Calcium Regulation of Myosin3B

A Thesis in
Anatomy

by

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Abstract:

Purpose: Roughly 37.5 million people in the United States are affected by hearing loss (Blackwell, Lucas, & Clarke, 2014). Much of this comes from either hereditary or age-related conditions. Class III myosin have known mutations which can lead to the progression of non-syndromic delayed onset deafness. In this study we investigate the calcium regulation of a class III myosin in order to have a better understanding of its function in inner ear hair cells.

Methods: There are two isoforms of class III myosins, Myo3A and Myo3B. Using the baculovirus system we expressed Myo3B constructs with different numbers of calmodulin binding sites (IQ motifs) to determine which binding site was regulatory. We performed ATPase assays, in vitro motility assays, and cell biological studies in order to examine how this myosin was regulated by calcium. We assessed ATPase activity as a function of actin concentration, and as a function of calcium concentration. We also studied the effect of calcium on actin sliding velocity in the in vitro motility assay. Finally, we performed imaging of COS7 cells transfected with different Myo3B constructs in order to examine the calcium dependence of localization to actin-based structures in the cell. For ATPase and in vitro motility, the specific constructs designed contained the motor domain and the IQ motifs but lacked the kinase and tail domains. The baseline construct had both IQ motifs, while the other constructs consisted of deletions of the second IQ motif, both IQ motifs, and another construct with a deletion of the second IQ motif along with a mutation of the first IQ motif.

Results: Calmodulin binding assays demonstrated that all constructs had an increased ability to bind calmodulin in the presence of calcium except for Myo3B.2IQ, which demonstrated similar binding in the presence and absence of calcium. ATPase assays showed a 3-fold increase in the maximum ATPase (kcat) of Myo3B containing 2 IQ domains in the
presence of calcium. The calcium dependence of ATPase activity was less pronounced in the constructs containing only the first IQ motif and was unregulated when there were no IQ motifs. In vitro motility of Myo3B containing 2 IQ motifs was not calcium dependent but was higher than Myo3B with only the first IQ motif. In cell biology experiments we observed that mutating the first IQ motif decreased localization to the tips of filopodia, suggesting an intact first IQ motif is important for Myo3B based transport.

Conclusion: Our findings showed that Myo3B ATPase is calcium regulated, while the in vitro motility is not calcium sensitive. This suggests that calcium increases the actin affinity, which may play an important role in its ability to be retained at the tips of the stereocilia. When the second IQ motif is removed, there is a change in the first IQ motifs affinity for calmodulin, demonstrating cooperativity between the IQ motifs. Thus, both IQ motifs likely play a role in calcium regulation of Myo3B.
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Abbreviations:

ADP  Adenosine Diphosphate
Alexa Alexa Fluorescent Label
ATP  Adenosine Triphosphate
BSA  Bovine Serum Albumin
CaCl  Calcium Chloride
DAPI  4’,6-diamidino-2-phenylindole
DMEM Dulbecco’s Modified Eagle’s Medium
DNA  Deoxyribonucleic Acid
DTT  Dichloro Diphenyl Trichloroethane
EGTA Egtazic Acid
FBS  Fetal Bovine Serum
GFP  Green Fluorescent Protein
KATPase ½ Maximum ATPase Rate
Kcat Maximum ATPase Rate
KCl  Potassium Chloride
KMg50 Magnesium Potassium
MgCl2 Magnesium Chloride
mL  Milliliter
mM  Millimolar
Myo3A Class III Myosin Isoform A
Myo3B Class III Myosin Isoform B
nM  Nanomolar
PBS Lead Sulfide
pCa  Calcium Concentration
pCa50 Calcium Concentration at ½ Activation
PCR Polymerase Chain Reaction
PEP Phosphoenolpyruvate
PKA  Protein Kinase A
SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel
Electrophoresis
THDI Tail Homology Domain I
THDII Tail Homology Domain II
uL  Microliter
uM  Micromolar
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Chapter 1: Review of Literature
What is Myosin?

There are two major categories of myosin, conventional and unconventional. These categories separate myosin by their functions. While all myosin bind to actin, conventional myosin is used in muscle fibers where they interact with actin filaments during contraction, unconventional myosins serve a variety of functions in non-muscle cells where they interact with actin to perform a variety of mechanical functions. For example, unconventional myosins function in mitosis, intracellular transport, and sensory transduction as well as membrane tension, exocytosis, and endocytosis. (Adikes, Unrath, Yengo, & Quintero, 2013; Fath & Burgess, 1994; Hartman & Spudich, 2012; Ikebe et al., 2003; Woolner & Bement, 2009)

Figure 1. General myosin structure. Myosin consists of three general domains. The motor domain (in red), contains an ATP and actin binding site. The neck domain (blue), is considered the lever arm of myosin and is the location of light chain binding. The tail domain (green) has been found to be a main site for binding cargoes to transport and has been shown to affect the motor activity of myosin.

Domain Structure of Myosin

Each myosin has at least three main domains; head, neck and tail (Figure 1). The head domain contains the actin and ATP binding sites, which is referred to as the motor domain. The actin binding region allows myosin to interact with actin filaments, while the ATP binding region allows ATP hydrolysis. These two regions are coordinated to allow myosin to cyclically interact with actin in an ATP-dependent manner which allows force and motion.

