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Microtubule Motors Drive Nuclear Dynamics and Positioning in Developing Skeletal Muscle Cells

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Abstract
Dynamic interactions with the cytoskeleton are essential to move and anchor nuclei during tissue development, and defects resulting in nuclear mispositioning are often associated with human disease, such as muscular dystrophy and myopathy. Skeletal muscle cells are large syncytia formed by fusion of myoblasts, and contain hundreds of nuclei positioned regularly along the length the cell. During muscle cell development, nuclear movement in myotubes requires microtubules, but the mechanisms involved have not been elucidated. Here, we find that nuclei actively translocate through myotubes. As they translocate, they also rotate in three-dimensions. These movements require an intact microtubule cytoskeleton, which forms a dynamic bipolar network around the nuclei, and are driven by the microtubule motor proteins, kinesin-1 and cytoplasmic dynein. Depletion of the plus-end directed motor kinesin-1 abolishes nuclear rotation and significantly inhibits nuclear translocation, resulting in the abnormal aggregation of nuclei near the midline of the myotube. Loss of the minus-end directed dynein motor also inhibits nuclear dynamics, but to a lesser extent, leading to altered spacing between adjacent nuclei. The motors are found throughout the cytoplasm, but also decorate the nuclear envelope. To test whether kinesin-1 on the nucleus is essential for nuclear distribution, we controlled the recruitment of truncated, constitutively active kinesin-1 motors to the nuclear envelope. We show that nuclear-based kinesin-1 is necessary to prevent nuclear aggregation. Additionally, we show that kinesin-1 localization to the nuclear envelope in myotubes is mediated at least in part by interaction with the nuclear envelope protein, nesprin-2. We identify a conserved kinesin light chain-binding motif in nesprin-2 and show that recruitment of the motor complex to the nucleus via this motif is essential for proper nuclear distribution. Thus, our work indicates that oppositely directed motors acting from the surface of the nucleus drive nuclear motility in myotubes. The variable dynamics observed for individual nuclei within a single myotube likely result from the stochastic activity of competing motors interacting with a complex bipolar microtubule cytoskeleton. The three-dimensional rotation of myotube nuclei may facilitate their motility through the complex and crowded cellular environment of the developing muscle cell, allowing for proper myonuclear positioning.

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MICROTUBULE MOTORS DRIVE NUCLEAR DYNAMICS AND POSITIONING IN DEVELOPING SKELETAL MUSCLE CELLS

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ABSTRACT

MICROTUBULE MOTORS DRIVE NUCLEAR DYNAMICS AND POSITIONING IN DEVELOPING SKELETAL MUSCLE CELLS

Meredith Hayley Wilson

Dr. Erika L.F. Holzbaur

Dynamic interactions with the cytoskeleton are essential to move and anchor nuclei during tissue development, and defects resulting in nuclear mispositioning are often associated with human disease, such as muscular dystrophy and myopathy. Skeletal muscle cells are large syncytia formed by fusion of myoblasts, and contain hundreds of nuclei positioned regularly along the length the cell. During muscle cell development, nuclear movement in myotubes requires microtubules, but the mechanisms involved have not been elucidated. Here, we find that nuclei actively translocate through myotubes. As they translocate, they also rotate in three-dimensions. These movements require an intact microtubule cytoskeleton, which forms a dynamic bipolar network around the nuclei, and are driven by the microtubule motor proteins, kinesin-1 and cytoplasmic dynein. Depletion of the plus-end directed motor kinesin-1 abolishes nuclear rotation and significantly inhibits nuclear translocation, resulting in the abnormal aggregation of nuclei near the midline of the myotube. Loss of the minus-end directed dynein motor also inhibits nuclear dynamics, but to a lesser extent, leading to altered spacing between adjacent nuclei. The motors are found throughout the cytoplasm, but also decorate the nuclear envelope. To test whether kinesin-1 on the nucleus is essential for nuclear distribution, we controlled the recruitment of truncated, constitutively active kinesin-1 motors to the nuclear envelope. We show that nuclear-based

kinesin-1 is necessary to prevent nuclear aggregation. Additionally, we show that kinesin-1 localization to the nuclear envelope in myotubes is mediated at least in part by interaction with the nuclear envelope protein, nesprin-2. We identify a conserved kinesin light chain-binding motif in nesprin-2 and show that recruitment of the motor complex to the nucleus via this motif is essential for proper nuclear distribution. Thus, our work indicates that oppositely directed motors acting from the surface of the nucleus drive nuclear motility in myotubes. The variable dynamics observed for individual nuclei within a single myotube likely result from the stochastic activity of competing motors interacting with a complex bipolar microtubule cytoskeleton. The three-dimensional rotation of myotube nuclei may facilitate their motility through the complex and crowded cellular environment of the developing muscle cell, allowing for proper myonuclear positioning.

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CHAPTER 1: Introduction

I. Nuclear Positioning – An Introduction

The nucleus is the largest and perhaps the most important organelle in a eukaryotic cell. Essential functions of the nucleus include the storage, organization and replication of the genome, as well as the distribution of genetic information through both synthesis and processing of RNAs and the assembly of the translational machinery. While these functions define the nucleus as the control center of the cell, the nucleus is not necessarily always located in the geometric center of the cell as is typically depicted in textbooks. The position of a nucleus may vary with cell type, and may change during processes such as cell division, cell migration, tissue differentiation, and regeneration.

For example, in the budding yeast *Saccharomyces cerevisiae*, migration of the nucleus to the bud neck during cell division is required for proper segregation of genetic material to daughter cells (Yeh et al., 1995). Similarly, during zygote formation in many species, the male and female pronuclei must move toward one another following fertilization before undergoing the first mitosis, thereby ensuring that the daughter cells each receive equal amounts of paternal and maternal chromatin (Wilson, 1896).

In developing zebrafish retinas, the position of the nucleus within neural precursor cells regulates cell-cycle exit and cell fate determination, such that defective nuclear migration results in a lack of photoreceptor cells (Del Bene et al., 2008). Furthermore, during neocortical development, newly-born neurons migrate from the ventricular zone outward along radial glial fibers to more superficial layers of the cortex (Nadarajah and Parnavelas, 2002). In these examples, effective migration requires carefully coordinated movement of

the nucleus, and defects have dire consequences for brain architecture and function (Shu et al., 2004; Tsai et al., 2007).

In the mechanosensory hair cells in the vertebrate ear, the nucleus resides at the basal cell surface. Aberrant localization of the nucleus to the apical surface is associated with degeneration of the hair cells and progressive high-frequency hearing loss (Horn et al., 2013). In a last example, a cluster of 3-8 nuclei is specifically positioned under the membrane at the neuromuscular junction in skeletal muscle cells (Bruusgaard et al., 2003; Kelly and Zacks, 1969; Nakai, 1969). Specialized gene expression in these nuclei is necessary for synaptic maintenance and transmission (Jevsek et al., 2006; Merlie and Sanes, 1985), and loss of the synaptic nuclei is correlated with neuromuscular disease in mice (Mejat et al., 2009; Zhang et al., 2007).

From these examples, it is not only clear that nuclear positioning is essential to development and proper cell function, but also that nuclear positioning is an active process that involves two related mechanisms: nuclei must move or migrate through the cytoplasm to first localize to the appropriate location, and then nuclei are anchored in order to maintain this position.

Furthermore, nuclear movement can be subdivided into both translational components and rotational components. Translation/translocation implies that a net force acts on the nucleus causing movement from one point of space to another, whereas during rotation, forces act on the nucleus to cause it to spin about its axis, but with no net change in space. These components of nuclear movement can occur separately or concurrently, with the relative contribution of each depending on the cellular context. For example, in the developing vertebrate neuroepithelium, nuclei undergoes interkinetic nuclear migration, consisting of oscillatory translational movements along the apico-basal axis of the cell (Chenn and McConnell, 1995; Sauer, 1935), whereas in rapidly migrating fish epidermal

keratocytes, the nucleus rolls forward along with the cell body during translocation (Anderson et al., 1996).

