

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FORMATION OF HIRANO BODIES

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DEDICATION

I would like to dedicate this manuscript to my loving grandfather, Cleveland Alfonso Piggott. His battle with Alzheimer's disease has forever changed my outlook on life. Watching the effect of this disease on him and my family has inspired me to do research and fueled my passion to pursue a career in medicine.

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CHAPTER 1
ABBREVIATIONS

AICD – Amyloid Precursor Protein Intracellular C-terminal Domain

GFP – Green Fluorescence Protein

TEM – Transmission Electron Microscopy

VASP - Vasodilator-Stimulated Phosphoprotein

CHAPTER 2
INTRODUCTION

Neurodegenerative disease and Hirano bodies

Neurodegenerative diseases affect millions of Americans and are becoming an increasing worry in our nation as our population begins to age. A wide variety of neurodegenerative diseases are characterized by the accumulation of intracellular and/or extracellular protein aggregates in the brain. Protein with polyglutamine repeats are associated with inclusion bodies in Huntington's disease (Lunkes, Trottier et al. 1998), α-synuclein aggregates form Lewy bodies which are associated with Parkinson's disease (Spillantini, Schmidt et al. 1997), and misfolded prion proteins cause Creutzfeldt-Jakob disease (Aguzzi and Haass 2003). In addition, intracellular aggregates of actin and actin-associated proteins have also been demonstrated to play a role in neurodegenerative disease (Bamburg and Bloom 2009). In Alzheimer's disease, the presence of actin-cofilin rods and thread-like inclusions are associated with cognitive decline (Bamburg and O'Neil 2002). Cells from patients with Alzheimer's disease frequently contain actin-rich inclusions called Hirano bodies (Hirano 1994).

Hirano bodies are intracellular, paracrystalline, eosinophilic, F-actin enriched structures most commonly found in the autopsied brains of humans with neurodegenerative diseases (Figure 2.1). These highly ordered rod-shaped inclusion bodies were first characterized and identified in the post-mortem tissue of Guamanian patients with Parkinson's disease and amyotrophic lateral sclerosis (Hirano, Dembitzer et al. 1968). Hirano bodies have been found in patients suffering from a number of other diseases including Alzheimer's disease (Schmidt, Lee et al. 1989), Pick's disease (Schochet, 1968), Creutzfeldt-Jakob disease (Cartier, Galvez et al. 1985), and chronic

alcoholism (Lass and Hagel 1994). In addition, Hirano bodies have also been associated with general aging (Hirano 1994). Hirano bodies are predominantly found in the central nervous system, especially in the pyramidal neurons of the CA1 region of the hippocampus (Hirano 1994). However, they have also been found in the peripheral nervous system (Atsumi, Yamamura et al. 1980), muscle cells (Fernandez, Fernandez et al. 1999), and even testis (Setoguti, Esumi et al. 1974). Despite their strong association with disease, questions concerning their physiology, structure, and function still remain due to the fact that much of what is known about these structures comes from post-mortem cells.

Recently, our laboratory developed a cell model system to induce formation of Hirano bodies in living cells based on the expression of the carboxy-terminal fragment (CT: aa124-295) of a 34 kDa actin bundling protein in *Dictyostelium discoideum* and mammalian cells (Maselli, Davis et al. 2002). Based on characterization of the CT fragment, it is believed that Hirano body formation is induced as a result of 34 kDa protein's increased F-actin binding and a loss of calcium regulation (Maselli et al. 2002). Subsequently, two other modes for inducing the formation of Hirano bodies have been discovered through altered forms of the 34 kDa actin bundling protein (Figure 2.2). This model for Hirano body formation allows questions concerning the biochemistry, genetics, and physiology of these structures to be further elucidated in living cells.

Through the use of our model system, we now know that Hirano bodies are not necessarily related to cell death (Maselli et al. 2002). In addition, immunohistochemistry has revealed the composition of Hirano bodies and the presence of several proteins including amyloid precursor protein intracellular domain (AICD) and the nuclear adapter protein Fe65 (Davis, Furukawa et al. 2008). The role of AICD and Fe65 is under investigation due to their association

with neurodegeneration in Alzheimer's disease. Hirano bodies sequester these proteins and impede AICD- and Fe65-dependent transcriptional activity and AICD-dependent apoptosis (Ha, Furukawa et al. 2010). In addition, model Hirano bodies are degraded both through the microtubule-dependent autophagy pathway and via the proteosome (Kim, Davis et al. 2009). Based on these findings, it was hypothesized that Hirano bodies may play a protective role in the cell by preventing cell death (Kim et al. 2009; Ha et al. 2010).