The neck domain contains the light chain binding sites, which are calmodulin and calmodulin-like light chains. The neck domain is believed to function as a lever arm which
generates force by changing conformation while the motor is tightly bound to actin. (De La Cruz & Ostap, 2004)

The tail domain is the most diverse region of myosin and its structure and sequence vary tremendously among the myosin family. The tail of myosin is known to have the ability to bind to cargoes for intracellular transport and also allows some myosins to dimerize and form filaments.(Akhmanova & Hammer, 2010; Oliver, Berg, & Cheney, 1999)

**How Myosin Utilizes Energy**

All myosins are able to generate force and motion by using the energy from ATP hydrolysis (De La Cruz & Ostap, 2004). ATP hydrolysis is a chemical reaction where an ATP molecule is hydrolyzed at its gamma phosphate and this chemical energy can be used to generate a conformational change. Specifically, the energy of ATP binding and hydrolysis is used to cock the myosin head after it has dissociated from the actin filament. When the ATP is hydrolyzed, ADP and phosphate are trapped in the active state. Myosin can re-associate with the actin in this state. Once the myosin has been re-associated, the release of the phosphate group causes the myosin power stroke to flex the myosin head. After the power stroke the ADP release must occur first before ATP binding can induce another ATPase cycle (Figure 2).

All identified unconventional myosins have been shown to contain a neck domain with at least 2 IQ motifs which bind calmodulin light chains (Wolenski, 1995). These calmodulin light chains have been
shown to affect the motor activity of myosins, mostly through calcium regulation of the calmodulin binding (Cheney et al., 1993; Lu, Krementsova, & Trybus, 2006; Wolenski, 1995; Wolenski, Hayden, Forscher, & Mooseker, 1993). In some myosins, when calcium binds calmodulin, calmodulin dissociates from the myosin causing the in vitro motility to decrease based on the lever arm theory (Anson, Geeves, Kurzawa, & Manstein, 1996; Uyeda, Abramson, & Spudich, 1996). Calmodulin contains four calcium binding sites, two low affinity binding sites at the N-terminal domain and two high affinity binding sites at the C-terminal domain (Thulin, Andersson, Drakenberg, Forsen, & Vogel, 1984). Mutations of the C-terminal domain have been shown to abolish calcium regulation of calmodulin, however amino acid mutations of the N-terminal domain have shown little difference from the wild type calmodulin (Zhu, Beckingham, & Ikebe, 1998).
Class III Myosin

Class III myosin consists of two vertebrate isoforms, Myosin3A and Myosin3B (Dose et al., 2003). These two isoforms are encoded by separate genes and are predominantly expressed in the retina and cochlea where they are believed to function as actin-based transporters in actin bundles (Dose, Ananthanarayanan, Moore, Burnside, & Yengo, 2007). Class III myosins are also found in the brain, testes and the gastro-intestinal tract, however their function in these organs is unknown (Dose et al., 2003). While they contain the typical motor, neck and tail domains, they also contain an N-terminal kinase domain. Class III myosins are the only known kinase-motor hybrid making them quite unique (Mooseker & Cheney, 1995)(Figure 3).

![Figure 3. Myo3 domain structure. A)Stick figure representation of the Myo3B structure. The green section represents the motor domain, the two dashes along the right end represent the IQ motifs, while the pear green and red boxes represent the tail domain and tail homology domain, respectively. B: Stick figure representation of Myo3A domain structure. Purple domain represents the motor domain, with the two dashes along the right end representing both IQ domains. The orange and red represent the tail domain and tail homology domain one and two, respectively.

Modified from(Raval et al., 2016)](image)

The kinase domain of class III myosin has been shown to regulate the motor domain by autophosphorylating sites on the motor domain (Quintero et al., 2010). The kinase domain has an activation loop which when it is phosphorylated, turns on kinase activity similar to what occurs in PKA kinase family members. In an experiment studying Myo3A’s ability to properly localize in the cell it was found that mutations that prevent phosphorylation of the kinase activation loop or remove the kinase domain increased localization to actin protrusions (Quintero et al., 2010). It
was proposed that the kinase domain regulates motor activity which in turn mediates cellular function (Quintero et al., 2013).

Myo3A and Myo3B have some structural differences, the majority of these are in the tail domain (Figure 3). Myo3A contains a much longer tail and tail homology domain 2 (THD2). Interestingly, THD2 is essential to allow Myo3A to walk along actin filament bundles such as filopodia, microvilli, or stereocilia (Merritt et al., 2012; Raval et al., 2016). This is especially useful in the stereocilia, where Myo3A transports cargoes to the tips of the stereocilia aiding in stabilization and elongation of actin filaments. While Myo3B also localizes to the tips of the stereocilia, it requires ESPN-1 to bind to its tail to allow it to walk along the actin filaments. ESPN-1 is an actin bundling protein, whose function is critical in the stereocilia. ESPN-1 binds to a region in the tail domain called tail homology domain I (THDI), which has been found in the tail domain of both Myo3A and Myo3B (Manor, Grati, Yengo, Kachar, & Gov, 2012; Merritt et al., 2012; Salles et al., 2009). While THD2 has been studied the function of other regions of the tail domain are relatively unknown. However, it has been shown that deletions of different portions of the tail domain each can impact cellular localization, which suggests that THD2 is not the only important region of the tail (Raval et al., 2016).