Central to both nuclear movement and anchorage is the interaction between the nuclear envelope and the cytoskeleton. All three components of the cytoskeleton, the microtubules, actin filaments and intermediate filaments, are involved in nuclear positioning to varying degrees depending on cell type and differentiation state. These cytoskeletal elements, including associated molecular motors, generate force or provide scaffolding, whereas protein complexes on the nuclear envelope mediate force transmission to the nucleoskeleton to effectively move or anchor the nuclei.

While our knowledge about nuclear positioning in many different cell types and different organisms is growing, there is still a great deal more to learn, especially with regards to the mechanisms of nuclear movement and the relationship between nuclear position and disease. With these questions in mind, the work contained within this thesis was undertaken in order to further our understanding of the mechanisms driving nuclear movement and positioning in developing skeletal muscle cells.

II. The Microtubule Cytoskeleton

The cytoskeleton is a network of protein filaments that extends throughout the cytoplasm of eukaryotic cells. It provides a structural framework for the cell that determines cell shape and acts as a scaffold for organization of organelles. Importantly, the cytoskeleton is a dynamic network that is continuously reorganized as cells move and change shape. The cytoskeleton is composed of three main types of protein polymers: microtubules and actin filaments, which are inherently polarized, and intermediate filaments, which consists of a large family of non-polarized filaments. Although cells most often employ

microtubules and/or the actin cytoskeleton to move nuclei, intermediate filaments may also play a role in nuclear positioning in certain cells. As the focus of this dissertation is on a cell system utilizing microtubules for nuclear movement, mechanisms of nuclear positioning by actin and intermediate filaments will only be discussed briefly.

Microtubules

Microtubules are not only structural elements within the cell, but also serve as tracks for intracellular transport. Microtubules are polymers composed of heterodimers of two closely related polypeptides, α- and β-tubulin. These tubulin subunits self-assemble head-to-tail to form protofilaments, and protofilaments interact laterally to form the walls of the microtubule (for review see (Wade, 2009)). Typically, microtubules are composed of 13 protofilaments arranged radially to form a hollow tube approximately 25 nanometers in diameter (Tilney et al., 1973). Because of both longitudinal and lateral contacts between heterodimers within the microtubule lattice, the addition or loss of subunits occurs almost exclusively at the microtubule ends. Additionally, these contacts make microtubules stiff and difficult to bend, properties that are important for many of the microtubule's cellular roles.

The subunits in each microtubule protofilament are uniformly oriented, such that α-tubulin is at the more stable microtubule minus-end, and β-tubulin faces the dynamic plus-end. This arrangement of α- and β-tubulin confers overall polarity to the microtubule (Hoenger and Milligan, 1996; Mitchison, 1993). Microtubules grow mainly through addition of tubulin heterodimers to the plus-end, although slow growth can also occur at the minus-end *in vitro* and in cells (Jiang et al., 2014). Additionally, microtubules alternate between periods of slow growth and rapid depolymerization (Horio and Hotani, 1986; Mitchison and Kirschner, 1984).

This phenomenon, known as dynamic instability, is the consequence of enzymatic activity of the tubulin monomers. Both α- and β-tubulin monomers bind guanosine

triphosphate (GTP) in their guanine nucleotide binding site, however, only the GTP in the β-tubulin site is hydrolyzed to guanosine diphosphate (GDP) (Jacobs et al., 1974; Weisenberg et al., 1976). Hydrolysis proceeds slowly in the unbound state, so soluble tubulin heterodimers are typically in the GTP-bound state. However, GTP hydrolysis is accelerated when the tubulin incorporates into a microtubule, and GDP no longer exchanges for the GTP in solution (David-Pfeuty et al., 1977; Kobayashi and Simizu, 1976; Weisenberg et al., 1976). The more time a subunit is in the microtubule, the more likely it is to have hydrolyzed the bound nucleotide. In the GDP-bound state, tubulin heterodimers are more likely to dissociate from the end of the microtubule lattice, whereas GTP-tubulin is more stable and resists depolymerization. The GTP or GDP-state of the tubulin subunits at the end of the microtubule depends on the rate of hydrolysis compared to the rate of subunit addition. If the rate of polymer addition exceeds the rate of hydrolysis, as the microtubule grows, a GTP cap forms, which protects the microtubule from undergoing depolymerization (Drechsel and Kirschner, 1994). However, if the rate of hydrolysis catches up to the rate of subunit addition, the GDP-bound tubulin is exposed at the microtubule end, enhancing the probability of 'catastrophe', the switch to a fast-shrinking state. The difference in rates can again switch, leading to rescue and resumed growth of an individual microtubule. Therefore, in a population of microtubules, some microtubules may be growing and some depolymerizing, with the ratio depending on the hydrolysis rate and free subunit concentration (Horio and Hotani, 1986; Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984). Dynamic instability, when combined with additional cellular mechanisms for nucleating, stabilizing or destabilizing microtubules, allows for rapid rearrangements of the microtubule network that are essential for cellular re-organization as well as cell migration.

Microtubule Nucleation

Though a solution of tubulin subunits at a sufficiently high concentration will polymerize into microtubules *in vitro* via spontaneous nucleation, cells have evolved mechanisms for nucleating microtubules. Most animal cells have a single, well-defined microtubule organizing center (MTOC) known as the centrosome, which is typically located near the nucleus. The centrosome, which was first observed over 100 years ago, is composed of two orthogonally arranged centrioles, surrounded by a dense amorphous material (referred to as the pericentriolar material or the centosome matrix), from which microtubules emanate (for review see (Mazia, 1984; Sathananthan et al., 2006)). Electron microscopy has revealed that centrioles are composed of nine triplet microtubules and accessory proteins arranged to form a short cylinders (Vorobjev and Chentsov Yu, 1982). However, the ultrastructure, duplication, and function of the centrioles remains an area of active investigation (Jana et al., 2014). The pericentriolar material contains proteins responsible for microtubule nucleation and anchoring, including γ-tubulin, pericentrin and ninein (Dictenberg et al., 1998; Doxsey et al., 1994). The γ-tubulin ring complex, is composed of γ-tubulin subunits and two accessory proteins that form a capped ring-like structure that is thought to serve as a template for the 13 protofilament microtubule (Kollman et al., 2010; Moritz et al., 2000).

Microtubule nucleation at the centrosome is most easily visualized in re-growth experiments where the cellular microtubules are first depolymerized with drugs like colchicine, and upon drug withdrawal, microtubules grow outward from the centrosome (Frankel, 1976; Spiegelman et al., 1979). If detergent-extracted cells are provided with increasing amounts of purified tubulin, the number of microtubules nucleated by the centrosome reaches a plateau, suggesting a saturable number of defined microtubule-nucleating sites in the pericentrosomal material (Brinkley et al., 1981). Given that the minus-ends of microtubules are composed of GDP-tubulin subunits, they will readily depolymerize if

not protected; therefore, the γ-TuRC, or other minus end capping proteins, such as ninein may serve to cap and anchor the microtubules at the centrosome (Mogensen et al., 2000).

Microtubules are known to detach from the centrosomes in mitotic cells suggesting that it is a cell-cycle dependent event that may be important for the structure of the mitotic spindle and cell division (Yang et al., 2010). Detachment is also frequent in migrating cells, and prevention of microtubule release is associated with inhibition of cell migration (Abal et al., 2002). Additionally, release of microtubules from the centrosome in epithelial cells, followed by microtubule translocation and capture at apical sites, has been suggested as a mechanism by which epithelial cells re-organize their microtubules to form a predominantly-non-centrosomal apical-basal array (Keating et al., 1997; Mogensen, 1999).