The cytoskeleton and Hirano bodies

There are still many questions concerning the role of Hirano bodies in disease progression such as how and why they form. The focus of this study was to examine, identify, and characterize the role in the formation of Hirano bodies of two major cytoskeletal systems, actin filaments and microtubules.

Actin is a highly abundant and conserved cytoplasmic protein in eukaryotic cells that is involved in determination of cell shape and polarity, movement of cells, and intracellular transport. Actin monomers, G-actin, can polymerize to form helical filamentous actin (F-actin). Intracellular transport on actin filaments is achieved by myosin protein. Myosins are actin-dependent motor proteins that hydrolyze ATP in order to produce movement along actin filaments. There are many superfamilies of myosins, such as myosin II, and they each serve a different function in actin-dependent motility (Lodish, Berk et al. 2008).

In addition to actin and its associated motor proteins, microtubules also play a very important role in the cytoskeleton and cellular transport. Microtubules are assembled from α/β-tubulin dimers and have a significant role in providing mechanical support, aiding cell division and allowing intracellular transport of vesicles. Motor proteins also play a key role in the active

transport of proteins and vesicles along microtubules. Kinesin and dynein are both ATP-dependent motor protein families that move cargo along microtubule tracks (Lodish et al. 2008).

Hirano bodies have been seen to form as small actin inclusions inside cells but coalesce into one large structure over time (Davis et al. 2008). Actin and/or microtubules and their associated proteins may be responsible for the formation of this single large Hirano body structure. Interestingly, previous studies performed in our laboratory were unsuccessful in recovering living cells with Hirano bodies when Hirano body formation was induced in the absence of functional myosin II (Maselli unpublished), suggesting a role of myosin II in the formation of Hirano bodies. Therefore, this study focuses specifically on the role of myosin II and microtubules on the formation of Hirano bodies, and an experiment was designed to test the hypothesis that these cytoskeletal transport systems contribute to Hirano body formation.

Experimental design

In order to determine the roles that the microtubule and actin cytoskeleton play in the formation of Hirano bodies in *Dictyostelium*, temporal control of the expression of the mutated form of the 34 kDa protein and a method to regulate Hirano body degradation were required. We used an expression vector with an inducible discoidin promoter (pVEII) for the expression of E60K, 34 kDa actin bundling protein mutant, fused to green fluorescence protein (GFP). The discoidin promoter is repressed in the presence of folate (Blusch, Morandini et al. 1992), and this enables E60K-GFP expression to be regulated in *Dictyostelium*. The E60K protein is a result of a point mutation at the 60^{th} amino acid codon, which changes a negative charged glutamate (E) to a positive charged lysine (K) in the N-terminal inhibitory region of the 34 kDa protein (Lim, Furukawa et al. 1999a). F-actin co-sedimentation assays of E60K have shown that the E60K protein is calcium sensitive and has a nearly 2.5-fold higher F-actin binding affinity than wild-

type 34 kDa protein (Griffin 2010) (Figure 2.3). In addition, when E60K-GFP is expressed in *Dictyostelium discoideum*, it has been characterized by both fluorescence and transmission electron microscopy to form Hirano bodies consistent with the structure seen in mammalian tissue (Griffin 2010) (Figure 2.4 and 2.5). E60K-GFP was chosen because it was observed to form more small aggregates versus other mutations of the 34 kDa protein. This inducible vector for E60K-GFP expression allowed the establishment of an approximate start time for Hirano body formation and enabled time-lapse fluorescence microscopy studies.

The process of Hirano body formation must be distinguished from its degradation, for Hirano body formation and degradation have been shown to be a dynamic process (Kim et al. 2009). Protein degradation in a eukaryotic cell is achieved by the proteosome and autophagy pathways. Proteosomes are large protein complexes that degrade ubiquitin tagged proteins (Goldberg 2007). Autophagy, the other degradation pathway in cells, is a catabolic process that uses lysosomal enzymes to degrade cellular components (Mizushima, Levine et al. 2008). In this process, an autophagosome forms, with the aid of microtubules, and fuses with a lysosome to cause degradation. A *Dictyostelium* autophagy knockout mutant, ATG5- (Otto, Wu et al. 2004), was used so that all Hirano bodies observed by microscopy were not going through autophagy. Hirano bodies were observed over nine hours in these autophagy mutants and degradation by the proteosome was found to be negligible to undetectable (data not shown).