Another difference between Class III myosins is the efficiency of the motor domain. A study was performed using constructs which had deleted the tail domain and allowed examination of the motor activity of Myo3A and Myo3B. These constructs referred to as “myosin3A 2IQ” and “myosin3B 2IQ”, contained the motor domain and two IQ domains in the neck region. While both are capable of motility, Myo3A 2IQ has a 3-4 fold higher ATPase activity, a 20-fold higher actin affinity and roughly a 2-fold faster in vitro motility velocity compared to Myo3B 2IQ.
**Hearing in Vertebrates**

Vertebrates use an intricate process of converting sound waves into electrophysiological signals that cause the release of neurotransmitters from the inner ear hair cells in the cochlea. This is called mechanotransduction because mechanical changes to hair cells lead to the release of neurotransmitters that cause an efferent action potential in the auditory nerve which is sent to the brain. Thus, mechanotransduction can turn a sound wave into a neural signal which is interpreted by the brain. This process gives us the ability to hear, however when the mechanosensors called the stereocilia degrade, they are unable to perform mechanotransduction correctly (Ebrahim et al., 2016). Therefore, this process of maintaining stereocilia in the inner ear hair cells is critical for the auditory system in vertebrates. When a sound is heard, it starts as a vibration in the air. It is then captured by the pinna of the outer ear and funneled into the external auditory meatus. Here, these vibrations transfer to the tympanic membrane, which send them through the three smallest bones in the human body; the malleus, incus and stapes. These bones transmit the signal to the oval window, which converts the vibration into endolymph fluid waves. As the endolymph of the inner ear reverberates the sound waves, it creates a shearing of the tectorial membrane and the basilar membrane deflecting the stereocilia, which are organized in a “staircase” manner. When the shortest stereocilia is pushed towards the tallest, the ion channels are opened allowing for potassium to enter the hair cell. This process is referred to as mechanotransduction (Figure 4). The influx of potassium depolarizes the cell and causes the release of neurotransmitters that synapse with the efferent auditory nerve (Nambiar, McConnell, & Tyska, 2010). Thus, mechanotransduction is a very elegant process that requires precise structure of the stereocilia. The stereocilia are organized in a staircase pattern and are made up of
parallel actin bundled structures, with the plus end of the actin filaments oriented at the stereocilia tips.

Physiological Role of Class III Myosin

Class III myosin are important for the function of the cochlea, since they are critical in the maintenance, growth, and stability of stereocilia (Pollock, Chou, & McDermott, 2016). When there is a mutation in class III myosins, the stereocilia have been shown to degrade which disrupts their function (V. L. Walsh et al., 2011). The mechanotransduction process requires proper organization of the stereocilia and any disruption in the stereocilia length causes malfunctions in the process of mechanotransduction, leading to hearing defects.

The retina has also been found to contain class III myosins. Specifically, class III myosins are found in vertebrate photoreceptors and the Drosophila rhabdomere. In Drosophila,
Myo3A is thought to participate in phototransduction and light adaptation, but its role in vertebrates is unclear. For example, humans with a mutation in Myo3A causing non-syndromic deafness had no reports of visual symptoms, leading us to believe the class III myosins do not play an essential role in retinal development in vertebrates.

**Transport Cargoes and Their Function**

Class III myosin are capable of binding cargo in their tail domain and transporting the cargo in the cell. Some of the studied cargoes that are known to bind to class III myosin are ESPN-1, ESPN-L, and MORN4 (Raval et al., 2016). Both ESPN isoforms have been found to enhance stereocilia elongation and stability (Salles et al., 2009). It is believed that ESPN-1 prefers to bind to Myo3A while ESPN-L prefers to bind Myo3B, and they both transport to different rows of stereocilia. While ESPN is known to bind to Myo3B, it is believed to act as a

![Figure 5. Current model of Myo3B motility](image)

*Figure 5. Current model of Myo3B motility.* Myo3A is able to bind actin filaments via the Tail Homology Domain II (THDII). This allows it to walk using an inchworm like fashion. Myo3B is required to bind a cargo such as ESPN in order to tip localize due to the lack of a Tail Homology Domain II. ESPN is able to bind actin via its actin bundling module (ABM), allowing Myo3B to use ESPN as a crutch and localize to the tips of the filapodia.

*(MERRITT ET AL., 2012)*
crutch, allowing Myo3B to walk along actin filaments in an inchworm-like fashion (Figure 5). This is critical in Myo3B’s ability to tip localize in stereocilia and explains Myo3Bs inability to tip localize in the absence of ESPN.

MORN4 is specific for the Myo3A tail and was found to interact with the exon 30-31 region (Mecklenburg et al., 2015). MORN4 also enhances tip localization in Myo3A. Interestingly, MORN4 may anchor Myo3A to the tips of stereocilia by linking it with the plasma membrane. Since MORN4 is found in both photoreceptors and inner ear hair cells this cargo may localize Myo3A to the membrane domains of actin protrusions in these specialized cells.

Mutations of Myosin3A

One of the many ways to classify mutations is by genetic inheritance. There are four general classifications, autosomal dominant, autosomal recessive, X-linked, and mitochondrial. Some mutations of Myo3A are autosomal recessive meaning that in order for the mutation to manifest, the carrier must have two copies of the mutated gene. If there is one copy of the gene unaffected, then the subject is considered a “carrier” who shows no signs or symptoms. The location of the mutation may dictate whether it is dominant or recessive. Mutations of the motor domain will cause the motor to not function properly or possibly not at all. While a mutation of the tail domain will prevent binding to cargoes.