In most proliferating animal cells, the centrosome-nucleated microtubules form a radial array with the plus-ends extending to the cell periphery. In contrast, in most differentiated animal cells, including epithelial cells, neuronal cells and muscle cells, as well as in most fungi and plant cells, microtubules are arranged in noncentrosomal arrays. (Bartolini and Gundersen, 2006). In addition to release from the centrosome, microtubules in these arrays may be generated either by nucleation at noncentrosomal sites, such as the Golgi (Efimov et al., 2007), or by breaking or severing of microtubules at sites distant from the centrosome by proteins such as katanin or spastin (Evans et al., 2005; McNally et al., 1996).

Different types of cells seem to have evolved different mechanisms for microtubule nucleation and organization. For example, plant cells lack centrosomes and nucleation occurs from the nuclear envelope as well as from cortical sites (Hasezawa et al., 2000; Kumagai et al., 2001; Stoppin et al., 1994). In neurons, some microtubules are nucleated at the centrosome in the cell body and initial evidence suggested that these microtubules were released or severed and transported into the axons and dendrites (Ahmad and Baas, 1995; Ahmad et al., 1999). However, more recent reports indicate that axonal extension can occur

even after centrosome ablation (Nguyen et al., 2011; Stiess et al., 2010), and suggest that noncentrosomal nucleation of microtubules occurs in the neuronal processes, both from Golgi outposts and regions devoid of Golgi (Nguyen et al., 2014; Ori-McKenney et al., 2012; Yau et al., 2014). In most, if not all of these examples of microtubule nucleation, γ-tubulin and the γ-TuRC are still responsible for nucleating microtubules. However, other proteins, such as XMAP215 or doublecortin, may also act as nucleation factors in cells (Bechstedt and Brouhard, 2012; Brouhard et al., 2008; Popov et al., 2002).

Microtubule Modifications and Microtubule Associated Proteins

Tubulin can undergo a number of types of post-translational modification (PTM), which can affect microtubule stability, motor binding and activity, as well as the binding of microtubule associated proteins or MAPs. The better-studied PTMs include detyrosination, acetylation and polyglutamylation, however, tubulin can also be polyglycylated, phosphorylated, ubiquitylated, sumoylated and palmitoylated (Magiera and Janke, 2014).

The cloning of tubulin genes revealed that most α-tubulin genes contain a carboxy-terminal tyrosine (Tyr) residue (Paturle-Lafanechere et al., 1991). This Tyr can be removed (detyrosination) by a yet unidentified enzyme and can be replaced (tyrosination) by an enzyme known as tubulin Tyr ligase (TTL) (Schroder et al., 1985). While the carboxypeptidase responsible for detyrosination is unknown, it is known to function preferentially on polymerized tubulin (Kumar and Flavin, 1981), while TTL acts on soluble tubulin dimers. Detyrosinated tubulin (also known as Glu tubulin) can also be converted to Δ2-tubulin by the removal of the exposed glutamic acid (Paturle-Lafanechere et al., 1991). Detyrosination is a marker of long-lived microtubules, however the modification acts indirectly to stabilize microtubules by decreasing the affinity of microtubule depolymerizing kinesins, such as MCAK and Kif2A (Peris et al., 2009). Additionally, there is evidence that the Δ2-modification locks the microtubule in the detyrosinated state, helping to further

stabilize them (Paturle-Lafanechere et al., 1994). Detyrosination has been shown to enhance kinesin-1 transport in neurons (Dunn et al., 2008), while it inhibits the microtubule plus-end localization of plus-tip proteins containing CAP-Gly domains (Peris et al., 2006).

The α-tubulin subunit can be acetylated on lysine 40 (K40) (L'Hernault and Rosenbaum, 1985), which is peculiar since it is positioned on the inside of the microtubule lumen, and it is unclear how this modification would influence binding of MAPs and motors. Immunostaining with an anti-K40-acetylation antibody staining reveals that certain populations of microtubules show strong acetylation, including microtubules in cilia and flagella, as well as stable populations of neuronal microtubules (Cambray-Deakin and Burgoyne, 1987; Lim et al., 1989; Piperno and Fuller, 1985). While initial observations suggested that plus-end directed kinesin-1 motors preferred acetylated microtubules, more recent reports suggest that this cannot be explained by acetylation alone (Kaul et al., 2014; Reed et al., 2006; Walter et al., 2012). Acetylation is especially abundant on old microtubules that are resistant to depolymerization and has thus been thought to promote microtubule stability. While recent work indicates that tubulin acetyl transferase has slow catalytic activity, so that acetylation acts to preferentially mark long-lived microtubules (Szyk et al., 2014), there is also evidence that acetylation stabilizes microtubules by promoting salt bridge formation between adjacent protofilaments (Cueva et al., 2012).

The carboxy-terminal tails of both α- and β-tubulin can be polyglutamylated (L'Hernault and Rosenbaum, 1985). This entails enzymatic addition of glutamate side chains onto gene-encoded glutamate residues. Polyglutamylation occurs on several sites within the tails and side chains of various lengths are generated (Edde et al., 1991). The addition of the side chains affects the charge of the tails and is believed to regulate electrostatic microtubule-MAP interactions. Polyglutamylated microtubules are preferred by the microtubule-severing enzymes katanin and spastin (Lacroix et al., 2010; Sharma et al., 2007). Interestingly, high levels of polyglutamylation are found on microtubules in centrioles,

cilia, flagella and in neurons (reviewed in (Janke et al., 2008)), suggesting that these microtubules must be shielded from severing activity, possibly by MAPs such as tau (Bonnet et al., 2001; Qiang et al., 2006).

Microtubule associated proteins or MAPs, were originally identified as proteins that co-purified with tubulin through repeated cycles of microtubule polymerization and depolymerization and were shown to stimulate microtubule assembly (for review see (Olmsted, 1986)). However, this definition has now broadened to include proteins that are found at least transiently associated with microtubules. A number of diverse types of MAPs can be found in eukaryotes, including structural MAPs, centrosome-associated proteins, end binding proteins, and enzymatically active MAPs such as severing proteins and microtubule motors. A number of enzymatically active MAPs and centrosome-associated proteins, such as katanin, spastin, pericentrin, and ninein, were introduced in the preceding sections, and microtubule motors will be discussed in the subsequent section.

Structural MAPs, such as MAP1, MAP2, MAP4 and tau, are classical microtubule associated proteins in that they bind to, stabilize, and promote the assembly of microtubules (Mandelkow and Mandelkow, 1995). MAP1A are MAP1B are predominantly expressed in neurons, where they are thought to be important in stabilizing microtubules for the formation and development of axons and dendrites. MAP1 proteins can also interact with other cellular components, including filamentous actin and a number of signaling proteins (Halpain and Dehmelt, 2006). Although they have different microtubule interaction motifs from the MAP1 proteins, the MAP2/tau family, which also includes MAP4, all have a conserved carboxy-terminal domain containing microtubule-binding repeats, and an amino-terminal projection domain of varying size (Dehmelt and Halpain, 2005). Mammalian MAP2 and tau are predominantly found in the brain, with tau present mainly in axons and MAP2 restricted to cell bodies and dendrites of mature neurons. MAP4 is expressed in various organs, including lung, muscle and liver (Kotani et al., 1988; Mangan and Olmsted, 1996). Although

it is also found in the brain, it is only expressed in non-neuronal cells and is absent from neurons (Parysek et al., 1985). Similar to MAP1 proteins, the MAP2/tau proteins bind to and stabilize microtubules (Gamblin et al., 1996). They also increase microtubule rigidity (Felgner et al., 1997), bundle microtubules through cross-linking (Chen et al., 1992; Takemura et al., 1995) and regulate microtubule-mediated transport (Dixit et al., 2008; Ebneth et al., 1998; Hagiwara et al., 1994; Seitz et al., 2002).