In order to understand the role microtubules and myosin II contribute to Hirano body formation, pharmaceuticals were utilized to disrupt their function. Microtubules were depolymerized by treating cells with nocodazole (Roos, De Brabander et al. 1984). Nocodazole was used because it is the most effective at causing microtubule depolymerization in *Dictyostelium* cells (Borgers, De Nollin et al. 1975). Myosin II function was halted by

blebbistatin, an inhibitor specific for non-muscle myosin II ATPase activity in *Dictyostelium* (Shu, Liu et al. 2005; Takacs, Billington et al. 2010). Data was collected on Hirano body size at fixed time points over 24 hours by fluorescence microscopy and relative areas were compared.

Fig. 2.1: Electron micrograph of a Hirano body found in the hippocampal region of an autopsied human brain (Schochet and McCormick 1972).

Fig. 2.2: 34 kDa actin bundling protein schematic (Griffin 2010). Stars represent where 34 kDa protein is cross-linked to actin as found by mass spectroscopy. Different mutations in this protein have led to the formation of Hirano bodies in *Dictyostelium* and mammalian cells. The mutants are CT (Maselli et al. 2002), ΔEF1 (Maselli, Furukawa et al. 2003), and now E60K (Griffin 2010).

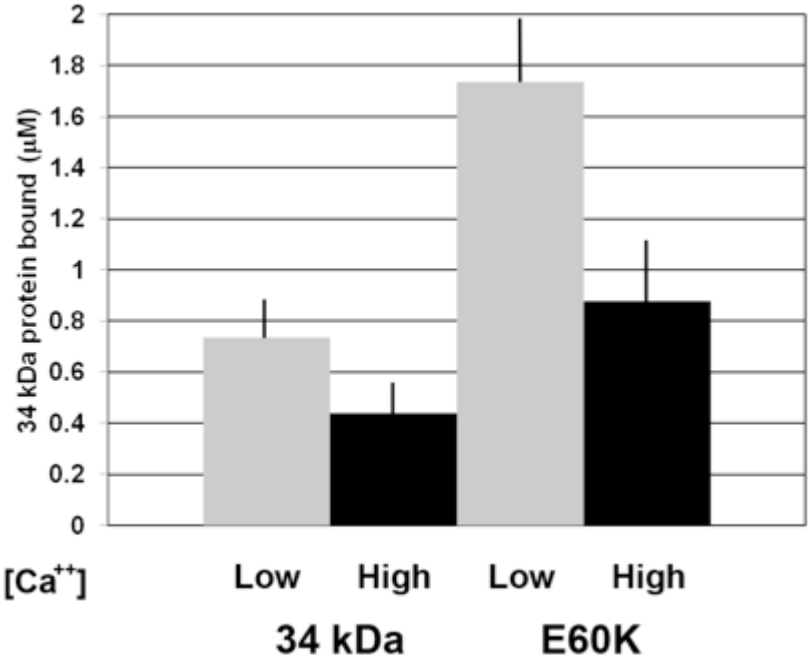

Fig 2.3: Determination of the calcium sensitive binding for 34 kDa and E60K proteins. Figure is shown with error bars. Equimolar concentrations of actin (3 μM) and either 34 kDa or E60K protein were compared in the presence of low and high calcium. The E60K protein had similar calcium sensitive F-actin binding to 34 kDa protein (Griffin 2010).