In Myo3A, several mutations associated with non-syndromic deafness have been described (T. Walsh et al., 2002), which are associated with loss of hearing without any other reported symptoms. The majority of these mutations are delayed onset. The delayed onset phenotype may be caused by a mutation that reduces motor function, disrupting transport and impacting hearing (T. Walsh et al., 2002). This is because as mentioned earlier, class III myosins
are responsible for intracellular transport that is essential for stereocillia length and stability. Since they have overlapping functions, Myo3B is thought to compensate for Myo3A in people that have Myo3A mutations. However, the less efficient Myo3B motor that contains a shorter tail is believed to lack the ability to completely compensate for the loss of Myo3A. It is important to understand how Myo3A and Myo3B are regulated. This is especially important for Myo3B since it would have to compensate for the lack of Myo3A when it contains loss of function mutations.
Chapter 2: Rationale, Aims and Hypothesis

Rationale:

The goal of this study was to investigate the calcium regulation of Myo3B. Calcium binds to calmodulin, which interacts with the neck domain of Myo3B, also referred to as IQ motifs. We hypothesize that the first IQ motif is where calmodulin regulates the Myo3B motor. We propose this is the regulatory site because not only is the IQ motif closest to the motor domain, it is also the case in other myosins such as MyosinV. The hypothesis can be tested by studying Myo3B with and without certain parts of the neck domain. If the first IQ motif is the regulatory site, then there will be little to no effect when the second IQ motif is deleted. However, there will be a significant effect when the first IQ motif is mutated and both IQ motifs are deleted. To show this effect of calcium regulation, we propose to examine Myo3B constructs lacking the kinase domain and tail domain and thus only including the motor and variable number of functional IQ domains.

Hypothesis:

We hypothesize that the first IQ motif of Myo3B serves as a regulator of calcium dependent motor activity. In the case of a mutation or deletion of the first IQ motif, there will be a lack of calcium regulation of motor and ATPase activity.

Aim1:

Test the ATPase activity as a function of free calcium in a construct containing the full neck domain. We tested this using the construct containing both IQ domains in order to have a
baseline of activity for Myo3B without its kinase domain or tail domain (Figure 6A). All constructs engineered for this project contained a N-terminal Flag tag and C-terminal GFP.

We expected that as free calcium increased, so will ATPase activity and sliding velocity until calcium binding is saturated. We did not examine a construct containing the kinase domain and tail domain, while future studies will need to perform these important experiments.

Aim2:

The goal of Aim 2 is to test the hypothesis that deleting the 2nd IQ motif will have little to no effect on calcium regulation. However, deleting both IQ motifs or disabling the first IQ motif will cause ATPase activity to be unregulated by calcium. To test this, we used a variety of constructs which had differences in the neck domain, while none of the tested constructs contained a kinase domain or a tail domain. We measured the in vitro sliding velocity and ATPase activity of these constructs (Figure 6)

The first construct Myo3B.1IQ has a deletion of the second IQ motif in the neck domain. The second construct has a deletion of the entire neck domain. The third and final construct, Myo3B.1IQKO has a deletion of the second IQ motif, and a mutation of the amino acids L1067E, W1074D, and Y1080D which are calmodulin binding sites in the first IQ motif. We expected there would be little if any decrease in calcium regulation with the deletion of the second IQ motif, but that there would be a significant decrease in calcium regulation with the deletion of both IQ motifs or mutation of the first IQ motif.
Aim 3 is designed to examine the ability of Myo3B to localize to the tips of actin protrusions and to determine the impact of mutating the first IQ motif. We will transfect COS7 cells using a Myo3B construct with the first IQ motif mutated (Figure 6E) and compare it to a Wild-Type (WT) construct. In order for Myo3B to tip localize in filopodia it requires being bound to an ESPN isoform, thus we will co-express ESPN in all experiments. We will also determine the impact of intracellular calcium on the tip localization of mutant and WT Myo3B.

A - Myo3B.2IQ
B - Myo3B.1IQ
C - Myo3B.0IQ
D - Myo3B.1IQKO
E – Myo3B.1IQKO.FL

Figure 6 All baculovirus expressed constructs contain an N-terminal FLAG tag, and a C-terminal GFP tag (A-D). Each construct has the kinase domain deleted. A: Myosin3B.2IQ, B: Myosin3B.1IQ has the second IQ motif deleted, C: Myosin3B.0IQ has both IQ motifs deleted, D: Myosin3B.1IQKO has the second IQ motif deleted along with the first IQ motif mutated to be inactive. For cell biology experiments we utilized E: Myosin3B.1IQKO.FL construct that contains the full tail domain with a mutation of the first IQ motif.
Chapter 3: Methods:

Introduction:

To study the regulation of calcium at the neck domain we engineered Myo3B by deleting or mutating different portions of the neck domain which we believed would affect its ability to be regulated by the calcium/calmodulin complex and thus changing its motor properties. To generate the constructs, we used polymerase chain reaction (PCR), ligation, and quick-change site directed mutagenesis. We generated oligonucleotides which ended at the preferred domain section that were flanked by BamH1 and Not1 sites to cut the end of the PCR fragments, and then ligated the appropriate fragments into the pFastBac expression vector (Dose et al., 2008). To assess the ligation products, we used restriction digest and agarose gel electrophoresis, which allowed us to assess which plasmids contained the inserts of interest. The constructs were also verified by Sanger sequencing. We transformed these plasmids into DH10Bac cells to generate baculovirus expression bacmids. The bacmid constructs were then used to transfected SF9 cells to generate recombinant baculovirus (Dose et al., 2008; Merritt et al., 2012). The baculoviruses were amplified several times before using them for protein expression in SF9 cells. We then purified the protein using anti-FLAG affinity chromatography (Merritt et al., 2012; Quintero et al., 2010). The purified protein was assessed by SDS-PAGE and found to be 90-95% pure. All experiments were performed in KMg50 DTT buffer (10mM imidazole pH 7.0, 50mM KCl, 1mM MgCl2, 1mM EGTA 1mM DTT) at 25°C.

Calmodulin Binding Assay:

The ability of Myo3B constructs to bind calmodulin in the presence and absence of calcium was assessed with the calmodulin binding assay. First a sample of Myo3B and actin was
equilibrated with excess CaM in the presence and absence of calcium for 20 minutes on ice. Samples were then spun down in an ultracentrifuge with a TLA120.2 rotor at 95K for 15 minutes. The actomyosin pellets were then resuspended using KMg50 either with or without calcium and incubated for another 20 minutes on ice. To dissociate the Myo3B from actin we added 150mM KCl along with 2mM ATP and spun in the ultracentrifuge again for 10 minutes (95K in TLA120.2 rotor). The supernatant and pellet were examined by SDS-PAGE and the ratio of calmodulin relative to myosin in the supernatant was examined using densitometry with Image-J software.

**ATPase Assay:**

We used a stopped-flow apparatus to measure the steady state NADH-linked ATPase activity of the Myo3B constructs in KMg50 buffer (10mM imidazole pH 7.0, 50mM KCl, 1mM MgCl2, 1mM EGTA 1mM DTT) and 1mM ATP at 25°C. The ATPase assay allows us to determine if the deletions/mutations had an effect on actin-activated ATPase activity. We observed the ATPase activity with a range of actin concentrations and used the Michaelis-Menton equation to determine the maximal actin-activated ATPase rate ($k_{cat}$) and the actin concentration at which the ATPase rate is half maximal ($K_{ATPase}$). We used non-linear least squares fitting with a hyperbolic function to determine the values of $K_{ATPase}$ and $k_{cat}$. A pCa curve was performed in which the concentration of calcium was varied during repeated runs from 1nm to 100µM in order to observe the effects calcium had on ATPase activity in the presence of 40µM actin. The Hill equation was then used to graph the ATPase activity curve and determine the concentration of calcium of which the ATPase activity is 50% of maximal (pCa$_{50}$).
**In Vitro motility Assay:**

We used a modified protocol for our in vitro motility assay, as described in (Kron, Toyoshima, Uyeda, & Spudich, 1991; Yengo, Takagi, & Sellers, 2012). We created a flow cell using a nitrocellulose coated coverslip and a glass microscope slide. We first washed with KMg50 buffer without DTT, then introduced the GFP antibody, and incubated for 3 minutes to allow it to attach to the coverslip. We then treated the flow cell with 1mg/mL BSA two times, using 2-minute incubations which prevented non-specific binding. The Myo3B protein was then added and allowed to incubate for 3 minutes in order for it to bind to the GFP antibody, followed by a wash step using KMg50DTT. We then added sheared actin, followed directly by 1mM ATP, to prevent any “dead heads” (myosin molecules which are too damaged to perform their function) from interfering with the “active-heads” (functional myosin molecules). We then added Rhodamin-Phalloidin labeled actin, (10nM) to the flow cell. Finally, we added 50µL of activation buffer (50µL 10xBSA, 5µL 100xglucose, 2.3µL PEP, 4.6µL PK, 125µL of 1.4% Methylcellulose, 263.1µL dH2O) in KMg50 buffer containing the appropriate amount of calcium and balanced with KCl to keep ionic strength constant. We quickly brought the slide to be observed in a NIKON TE2000 microscope equipped with shutter controlled time-lapse imaging. Images were collected every 10 seconds for 10 minutes. The Image-J software was used to track the filaments paths over the course of 10 minutes with the plug-in called M-track. We analyzed 50 filaments per condition and compared 1-3 preps of each construct.

**COS7 Transfection and Cell Confocal Imaging:**

We also examined the calcium regulation of Myo3B in a cellular context. We transfected a N-terminal GFP tagged full length Myo3B.1IQKO construct and compared it to Wild-type
Myo3B into COS7 cells in the presence of ESPN1 using an established protocol (Raval et al., 2016). Myo3B is known to localize to filopodia structures and reach the tips of the filopodia when the motor is active and ESPN is present.

A previously described protocol was used to culture and transfect COS7 cells (Quintero et al., 2010). In short, we maintained the cell cultures in DMEM with 10% FBS and 100 units of Penicillin-streptomycin. Before transfection the cells were plated onto acid-washed, 22mm², number 1.5 coverslips using roughly 35,000-45,000 cells per coverslip. These cells were incubated overnight in order to adhere to the coverslip. These cells were then transiently transfected using lipofectamine, which consists of 0.3µg of plasmid DNA being diluted into 100 µL of Opti-MEM media not including serum or antibiotics. The DNA was gently mixed with lipofectamine and left to incubate for 15 minutes at room temperature. The mixture was then added via drops to the coverslips containing cells, in a 6 well dish. After 24 hours the cells were treated with ionomycin for 30 minutes with excess calcium or EGTA in the media.