Microtubule plus-end binding proteins (+TIPs) are a structurally and functionally diverse group of proteins that predominantly accumulate at the growing microtubule plus ends. More than 20 different families of +TIPs have been identified, including the end-binding proteins (ex. EB1 and EB3), CAP-Gly domain-containing proteins (ex. CLIP170 and $p150^{Glued}$), SxIP proteins (ex. APC and STIM1) and TOG domain-containing proteins (ex. XMAP215 and CLASP) (Akhmanova and Steinmetz, 2008). A number of +TIP proteins contain features characteristic of several +TIP families and not all +TIP proteins autonomously track with growing microtubules, but hitchhike through interactions with other +TIP proteins. For example, the EB proteins track plus-ends independently of any binding partners and target other +TIPs to the growing microtubule ends (Bieling et al., 2007; Dixit et al., 2009). Enrichment at microtubule plus-ends allows +TIPs to regulate microtubule dynamics, by catalyzing growth (Brouhard et al., 2008), promoting rescue (Komarova et al., 2002), and by mediating microtubule stabilization at the cell cortex (Mimori-Kiyosue et al., 2005; Wen et al., 2004). Additionally, there is a growing appreciation of the involvement of +TIPs in many cellular processes, such as linking microtubule ends to cellular structures such as the ER (Grigoriev et al., 2008) and the cell cortex (Lansbergen et al., 2006; Schmidt et al., 2012), contributing to loading cargo for minus-end directed microtubule transport (Lomakin et al., 2009; Moughamian and Holzbaur, 2012; Moughamian et al., 2013) and promoting organization of specialized microtubule arrays (Goshima et al., 2005).

Since microtubules fulfill a wide range of functions in the cell, marking microtubules with specific post-translational modifications and decorating them with MAPs may provide a mechanism by which a cell defines a microtubule's identity and function. Therefore, current work is aimed at identifying the 'tubulin code' and defining the +TIP interaction network in the hopes of better understanding how cells use microtubules for so many diverse functions (Akhmanova and Steinmetz, 2008; Galjart, 2010; Parrotta et al., 2014; Sirajuddin et al., 2014; Vemu et al., 2014).

III. Microtubule Motors

Microtubule motors represent another family of microtubule-associated proteins. Kinesins and dyneins use polarized microtubules as railways for long-distance organelle transport in the cell. Motors are ATPases, using the energy from ATP hydrolysis to generate the force needed for their movement along the microtubule. Cargo transport by microtubule motors is essential for many cellular functions such as protein secretion, signaling, and organelle distribution, including the nucleus. Additionally, when motors are anchored to solid surfaces *in vitro* or to certain cellular structures *in vivo,* such as the cell cortex, motors actively move microtubules. Importantly, cells regulate motor function in various ways, including selective cargo binding, autoinhibition and activation mechanisms, and numbers and types of motors on a cargo. In this way, motor activity is coordinated with cellular demands which can vary greatly during cell migration, growth and differentiation, as well as changes in the cellular environment.

The Kinesin Family of Motors

The first kinesin motor to be identified, conventional kinesin or kinesin-1, was purified from preparations of cytoplasm from the giant axons of squid neurons (Lasek and Brady, 1985; Vale et al., 1985a). From these studies, it was determined that kinesin-1, which was also identified in bovine and chick brain, moves toward the microtubule plus-end in an ATP-dependent manner (Brady, 1985; Vale et al., 1985a; Vale et al., 1985b; Vale et al., 1985c). Since this initial discovery, the kinesin superfamily has grown to include at least 45 mammalian KIF genes that have been categorized into 14 subfamilies (Lawrence et al., 2004; Miki et al., 2005). Kinesins can be also classified into 3 major groups based on their domain architecture (Hirokawa et al., 2009). Generally, kinesins with motor domains at the N-terminus, such as kinesin-1, are plus-end directed motors, whereas kinesins with motor domains at the C-terminus, such as kinesin-14 family members, move toward the minus-end of microtubules. Additionally, the kinesin-13 family of motors, including the mitotic centromere-associated kinesin (MCAK), have motor domains in the center of the protein and depolymerize microtubules (Moores and Milligan, 2006).

Most kinesins contain coiled-coil segments that allow for oligomerization. Although many of the motors exist only as homodimers, Kinesin-1 motors can also form heterotetramers with 2 kinesin heavy chains (KHC) and 2 kinesin light chains (KLC). Additionally, some Kinesin-2 motors can form heterotrimers of Kif3A, Kif3B and kinesin-associated protein (Cole et al., 1993). Kinesin-3 motors can exist as monomers or homodimers and the Kinesin-5 family consists of homotetrameric motors that can cross-link and slide adjacent microtubules (Verhey and Hammond, 2009). In addition to their motor domain and the coiled-coil segments, kinesins contain unique non-motor domains that confer isoform-specific regulatory and/or functional properties (Hirokawa et al., 2009). The diversity of kinesin motors, together with the scaffolding and adaptor proteins they interact with, provides the cell with control over specificity of cargo recognition and transport regulation.

The motor domain, or head, of the kinesin motors is well conserved among various kinesins. Each head contains a binding site for the microtubule and a binding site for ATP. As the kinesin-1 motor walks in a hand-over-hand manner, it moves along a single microtubule protofilament in uniform steps of 8 nm, coupled to the hydrolysis of one ATP molecule (Hua et al., 1997; Schnitzer and Block, 1997; Svoboda et al., 1993; Wang et al., 1995). This stepping mechanism requires tight coupling of the biochemical cycles of both heads so that the front head remains bound to the microtubule while the rear head detaches. Binding of ATP to the lead head results in a conformational change in the motor domain that moves the detached head forward. This occurs by docking of the ~15 amino acid neck linker region on the motor domain in a position that extends toward the plus-end of the microtubule (Case et al., 2000; Rice et al., 1999). Strain between the two heads caused by this conformational change in the neck linker region leads to coupling of their ATPase cycles, allowing coordinated stepping and processive motility (Yildiz et al., 2008). The velocity at which a kinesin moves is directly correlated with its rate of ATP hydrolysis, and single molecule experiments indicate that this can vary for different families of kinesin motors (Friel and Howard, 2012).

Conventional Kinesin or Kinesin-1

Kinesin-1 drives nuclear movement and positioning in a number of cell systems as will be discussed further below. Kinesin-1 is composed of a dimer of kinesin heavy chains, of which there are three subtypes in mammals: Kif5A, Kif5B and Kif5C. Kif5B is expressed ubiquitously, whereas the expression of the 5A and 5C isoforms is restricted to neurons (Kanai et al., 2000). Typically, the KHC dimer is in a complex with two light chains, which serve as cargo adaptors and aid in regulation of motor activity (Figure 1.1). Four different KLC isoforms have been identified (KLC1-4), but kinesin-1 tetramers contain identical light chains (Gyoeva et al., 2004). Single kinesin-1 motors generate ~5-7 pN of force *in vitro*

(Svoboda and Block, 1994; Visscher et al., 1999) and have typical run lengths of ~1 μm (Kaul et al., 2014; Verbrugge et al., 2009), properties which make them very effective for long-distance transport in the cell. Evidence suggests that even when multiple motors are bound to small cargoes, teams of kinesin-1 are capable of producing much higher forces than a single kinesin (~17 pN for 2 motors), but on average detachment forces are similar to cargoes with a single kinesin, suggesting that kinesin-1 motors don't typically work together to move small cargoes (Jamison et al., 2010). However, at high applied loads, teams of kinesin-1 share the load to a greater degree (Jamison et al., 2011), suggesting that kinesin-1 motors may effectively cooperate to move large cellular cargoes.