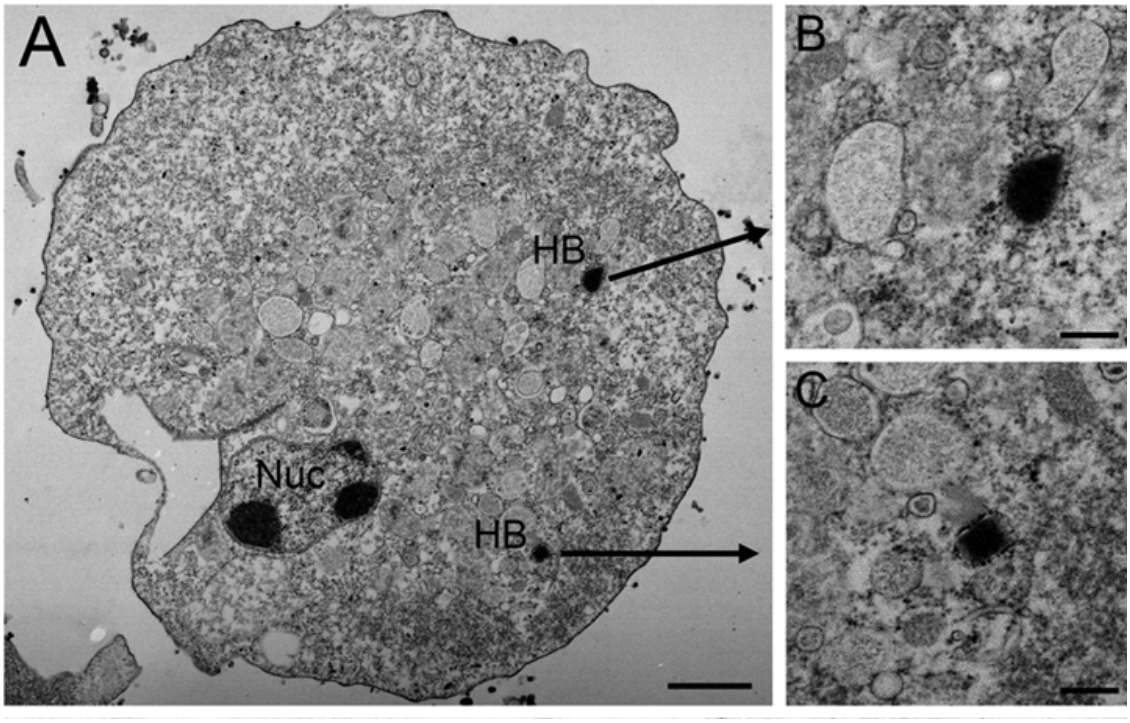

Fig 2.4: Electron micrograph of a cross section of a *Dictyostelium* cell expressing E60K-GFP (A). Inclusion bodies are marked and magnified (B, C). Scale bar = 250 nm. (Griffin 2010).

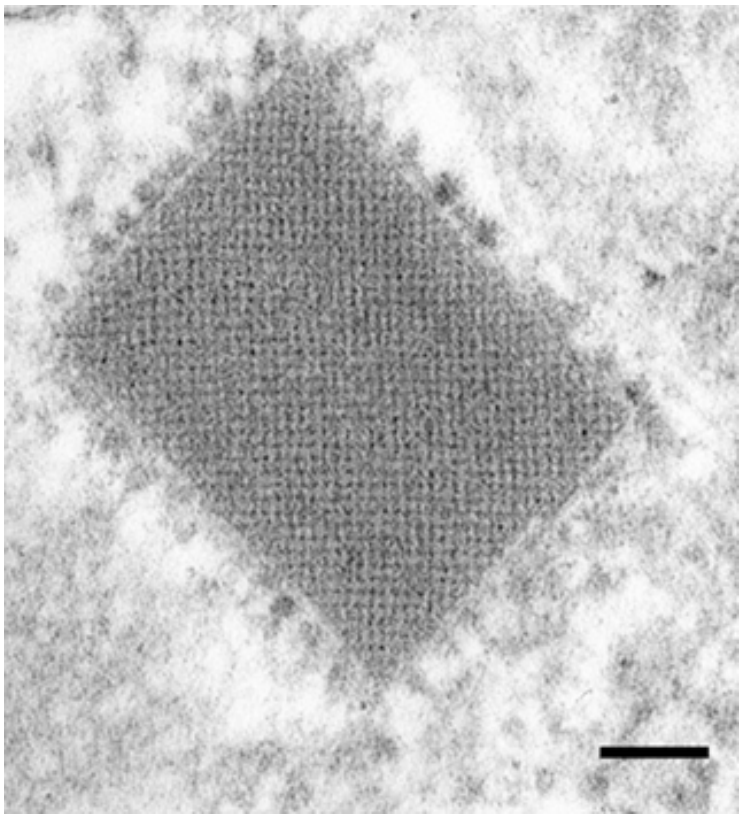

Fig. 2.5: High magnification TEM showing the paracrystalline structure of an inclusion body formed by E60K-GFP. Scale bar = 50 nm. (Griffin 2010).