The actin filaments were also stained with an Alexa 633 phalloidin in order to observe the actin filaments using a fluorophore that will not interfere with GFP. We began by washing the cells once with 1mL PBS before moving them to a humid chamber and added 200µL of 4% paraformaldehyde in PBS to each coverslip. We let them incubate for 10 minutes to allow for the paraformaldehyde to properly fix the cells. Then we washed the cells with 1mL PBS for 30 seconds before permeabilizing the cells using 200µL of 0.1% Triton-X in PBS. We allowed the Triton-X to incubate for 5 minutes before washing the cells twice with 1mL PBS for 30 seconds each wash. We pipetted 200µL of the Alexa 488 phalloidin and 100nM DAPI stain to allow visualization of nuclei. Antifade mounting media was used to mount the slides. The coverslips
were sealed using nail polish. We viewed the slides within the next 24 hours of fixing and mounting (Trybus, 2000).
Chapter 4: Results

Calmodulin Binding assay:

The calmodulin binding assay was performed in order to determine if calmodulin was able to bind the Myo3B constructs in a calcium dependent manner. Myo3B.2IQ showed a similar degree of binding to calmodulin in the presence and absence of calcium (Figure 6A). Myo3B.1IQ showed the ability to bind calmodulin in the presence and absence of calcium, however there was an increased binding at higher calcium. Myo3B.1IQKO showed the ability to bind calmodulin in the presence of calcium, however this construct was unable to bind calmodulin in the absence of calcium. The construct without IQ domains (Myo3B.0IQ) demonstrated that it was still able to bind calmodulin in the presence of calcium but was unable to bind calmodulin in the absence of calcium. Overall, each construct made was able to bind calmodulin in the presence of calcium, however some were unable to in the absence of calcium (Figure 7, B-D).
**Table 1:** Relative CaM binding values. Examined in 1mM EGTA and at pCa 4.
**Actin Activated ATPase assay**

Actin activated ATPase rates were plotted versus actin concentration and fitted with a Michaelis-Menten hyperbolic fit for the Myo3B.2IQ construct however the other construct were unable to be fit to a hyperbolic function (Figure 8). This experiment was performed for each construct, at actin concentrations ranging from 0-60µM. The Myo3B.2IQ construct demonstrated a $k_{cat}$ value of 0.2496s$^{-1}$ in 1mM EGTA and a $K_{ATPase}$ value of 47.23µM. When compared to the values after the addition of calcium (pCa 4) there was a 2-fold increase in $k_{cat}$ to 0.4172s$^{-1}$ along with a roughly 4-fold decrease in $K_{ATPase}$ to 12.3µM. This change demonstrates that Myo3B ATPase activity is calcium dependent and that there is a higher actin affinity in the presence of calcium (Figure 7A). We also tested the constructs containing different parts of the neck, Myo3B.1IQ, Myo3B.1IQKO, and Myo3B.0IQ. The ATPase activity of these constructs could not be fit using the Michaelis-Menten curve because they did not fully saturate, thus we reported ATPase activity at 40µM actin. Myo3B.1IQ demonstrated a 2-fold difference in ATPase activity between 1mM EGTA (0.23s$^{-1}$) and pCa4 (0.09s$^{-1}$). This difference shows that there was still calcium regulation after the deletion of the second IQ motif, although it was attenuated. The Myo3B.1IQKO construct demonstrated variable differences in ATPase activity in the presence and absence of calcium. The final construct we tested was Myo3B.0IQ, which demonstrated little effect with the addition of calcium. Indicating this construct does not seem to be regulated by calcium (Figure 8 B-D).
ATPase activity at 40μM actin

<table>
<thead>
<tr>
<th>Construct</th>
<th>pCa 4</th>
<th>EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo3B.2IQ</td>
<td>0.4172s⁻¹</td>
<td>0.2496s⁻¹</td>
</tr>
<tr>
<td>Myo3B.1IQ</td>
<td>0.09s⁻¹</td>
<td>0.23s⁻¹</td>
</tr>
<tr>
<td>Myo3B.1IQKO</td>
<td>2.0s⁻¹</td>
<td>0.7s⁻¹</td>
</tr>
<tr>
<td>Myo3B.0IQ</td>
<td>1.143s⁻¹</td>
<td>1.275s⁻¹</td>
</tr>
</tbody>
</table>

Table 2: Actin activated ATPase maximum values. Maximum rates at 60 μM actin were extracted at pCa 4 and 1 mM EGTA.