Kinesin-1 was originally identified as a transport motor within extended neuronal processes, where it is important for anterograde (away from the cell body) transport of many cargoes including endosomes, RNA granules, mitochondria and synaptic vesicle precursors, however, the ubiquitous Kif5B isoform is involved in similar trafficking functions throughout the body (Hirokawa et al., 2009). Kinesin-1 associates with cargo either directly or through scaffolding/adaptor proteins present on vesicular cargoes (Fu and Holzbaur, 2014; Gindhart et al., 2003). These cargo or adaptor proteins bind either to the tail domain of kinesin heavy chain or through interactions with kinesin light chain. For example, kinesin-1 associates through its tail domain with the adaptor protein syntabulin to link the motor to synaptic membrane precursors (Su et al., 2004), and also binds TRAK1 (trafficking protein kinesin binding, also known as GRIF-1; and Milton in *Drosophila*) or Ran-binding protein 2 (RanBP2) to connect to mitochondria (Cho et al., 2007; Smith et al., 2006). Alternatively, cargoes may associate with KLCs, which contain N-terminal heptad repeats that mediate binding to KHC stalk domain and interact with cargoes via the tetratricopeptide repeat (TPR) domain in the C-terminus. KLC-interacting proteins include the tubulin binding protein CRMP-2, which is required for tubulin movement in axons and neurite elongation (Kimura et al., 2005), and the vaccinia integral membrane protein A36, which binds KLC for kinesin-1-dependent transport

of newly formed virus particles to the cell periphery (Rietdorf et al., 2001). Another KLC-binding protein is the scaffolding protein JNK-interacting protein 1 (JIP1), which binds to the TPR domain through the last 11 amino acids at the JIP1 c-terminus (Verhey et al., 2001). JIP1 also interacts directly with kinesin heavy chain, and this complex is required for amyloid precursor protein (APP) transport in neurons (Fu and Holzbaur, 2013).

Interestingly, a number of proteins that bind the TPR domain of kinesin light chain have been found to contain similar tryptophan-acidic-based motifs. This was first noted in the neuronal transmembrane protein, calsyntenin-1 (also known as alcadein) where two conserved binding regions (897-KENEMDWDDS-906 and 966-ATRQLEWDDSTLSY-979) were identified (Araki et al., 2007; Konecna et al., 2006). Although mutation of the tryptophan residue in either motif reduced binding, mutating both tryptophans abolished binding to KLC *in vitro*, suggesting that one site is sufficient to mediate the interaction (Konecna et al., 2006). Similar tryptophan-acidic motifs have now been identified in other KLC binding proteins, such as the cayman ataxia protein, Caytaxin (ATCAY) (Aoyama et al., 2009), Gadkin/AP1AR (Schmidt et al., 2009), the vaccinia virus protein A36 and the SifA-kinesin interacting protein (SKIP), a host protein important in salmonella pathogenesis and lysosomal trafficking (Boucrot et al., 2005; Dodding et al., 2011). Recently, a crystal structure of the SKIP WD-peptide in complex with the TPR domain of KLC2 has revealed the binding pocket for the peptide, which is mediated by a combination of sequence specific elements and electrostatic interactions (Pernigo et al., 2013). Furthermore, a bioinformatic analysis found that similar bipartite WD-motifs are widespread throughout the human genome, implying that many KLC binding partners have yet to be discovered (Dodding et al., 2011). In the WD-containing KLC binding proteins examined thus far, single WD motifs can bind KLC and support transport, suggesting that the list of possible KLC-binding partners may be even larger than the initial search indicated. It is also important to note that not all known KLC binding proteins contain WD-motifs.

In the cell, kinesin-1 activity must be tightly regulated so as to prevent accumulation of the motor along microtubules and at the cell periphery as well as wasteful ATP hydrolysis in the absence of cargo. Kinesin-1, as well as other kinesins, achieves this regulation in part through autoinhibition. Kinesin-1 maintains a folded, inactive conformation that is mediated by a flexible hinge region in the stalk domain and association of the highly basic IAK motif in the tail domain with the cleft between the two motor heads of the KHC dimer (Coy et al., 1999; Dietrich et al., 2008; Friedman and Vale, 1999; Hackney et al., 2009; Kaan et al., 2011; Stock et al., 1999; Verhey et al., 1998)(Figure 1.1). Full-length KHC containing the autoinhibitory IAK motif has weak affinity for the microtubule and pronounced inhibition of its ATPase activity compared to the truncated motor construct, likely because IAK binding prevents ADP release (Hackney and Stock, 2000; Kaan et al., 2011). Additionally, there is evidence that KLC can also function to inhibit the motor activity of KHC. Full-length KHC/KLC heterotetramers have decreased microtubule-binding compared to KHC dimers (Verhey et al., 1998) and fluorescence resonance energy transfer (FRET) studies suggest that in the inactive state, the KLC separates the KHC motor domains (Cai et al., 2007).

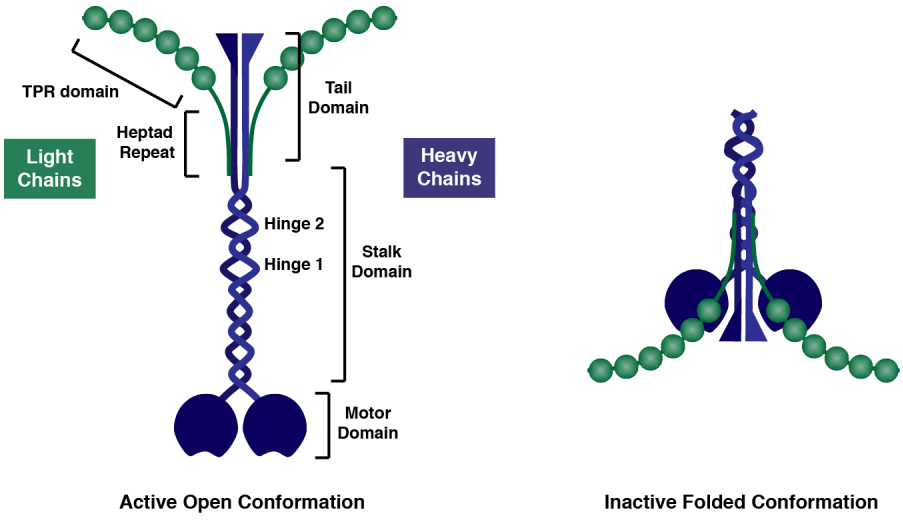

Figure 1.1. Depiction of the plus-end directed kinesin-1 microtubule motor protein.

Binding of cargo to KHC is thought to relieve autoinhibition, allowing the motor to engage with the microtubule. This has been demonstrated for recombinant KHC *in vitro* (Cho et al., 2009; Fu and Holzbaur, 2013), and *in vivo* (Blasius et al., 2007; Kawano et al., 2012). However, there is evidence that kinesin-1 motors can be inactive while attached to membrane cargo (Wozniak and Allan, 2006), suggesting that additional regulation may be needed to activate/inactivate cargo-bound motors. This may involve phosphoregulation of kinesin and/or associated adaptor complexes (De Vos et al., 2000; Fu and Holzbaur, 2013; Gindhart et al., 2003; Morfini et al., 2002; Sato-Yoshitake et al., 1992), regulation by Rab GTPases (Grigoriev et al., 2007), or calcium signaling (Macaskill et al., 2009; Wang and Schwarz, 2009).

An additional mechanism of activation that has been suggested involves the microtubule associated protein ensconsin (also known as MAP7 or E-MAP-115). Ensconsin binds the microtubule lattice through its N-terminal microtubule-binding domain and the C-terminal domain has been shown to be essential for efficient kinesin-1 transport *in vitro* and *in vivo* (Barlan et al., 2013; Sung et al., 2008). This effect is thought to involve the relief of kinesin-1 autoinhibition by binding to ensconsin, because a hinge-less, constitutively active KHC rescues the motility defects in ensconsin-null neurons (Barlan et al., 2013). However, while binding of MAP7 to kinesin-1 has been reported (Metzger et al., 2012), this binding has yet to be shown for fly ensconsin, suggesting that the interaction is transient, or highly regulated and therefore difficult to detect (Barlan et al., 2013; Sung et al., 2008).