CHAPTER 3
METHODS AND MATERIALS

E60K vector and transformation

A vector with an inducible promoter for the formation of Hirano bodies (pVEII-E60K) fused to a fluorescent tag (GFP) was used in this study. pE60K-GFP was serendipitously generated during the construction of pVEII-34 kDa-GFP for a previously published study (Kim et al. 2009). Transformation of pE60K-GFP into *Dictyostelium* cells bearing a knockout in the autophagy gene ATG5 ($atg5^-$) was performed by Dong Hwan Kim (unpublished) by electroporation (Rivero, Furukawa et al. 1996). Selection of transformed clonal cells was performed with 16 μg/ml G418 and 1 mM folate to suppress the discoidin promoter and expression of E60K-GFP. Cells were maintained in RM (Rivero et al. 1996) supplemented with 16 μg/ml of G418 and 1 mM folate on tissue culture treated plates. Cells were continuously cultured for three weeks or less before new cultures were established from frozen stocks.

Culture growth

For experiments, cells were harvested approximately 24 hours prior to the start of induction and plated at 3.5×10^5 cells in HL-5 (Loomis 1996) on microscope coverslips in 6-well tissue culture treated plates. To induce the expression of E60K-GFP, cells were washed twice with Sorensen's phosphate buffer, pH 6.1 and twice with HL-5. Cells in HL-5 were treated immediately with 10 μg/ml nocodazole (Sigma-Aldrich Chemical Co., St. Louis, MO) or 100 μM blebbistatin (Sigma-Aldrich Chemical Co., St. Louis, MO). Cells were harvested 3.5, 5.5, 7.5, and 24 hours after induction of protein expression and in the presence or absence of drugs. The cells were fixed 25 minutes in 3.7% formaldehyde in Sorensen's buffer with 1 mM EGTA, pH 7.

The cells were permeabilized with acetone (-20°C) for 2 minutes. The coverslips were allowed to air dry and mounted with Crystal Mount (Biomeda, Crystal City, CA) on microscope slides.

Immunofluorescence microscopy

Images of fluorescent Hirano bodies were collected with a LSM 510 VIS/META confocal microscope using a 40x objective at the four previously mentioned time points. Some samples were stained with TRITC-rhodamine-labeled phalloidin (Sigma-Aldrich Chemical Co., St. Louis, MO) (Fechheimer 1987). Images of each field were selected randomly and at least 100 Hirano bodies were counted per condition in each experiment. Hirano body sizes were quantified with ImageJ (NIH). Phalloidin-labeled cells were photographed with T-Max 400 film and processed according to manufacturer's recommendations with HC-110 (Kodak, Rochester, NY). Images were digitized using a SprintScan35 (Polaroid, Minnetonka, MN) and processed using Photoshop CS.

Transmission electron microscopy

Transmission electron microscopy (TEM) was used to compare the structures of Hirano bodies formed by E60K protein to that seen in autopsied brains. *Dictyostelium* cells were prepared for TEM as previously described (Novak, Peterson et al. 1995). Cells were embedded in EPON 812 and sectioned on an RMC 5000 ultramicrotome (Tuscon, AZ) with a diamond knife. Micrographs were taken on a Phillips 400 TEM.

CHAPTER 4
RESULTS

In order to demonstrate that E60K-GFP forms Hirano bodies, transmission electron microscopy on cells expressing E60K-GFP in *Dictyostelium* cells was performed as shown in Figure 2.4. Two distinct inclusion bodies in the cytoplasm can clearly be observed. These inclusions were not membrane bound and their paracystalline structure is shown in Figure 2.5 (Griffin 2010). The structure of these inclusions was consistent with previous characterizations of Hirano bodies in *Dictyostelium* and mammalian cells (Hirano 1994; Maselli et al. 2002). In addition to TEM, the presence of F-actin in these structures was tested in order to further confirm that E60K-GFP is associated with Hirano bodies. TRITC-phalloidin staining revealed co-localization of F-actin with E60K-GFP as shown in Figure 2.3. Therefore, E60K-GFP is associated with Hirano bodies.

After confirmation of its viability as a cell model for Hirano bodies, E60K-GFP was expressed in ATG5$^-$ *Dictyostelium* cells to observe the role the cytoskeleton plays in Hirano body formation. The mean Hirano body size was measured at 3.5, 5.5, 7.5, and 24 hours after induction. In order to control for effects on Hirano body size caused by a drug induced decline in cellular function, data was not collected from cells expressing Hirano bodies for longer time points. It is important to note that the cells exhibit normal cell morphology at 24 hours after drug treatment (Figure 4.2).