Figure 8: The ATPase activity in the presence (pCa 4) and absence (1mM EGTA) of calcium. A-Actin activated ATPase activity of Myo3B.2IQ. B- Myo3B.1IQ. C- Myo3B.1IQKO D- Myo3B.0IQ
**pCa Curve ATPase assay:**

The Hill equation was used to evaluate the ATPase activity as a function of free calcium. The Myo3B.2IQ construct shows a clear calcium dependence of ATPase activity and a pCa$_{50}$ of 6.5 (3.145x10$^{-7}$ µM Ca$^{2+}$) (Figure 9A). Calcium saturation appears to occur around 10µM calcium as demonstrated by a plateauing of the ATPase activity after that concentration is reached. Myo3B.1IQ (Figure 9B) also showed calcium dependent ATPase activity however the curve did not saturate as well. In the Myo3B.1IQ construct a pCa$_{50}$ of 5.6 (2.35x10$^{-6}$ µM Ca$^{2+}$) was determined and the Myo3B.1IQKO construct (Figure 9C) demonstrated a similar trend with a pCa$_{50}$ of 6.2 (6.69x10$^{-7}$ µM Ca$^{2+}$). This curve had some variable values which suggest more experiments are necessary to further evaluate calcium dependence. The Myo3B.0IQ (Figure 9D) did not show any calcium regulation. We did not report the pCa$_{50}$ values of M3B.0IQ due to its lack of and regulation by calcium (Table 3).

<table>
<thead>
<tr>
<th>Construct</th>
<th>ATPase Assay</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCa 50</td>
<td>pCa 4</td>
</tr>
<tr>
<td>Myo3B.2IQ</td>
<td>6.50</td>
<td>36.7 nm/s</td>
</tr>
<tr>
<td>Myo3B.1IQ</td>
<td>5.63</td>
<td>24.8 nm/s</td>
</tr>
<tr>
<td>Myo3B.1IQKO</td>
<td>6.17</td>
<td>10.8 nm/s</td>
</tr>
</tbody>
</table>

**Table 3.** Calcium dependence of ATPase activity and in vitro motility
In vitro motility:

We determined the average sliding velocity in the in vitro motility experiments. Myo3B.2IQ had a velocity of roughly 40nm/s across its pCa curve, demonstrating no calcium regulation of motility (Figure 10). The next construct measured was the Myo3B.1IQ, which demonstrated a slight calcium dependence. At low calcium concentrations (1nM or pCa 9) the Myo3B.1IQ had a velocity of 20.3 nm/s, while at a calcium concentration of 10µM there was an average velocity of 29.9 nm/s. The velocity decreased however when the calcium concentration
was increased to 100µM (pCa 4) showing an average velocity of 24.8nm/s. The reduced velocity of the 1IQ constructs compared to Myo3B.2IQ may be a result of the shortened lever arm. The Myo3B.1IQKO construct showed an even slower motility (13.9nm/s in 1mM EGTA) and even slower velocity of 10.8 nm/s at pCa 4. We did not include results with the Myo3B.0IQ since it lacks a lever arm we didn’t expect it to be motile.

![Image](image1.png)

**Figure 10** A) Histogram of the distribution of in vitro motility velocities for the Myo3B.2IQ construct at different pCa values. B) Example of in vitro motility tracks for several given actin filament paths.

**Cellular Localization of Myo3B:**

We performed confocal imaging of COS7 cells transfected with full length GFP tagged Myo3B (lacking a kinase domain) and measured actin localization using ALEXA phallodin, as well as nuclei using DAPI staining. The images were captured using the Leica DMi8 confocal microscope. Focus was on observing the ability of Myo3B.1IQKO to tip localize when expressed with ESPN in the presence and absence of calcium. Wild-type full length Myo3B was the control as it is known to be capable of tip localizing in the presence of ESPN. Myo3B.1IQKO
demonstrated the ability to tip localize however it was visibly apparent that more of the Myo3B.1IQKO stayed in the body of the filopodia instead of reaching the tip (Figure 11).

**Figure 11** Calcium induced Myo3B formation and elongation of actin protrusions. Confocal images of fixed and stained COS7 cells expressing A) Myo3B.1IQKO and ESPN (-calcium), B) Myo3B.1IQKO and ESPN (+calcium), C) Myo3B.WT and ESPN (-calcium), D) Myo3B.WT and ESPN (+calcium).
Chapter 5: Discussion

Introduction:

This study was performed to examine the calcium regulation of Myo3B and specifically to determine the role of the neck domain. Calcium binds calmodulin in the neck domain, which in turn regulates the motor activity of many myosins. We proposed that the first IQ motif in Myo3B is the regulatory IQ motif, and that in the case of a mutation or deletion of that IQ motif, there would be a loss of regulation by calcium. In our study we found that altering or deleting Myo3B’s first IQ motif greatly affected the motor activity showing that the first IQ motif is indeed important. However, the second IQ motif may also play a role since removal of this motif also decreased calcium regulation and altered calmodulin binding.

Calmodulin Binding assay:

The calmodulin binding assay gave us insight into Myo3B’s ability to bind calmodulin in the presence and absence of calcium. The constructs all showed an increased in the ability to bind calmodulin in the presence of calcium except for the Myo3B.2IQ construct which had roughly the same degree of binding in the presence and absence of calcium. The Myo3B.1IQKO and Myo3B.0IQ constructs both did not have the ability to bind calmodulin in the absence of calcium. Our results suggest that removal of the second IQ motif causes the first IQ motif to bind calmodulin in a calcium dependent manner. This is clear from the results with Myo3B.1IQ and Myo3B.1IQKO, although more extreme in Myo3B.1IQKO. A puzzling result is the calmodulin still bound in the construct containing no IQ motif. Thus, some of the calmodulin binding to Myo3B in the presence of calcium may be non-specific. Future studies will need to explore if
there is a novel calmodulin binding site in Myo3B separate from the 1IQ motifs or if calmodulin binds non-specifically to Myo3B in the presence of calcium.