Cytoplasmic Dynein

Well before the discovery of kinesins, dynein was first isolated from cilia in 1965 as the potential ATPase driving microtubule sliding in the cilia of *Tetrahymena* (Gibbons and Rowe, 1965). Since then, sixteen genes in humans encoding dynein heavy chains have been identified, fourteen of which encode proteins that function within the axoneme, where

they drive coordinated beating of cilia and flagella, and one which drives retrograde intraflagellar transport (Wickstead and Gull, 2007; Yagi, 2009). The remaining gene encodes cytoplasmic dynein, which localizes throughout the cell and drives cargo transport toward the minus-ends of microtubules (Lye et al., 1987; Paschal et al., 1987; Paschal and Vallee, 1987). Cytoplasmic dynein is responsible for the bulk of the minus-end-directed cargo transport in the cell, with the exception of a few minus-end-directed kinesins. Cytoplasmic dynein function is essential in animals, primarily because dynein is needed for proper cell division and development (Gepner et al., 1996; Robinson et al., 1999; Vaisberg et al., 1993). In contrast, plants lack cytoplasmic dynein and instead rely on minus-end-directed kinesins (Wickstead and Gull, 2007).

Cytoplasmic dynein (referred to as dynein going forward) is a large 1.6 MDa complex of proteins that includes two heavy chains (DHC), which contain an N-terminal tail domain, AAA ATPase motor domain and microtubule-binding domain. The tail domain promotes dimerization of DHC and provides a scaffold for the association of the additional subunits in the complex. These subunits include two intermediate chains (DIC) that bind to the tail of DHC, two light intermediate chains (LIC) that bind independently to DHC and three different light chains (Tctex1, LC8 and LC7/roadblock) that bind to the intermediate chains. In vertebrates, these intermediate and light chains are each encoded by two genes and are subject to alternative splicing and phosphoregulation, which has been suggested to provide regulation of cargo recognition (Pfister et al., 2006).

Dynein is a member of the AAA+ (ATPase associated with various cellular activities) family of proteins, which are large, ring-like hexamers of ATPase domains that function to unfold proteins, dismantle DNA and RNA and take apart macromolecular complexes and aggregates (Neuwald et al., 1999). The DHC motor domain includes the six AAA+ modules that make up the ring, a linker domain that stretches over the ring, and a microtubule-binding domain (Burgess et al., 2003; Gee et al., 1997; Roberts et al., 2009). Only the first four AAA

modules contain functional nucleotide binding motifs for ATP, with evidence for AAA1 being the main site of ATP hydrolysis (Kon et al., 2004). The microtubule binding domain consists of a small globular domain at the end of a 10 nm antiparallel coiled-coil stalk domain that extends out from between AAA4 and AAA5 (Gee et al., 1997; Kon et al., 2011). Near its base, the stalk interacts with a coiled-coil region known as the buttress (or strut) that emerges from AAA5 (Carter et al., 2011; Kon et al., 2011)(Figure 1.2).

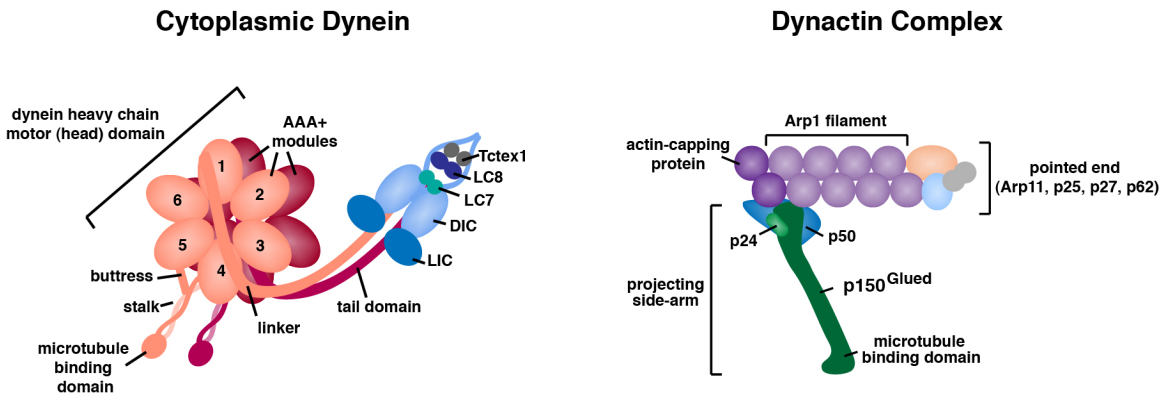

Figure 1.2. Depiction of the minus-end directed cytoplasmic dynein motor and its cofactor dynactin.

While the mechanical details of how the ATPase cycle transmits force over the large distance from the ring to the microtubule-binding domain are still emerging, current evidence suggests that conformational changes in the ring during ATP hydrolysis are transmitted to the stalk domain through the buttress (Carter et al., 2011). Sliding within the stalk domain may then couple the ATP-induced conformational changes to changes in microtubule binding affinity (Gibbons et al., 2005; Kon et al., 2009). More generally, ATP binding to AAA1 results in conformation changes in the ring, buttress and stalk that induce dissociation of the motor from the microtubule. The linker region then undergoes a rearrangement, priming it for the subsequent powerstroke, and increasing the search range of the microtubule-binding domain

along the microtubule. Re-binding of the motor to the microtubule stimulates release of phosphate (Pi), and ADP, which triggers the powerstroke – i.e. return of the linker to its original position and force generation (Burgess et al., 2003; Roberts et al., 2013; Roberts et al., 2012).

Single molecule studies of recombinant yeast dynein indicate that processive motion requires dimerization of the two DHC motor domains and that the dynein heads can take alternating steps, similar to processive kinesin motors (Reck-Peterson et al., 2006). However, although dynein predominantly takes 8-nm steps toward the minus-end of microtubules, its steps can vary in size 8-32 nm and it can also take steps toward the plus-end and sideways onto adjacent protofilaments (DeWitt et al., 2012; Gennerich et al., 2007; Mallik et al., 2004; Qiu et al., 2012; Reck-Peterson et al., 2006). Although coordination of stepping is not as tightly coupled as it is in kinesin motors, the heads are better coordinated as the distance between the motor domains increases and may be due to strain transmission between the two heads (Gennerich et al., 2007; Gennerich and Vale, 2009; Qiu et al., 2012). This flexibility in stepping may allow dynein to efficiently by-pass MAPs or other obstacles along the microtubule track (Dixit et al., 2008). While individual mammalian dynein motors are weak, stalling at only ~1 pN of force *in vitro* (Mallik et al., 2004; Schroeder et al., 2010), most dynein cargoes in the cell likely have multiple motors functioning in teams to generate higher forces for cargo transport (Hendricks et al., 2012; Hendricks et al., 2010; Welte et al., 1998).

As the predominant minus-end-directed microtubule motor in the cell, dynein is responsible for the transport of many organelles, including, but not limited to, endosomes (Driskell et al., 2007), autophagosomes (Maday and Holzbaur, 2012), lipid droplets (Gross et al., 2002), and ER-to-Golgi vesicles (Presley et al., 1997). This transport is not only important for ubiquitous organelle positioning and protein trafficking needs in the cell, but is also important for signaling in neurons (Heerssen et al., 2004; Perlson et al., 2009), efficient

release of lytic granules at the immunological synapse by lymphocytes (Mentlik et al., 2010), and is used by viruses for establishment of infection (Bremner et al., 2009). Furthermore, when anchored on cellular structures or the cell cortex, dynein can exert tension on microtubules (Hendricks et al., 2012). For example, during cell division, dynein pulls on astral microtubules emanating from the spindle poles to position the spindle properly (Collins et al., 2012) and in fibroblasts dynein acts from the leading edge to, pull microtubules and orient the centrosome (Dujardin et al., 2003). Dynein also has roles focusing the minus ends of microtubules at the spindle poles in the mitotic spindle (Merdes et al., 1996) and acts at the kinetochore to maintain spindle-kinetochore attachments, remove spindle assembly checkpoint proteins and drive rapid poleward chromosome movement (Foley and Kapoor, 2013).