Hirano bodies increased in proportion in cells with time, but there was no significant difference in average Hirano body size between the control and nocodazole-treated cells as a function of time as shown in Figure 4.1. In contrast, cells treated with blebbistatin were

significantly smaller than the control at all time points ($p<0.05$). Blebbistatin-treated cells had increasingly large differences from the control in the size of Hirano bodies as time increased. This is most evident at 24 hours after induction where it was clearly observed that the cells with ATPase defective myosin II contained smaller Hirano bodies in relation to the control ($p<0.0001$). These results for the nocodazole and blebbistatin experiments were consistent in multiple independent trials.

It is important to note that Hirano bodies were able to form in all experimental conditions, and all three conditions contained very large standard deviation in Hirano body area (Figure 4.1). However, blebbistatin-treated cells contained a higher proportion of cells with multiple small Hirano bodies than the control and nocodazole-treated cells. This is most evident 24 hours after induction as seen in Figure 4.1. The presence of large Hirano bodies in some cells treated with blebbistatin leads us to believe that one large Hirano body structure will form in the cell but at a slower rate. These findings suggest that myosin II is not required for Hirano body formation but facilitates the process of forming one large Hirano body aggregate.

We were surprised by the lack of a significant effect of nocodazole on Hirano body formation (Figure 4.1). Tubulin staining in the presence of nocodazole demonstrated that our stock and concentration of nocodazole was effective in degrading microtubules in *Dictyostelium* (not shown). In addition, further experiments were conducted to further probe if microtubules play a role in Hirano body formation. However, we continued to find no significant difference in Hirano body size in nocodazole-treated cells when compared with the control cells despite doubling nocodazole concentration and using different Hirano body expression vectors (not shown). Therefore, we concluded that microtubules play an insignificant role in Hirano body formation.