**Actin-Activated ATPase Assay:**

We clearly demonstrated that the ATPase activity of Myo3B.2IQ is regulated by calcium. The Myo3B.2IQ results suggest calmodulin binding is not dependent on calcium and thus the regulation is due to a calcium dependent conformational change. Removal of the second IQ motif, as shown in the Myo3B.1IQ, decreased its ability to be regulated by calcium. Myo3.1IQKO demonstrated an even more diminished ability to be regulated by calcium while the Myo3B.0IQ construct was completely unregulated by calcium. Overall our results suggest that both IQ domains contribute to calcium-calmodulin regulation, while further studies would be needed to investigate the role of the second IQ domain.

**pCa Curve ATPase assay:**

The results from the calcium dependent ATPase activity (pCa curve) demonstrate that Myo3B.2IQ is regulated by calcium in the physiological range (pCa$_{50}$ = 6.5). The Myo3B.1IQ and Myo3B.1IQKO constructs still showed an increase in ATPase activity as a function of calcium, demonstrating that there was still some regulation by calcium in the physiological range. Since we hypothesized that the first IQ motif was regulatory we were initially surprised by these results. This could be attributed to calmodulin binding to the first IQ motif in a calcium dependent manner in these constructs. The Myo3B.0IQ construct showed no regulation by calcium. As the IQ motifs are used for binding calmodulin and regulating motor activity, the 0IQ results agree with the hypothesis that the calcium regulation is associated with the neck domain.
**In vitro motility assay:**

The in vitro motility assay showed that the motility of all of the Myo3B constructs were mostly independent of calcium concentration. While there was minimal change in sliding velocity as a function of calcium, there was a stark difference between the sliding velocity of the Myo3B.2IQ and the Myo3B.1IQ. The velocity was essentially reduced 2-fold by removing the second motif. The reduction in the length of the lever arm is likely the cause of this reduction in velocity (Anson et al., 1996). Since the Myo3B.0IQ construct lacks a neck it is not surprising motility studies with this construct were unsuccessful. Future studies could introduce an artificial lever arm to avoid the structural issue of deleting the IQ motifs while still being able to study calcium regulation of the motor.

**Cellular Localization of Myo3B:**

The cell biological studies demonstrated that when the first IQ motif is mutated to prevent calmodulin binding, there is a decrease in Myo3B’s ability to tip localize. However, the construct was still capable of localizing to the tips, just not as effectively as wild-type Myo3B. Another take-away from the cell biology experiments is the effect that calcium had on the tip localization of Myo3B. Qualitatively, there seemed to be no effect of calcium on the ability of full length Myo3B.1IQKO. We will need to first determine if wild-type Myo3B has calcium dependent tip localization to evaluate these results. Assuming there is a calcium dependence of wild-type Myo3B tip localization, we would suggest that the loss of calcium regulation in Myo3B.1IQKO is because of the importance of the first IQ domain in regulating motor activity.
**Major Discoveries:**

In this study we found that when Myo3B contains a full neck domain, the addition of calcium effects only the ATPase activity and does not change the motility. Our results suggest that calcium effects Myo3B’s actin affinity and leads us to believe that calcium could play a role in retaining Myo3B at the tips of stereocilia. We also found that in the absence of the 2nd IQ motif there was a decrease in calcium dependent ATPase, which is evidence that the 2nd IQ motif plays a role in calcium regulation. When both IQ motifs were either deleted or nonfunctional, there was reduced calcium regulation further exemplifying that both IQ motifs play a role in regulation. Another finding from cellular biology studies, was that when we mutated the 1st IQ motif there was a decrease in filopodia tip localization. While more quantitation is needed to confirm these results, we believe our results indicate that calcium regulation plays a role in the ability of Myo3B to tip localize. This leads us to believe that patients suffering from a lack of functional Myo3A (DFNB30) may be prone to stereocilia damage/degeneration in the presence of calcium dysregulation.

**Future Directions:**

After the completion of our experiments there were a few additional experiments that may provide new insight into Myo3B calcium regulation. The first experiment would be to create a mutation of the 2nd IQ motif while still retaining the first IQ motif. This experiment would allow examination of calcium regulation while retaining the entire structure of the neck domain. This construct should theoretically have the same in vitro motility as Myo3B.2IQ but may have less calcium regulation. We could examine a Myo3B construct with both IQ motifs
mutated to prevent calmodulin binding. If there is still calmodulin binding to this construct it may demonstrate that there are other calmodulin binding sites in the motor.

The cellular biology showed interesting results in the 1IQKO construct seeming to have reduced tip localization. However, we need to ensure that the ESPN cargo is not localizing the Myo3B to the filopodia. Thus, a motor dead Myo3B would verify that Myo3B motor activity is required for localizing to filopodia. This would allow us to further evaluate the Myo3B.1IQKO results. Once further characterization is done in COS7 cells it would be interesting to determine the localization in inner ear hair cell stereocilia by transforming Myo3B constructs into isolated cochlea tissue cultures (Dantas et al., 2018).
References:


Dose, A. C., Ananthanarayanan, S., Moore, J. E., Corsa, A. C., Burnside, B., & Yengo, C. M. (2008). The kinase domain alters the kinetic properties of the myosin IIIA motor. Biochemistry, 47(8), 2485-2496. doi:10.1021/bi7021574