To perform most, if not all, of these roles, dynein functions with an activating factor known as dynactin (Figure 1.2). Dynactin was originally identified in preparations of dynein from rat liver and testis (Collins and Vallee, 1989) and was shown to activate dynein *in vitro* (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin is a large ~1.2 MDa complex of proteins that contains 11 subunits, and can be structurally divided into two parts, the Arp1 rod and the projecting side-arm (Schafer et al., 1994; Schroer, 2004). The side-arm of dynactin contains the largest subunit of the complex, $p150^{Glued}$, which not only mediates the interaction of dynactin with dynein, via dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), but it also interacts with microtubules directly through its N-terminal microtubule-binding domain (Waterman-Storer et al., 1995). Evidence suggests that microtubule binding by $p150^{Glued}$ enhances the recruitment of dynein to the microtubule (Ayloo et al., 2014) and is necessary for the efficient initiation of retrograde vesicular transport in neurons (Lloyd et al., 2012; Moughamian and Holzbaur, 2012). Additionally, $p150^{Glued}$ may increase the processivity of dynein following initiation of transport (Culver-Hanlon et al., 2006; King and Schroer, 2000). The C-terminal domain of $p150^{Glued}$ contains a

cargo-binding domain that binds various vesicular adaptor proteins such as Rab7-interacting lysosomal protein (RILP)(Johansson et al., 2007), huntingtin-associated protein 1 (HAP1)(Engelender et al., 1997), and JIP1 (Fu and Holzbaur, 2013). Additionally, the Arp1 rod of dynactin links to cargo. For example, the Arp1 subunit directly interacts with βIII-spectrin, which is found on the cytoplasmic surface of Golgi and other organelle membranes (Holleran et al., 2001; Holleran et al., 1996). However, not all cargo binding occurs through dynactin, many cargoes have also been shown to bind dynein directly through the intermediate, light intermediate and light chains (Allan, 2011).

In the cell, dynein also interacts with a number of other activating complexes. These include complexes of LIS1 (lissencephaly 1) and nuclear distribution protein E (NuDE) or NuDE-like (NuDEL). Loss-of-function mutations in these proteins result in mitosis defects in neuronal precursors and defects in neuronal migration during brain development, resulting in severe malformation of the brain cortex (Dobyns et al., 1993; Shu et al., 2004). Studies in fungi and metazoans have shown that these proteins are crucial to a number of dynein functions, including organelle transport and nuclear positioning (Lee et al., 2003; Liang et al., 2004; McKenney et al., 2010; Smith et al., 2000; Tanaka et al., 2004; Tsai et al., 2007; Xiang et al., 1994). Despite extensive investigation, the specific mechanisms by which these proteins function to regulate dynein activity are still unclear, but may involve regulation of dynein localization and/or ATPase activity (for review see (Kardon and Vale, 2009)).

Bicaudal D (BICD) is another dynein adaptor protein, but it is specific to metazoans. In *Drosophila*, BICD is involved in dynein-mediated mRNA localization during oogenesis and embryonic development (Bullock and Ish-Horowicz, 2001; Dienstbier et al., 2009; Swan et al., 1999). The mammalian homologues BICD1 and BICD2 recruit dynein for the transport of Golgi vesicles (Hoogenraad et al., 2001; Matanis et al., 2002), and bind RanBP2, a component of nuclear pore complexes, to localize dynein to the nuclear envelope (Splinter et

al., 2010). In addition to serving as a cargo adaptor, BICD2 may also enhance dynein processivity (Matanis et al., 2002; McKenney et al., 2014; Schlager et al., 2014).

Importantly, many cargoes in the cell have both minus-end directed dynein and plus-end directed kinesin motors attached simultaneously and undergo bidirectional movements along microtubules. These opposing motors may either undergo a stochastic tug-of-war (Hendricks et al., 2010; Muller et al., 2008), or cargo-based scaffolding proteins may more specifically regulate the activity of motors on the cargo (Fu and Holzbaur, 2014). These mechanisms are not mutually exclusive, and the strategy employed is likely to be cargo-specific.

IV. The Role of the Cytoskeleton and Molecular Motors in Nuclear Positioning

Microtubule-Mediated Nuclear Positioning

Nuclear positioning in many cell types involves the microtubule cytoskeleton. However, cells have evolved to utilize a number of different microtubule-based strategies for nuclear movement. Generally, these mechanisms fall into two major categories: MTOC-dependent nuclear positioning and MTOC-independent mechanisms. In MTOC-dependent movement, the microtubule organizing center is either embedded in the nuclear envelope (yeast spindle pole body) or tightly associated with it (the centrosome in animal cells) and the position of the nucleus follows the position of the MTOC. Conversely, if a nucleus is not tightly coupled to an MTOC, nuclei may track along microtubules, similar to how smaller organelles move along the microtubule network (Reinsch and Gonczy, 1998). Typically, both of these mechanisms involve the microtubule motor proteins, cytoplasmic dynein and/or the kinesin family of motors, yet non-motor microtubule-based mechanisms have also been described. Furthermore, a cell will often use more than one mechanism to ensure correct

nuclear positioning. For additional reviews, see (Dupin and Etienne-Manneville, 2011; Gundersen and Worman, 2013).

As discussed above, the MTOC serves to nucleate and anchor the minus-ends of microtubules and is typically embedded in or closely associated with the nuclear membrane (For review see (Kellogg et al., 1994)). In a number of systems, the centrosome can be positioned properly even when the nucleus is absent or its association to the nucleus is lost (Manes and Barbieri, 1977; Raff and Glover, 1988). This suggests that the MTOC normally determines the position of the nucleus in these cells. Experiments have also demonstrated that an intact, dynamic microtubule cytoskeleton is important for this MTOC positioning, as treatment with microtubule depolymerizing or stabilizing drugs prevents centrosome movement (Schatten and Schatten, 1981).

Positioning of the MTOC is an active process, with force generated by microtubules associating with cortical or cytoplasmic sites. In the simplest mechanism, forces resulting from microtubule polymerization and pushing on the fixed cell cortex can position the centrosome at the cell center, similar to how an aster composed of pure tubulin centers itself in a microfabricated chamber (Holy et al., 1997)(Figure 1.3A). For example, in fission yeast, interphase microtubules emanating from the spindle pole body form bundles arranged along the long axis of the cell and as growing microtubules interact with the cell cortex, transient pushing forces are generated that maintain the nucleus in the middle of the cell (Tran et al., 2001). This mechanism has also been proposed to move the male pronucleus to the center of sea urchin, sand dollar and *Xenopus* eggs following fertilization, however, because the force that can be exerted by a microtubule decreases with increasing length due to buckling, it is likely that microtubule pushing is not the sole mechanism positioning the centrosome in these large cells (*Xenopus* eggs are 1mm in diameter) (Dogterom et al., 2005; Reinsch and Gonczy, 1998).