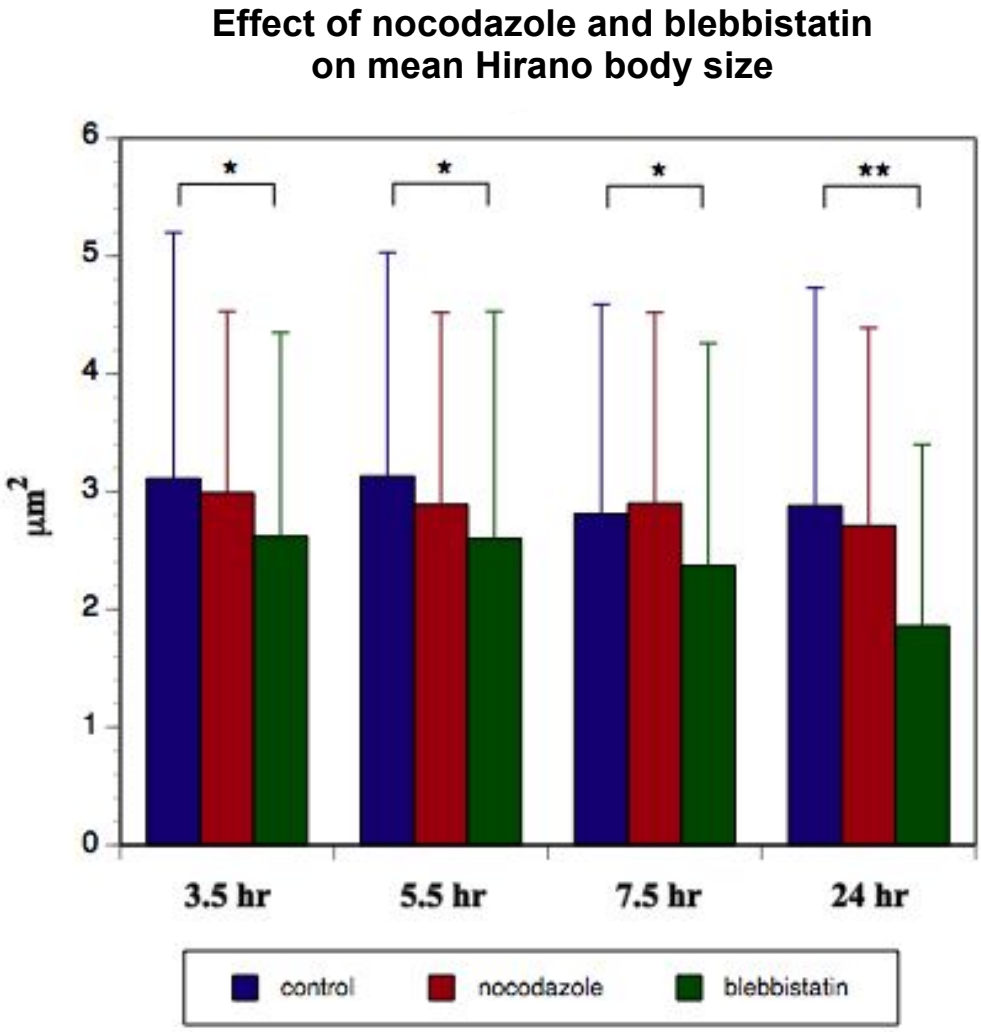

Fig. 4.1: Effect of nocodazole and blebbistatin on mean Hirano body size shown with standard deviation bars. Significance levels between control and blebbistatin conditions are represented by stars ($\star = p < 0.05$ while $\star\star = p < 0.0001$)

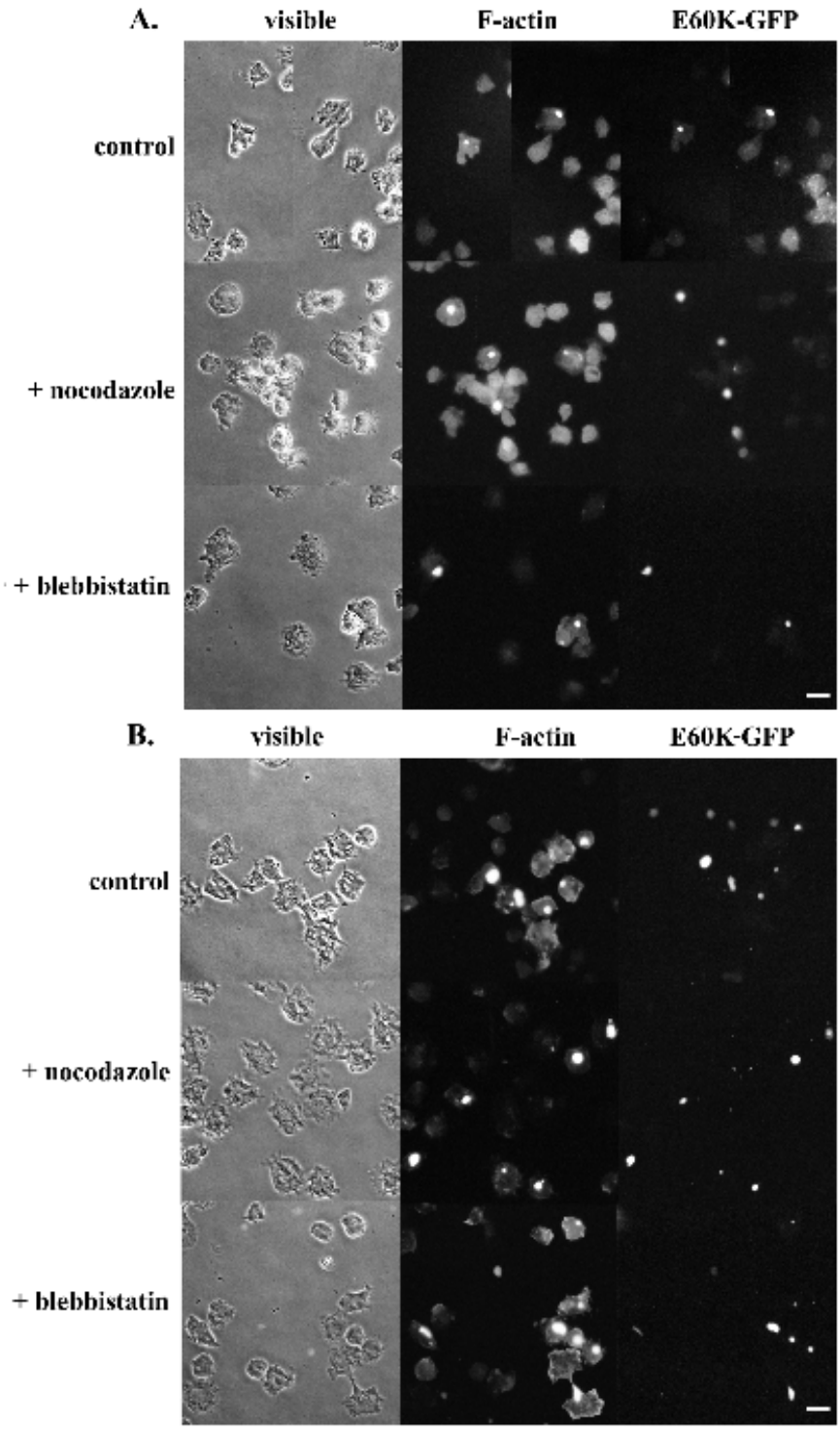

Fig. 4.2: Fluorescence microscopy of *Dictyostelium* cells expressing E60K-GFP. Images of E60K-GFP cells stained with TRITC-rhodamine-labeled phalloidin at 5.5 hours (A) and at 24 hours (B) after induction. F-actin is co-localized with the presence of E60K-GFP in the presence of all drug treatments. Scale = 10 μm.

CHAPTER 5
DISCUSSION

In 2006, the estimate for the worldwide prevalence of Alzheimer's disease was 26.6 million (Bookmeyer, Johnson et al. 2007). Alzheimer's disease is the 7th leading cause of death in the United States and the Alzheimer's Association estimates it costs the United States over 170 billion dollars annually. Alzheimer's disease is one of several diseases associated with Hirano bodies. However, there are still many questions concerning the role Hirano bodies play in disease and their biochemistry. This study focused on the role of the cytoskeleton in Hirano body formation, specifically the role of microtubules and myosin II.

Experiments performed using E60K-GFP and nocodazole revealed that microtubules play an insignificant role in Hirano body formation. This was surprising because studies on aggresomes and other inclusion bodies have shown the necessity of microtubules in their formation pathway (Kopito 2000; Hirano, Guhl et al. 2009). Though microtubules do not appear to play a significant role in Hirano body formation, they may play a small role in the transport of some of the other minor components of Hirano bodies. Hirano bodies are largely composed of actin, but they also contain cofilin, tropomyosin, vinculin, α -actinin, amyloid-precursor protein, and microtubule associated proteins such as tau (Maciver and Harrington 1995; Davis et al. 2008). Microtubules may play a role in transporting some of these components into the Hirano body, and further study of the composition of Hirano bodies in the presence of nocodazole is needed. In addition, microtubules may play a key role in Hirano body degradation by autophagy (Kim et al. 2009).

A large effect in Hirano body size was observed upon treatment with blebbistatin. On average, Hirano bodies seen in blebbistatin-treated cells were significantly smaller than the control cells but a large range in Hirano body size was seen in all treatments. We hypothesize this is due to a slower rate of formation of a single large Hirano body as opposed to a loss of inability for these large inclusions to form. As the expression rate of E60K-GFP increases with time, we see a higher rate of Hirano body formation. Control and nocodazole-treated cells maintain a fairly constant average size for Hirano bodies over time while blebbistatin-treated cells seem to exhibit an inverse relationship between time and average Hirano body size. If new small Hirano bodies were forming at a faster rate as time increased but the rate of single Hirano body formation remained constant or was slower due to inhibition, a gradual decrease in Hirano body size with time would be expected. This is exactly what we observed in this study (Figure 4.1). Therefore, we believe that myosin II plays a significant role in Hirano body formation, but is not required. Further study must be conducted on other myosin proteins to see which are required or aid in the formation of Hirano bodies.

We demonstrated that E60K-GFP produced characteristic Hirano bodies in *Dictyostelium* cells. This characterization was done both through TEM (Figure 2.4, 2.5) and fluorescence microscopy (Figure 4.2). TRITC-Phalloidin revealed co-localization of F-actin aggregates with Hirano bodies, but it is important to note that many F-actin aggregates cannot be classified as Hirano bodies. There are several ways to form F-actin aggregates in cells. The va sodilator-stimulated phosphoprotein (VASP) is a regulator of actin filament assembly, and when mistargeted to the endosome, it forms F-actin aggregates (Schmauch, Claussner et al. 2009). In addition, jasplakinolide, an F-actin stabilizing drug, has also been shown to form F-actin aggregages (Lázaro-Diéguez, Aguado et al. 2008). However, electron microscopy showed that

VASP and jasplakinolide both failed to create ordered structures that are characteristic of Hirano bodies. These studies further demonstrate that actin-binding proteins such as 34 kDa protein play a key role in the formation of Hirano bodies.

Understanding the necessary cytoskeletal components for Hirano body formation and what makes them different from other cellular aggregates will help us understand the role of Hirano bodies in aging and disease.

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