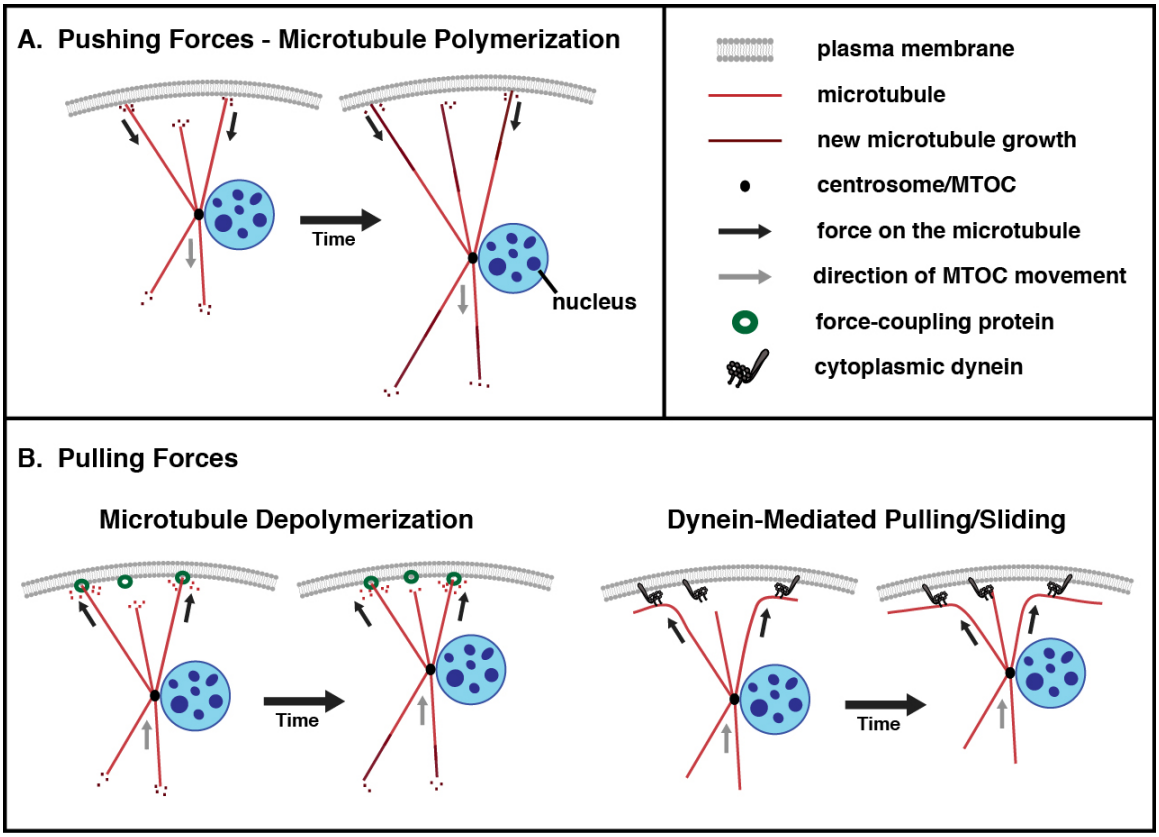

Figure 1.3. Force generating mechanisms driving MTOC-dependent nuclear movement

(A) Forces generated by microtubule polymerization can push the centrosome and attached nucleus away from the cell cortex. (B) Microtubule depolymerization forces (when coupled to proteins on the membrane), or dynein-mediated forces can pull the centrosome and nucleus toward the cell cortex.

Alternatively, microtubule pulling forces can contribute to MTOC and nuclear positioning (Figure 1.3B). In this strategy, microtubule plus-ends are captured at specific sites on the cortex, typically by cytoplasmic dynein motors, and as the motors attempt to move to the minus-end of microtubules, a pulling force is transmitted to the microtubule and the attached centrosome or spindle pole. Microtubule pulling forces can also result from

depolymerization of the microtubule itself (Grishchuk et al., 2005), and provided that a persistent linkage is present between the cortex and the shrinking microtubule, would also result in force transmission to the MTOC (Dogterom et al., 2005). An example of microtubule pulling occurs during mitosis in budding yeast, where the nucleus is moved into the bud-neck prior to spindle elongation and nuclear division, ensuring the appropriate distribution of genetic material to mother and daughter cells. Nuclear migration is mediated by astral microtubules which emanate from the spindle pole body; pulling forces are generated by microtubule depolymerization mediated by end-on contacts involving actin and cortical protein complexes (reviewed in (Pearson and Bloom, 2004; Yamamoto and Hiraoka, 2003)), and by dynein motors acting from the bud cell cortex to slide microtubules (Adames and Cooper, 2000; Yeh et al., 1995). To maintain the nucleus at the bud neck, antagonistic pulling forces must also applied to the astral microtubules contacting the cortex in the mother to balance the force.

Microtubule pulling forces are also responsible for positioning of mitotic spindles in early C. elegans embryos (Gonczy et al., 1999), and eukaryotic cells (O'Connell and Wang, 2000), as well as positioning of the centrosome in both migrating and non-migrating mammalian cells during interphase (Burakov et al., 2003; Koonce et al., 1999; Levy and Holzbaur, 2008). In these cells, cortically-anchored dynein motors are believed to be the predominant force driving microtubule pulling, though there may also be contributions by the acto-myosin network acting on microtubules as well (Rosenblatt et al., 2004).

Dynein-mediated microtubule pulling mechanisms also play a role in nuclear positioning in the hyphae of multinucleated filamentous fungi, including *Aspergillus nidulans*, *Neurospora crassa*, and *Ashbya gossypii*. In growing hyphae, nuclei migrate towards the growing tip and distribute evenly along the cell length. Though there are some differences between species, in general, an autonomous microtubule network emanates from the spindle pole body associated with each nucleus and dynein pulls nuclei by sliding microtubules along

the cell cortex (Yamamoto and Hiraoka, 2003). Careful observations of nuclear movements in *Ashbya gossypii* hyphae indicate bidirectional movements of nuclei, rotation of nuclei, as well as nuclear bypassing events (Grava et al., 2011; Lang et al., 2010). Almost all of these movements are led by the spindle pole body which is embedded in the nuclear envelope (Lang et al., 2010), and pulling on cytoplasmic microtubules by dynein is necessary to prevent nuclear aggregation (Grava et al., 2011). Interestingly, this microtubule-dependent nuclear movement is superimposed on bulk cytoplasmic flow resulting from osmotic pressure gradients, as nuclear translocation still occurs when microtubules are depolymerized (Lang et al., 2010).

In contrast to microtubule pushing and pulling mechanisms, nuclear tracking along microtubules occurs in cells in which the nucleus is not tightly coupled to the MTOC. Microtubule motors decorating the nuclear surface traffic the nuclei along microtubules in an analogous manner to smaller organelles (Figure 1.4). For example, in newly fertilized eggs of many species, the female pronucleus lacks both a centrosome and microtubule-nucleating activity. However, cytoplasmic dynein motors on the nuclear envelope move the female pronucleus toward the minus-ends of microtubules located at the centrosome associated with male pronucleus (Payne et al., 2003; Reinsch and Karsenti, 1997; Rouviere et al., 1994)(Figure 1.4A). Dynein is also responsible for nuclear movements in migrating neurons during development of the vertebrate central nervous system. Following extension of the leading process and movement of the centrosome forward, dynein acts from the nuclear surface to walk the nucleus into the leading process toward the centrosome along a cage of polarized microtubules (Rivas and Hatten, 1995; Shu et al., 2004; Tsai et al., 2007; Tsai and Gleeson, 2005).

In other systems, plus-end directed kinesin motors drive nuclear movements along microtubules (Figure 1.4B). For instance, the nuclei in *C.elegans* embryonic hypodermal precursor cells migrate toward the plus-ends of polarized microtubule bundles by the action

of kinesin-1 motors. Dynein is also present on these nuclei, and is responsible for back-steps or rotation, allowing more efficient movement of the nuclei around cytoplasmic organelles (Fridolfsson and Starr, 2010). Kinesin-3 (Kif1a) motors and dynein are also responsible for nuclear movements in neuroepithelial radial glial cells, which serve as neuronal precursors in vertebrate nervous system development. The centrosome remains at the apical cell surface while the nuclei move along the apico-basal axis in a cell-cycle dependent manner. The kinesin-3 motor, Kif1a, is implicated in the basal, plus-end directed nuclear movement and dynein drives the apical, minus-end directed movement along the polarized microtubules that extend the length of the cell (Tsai et al., 2010).

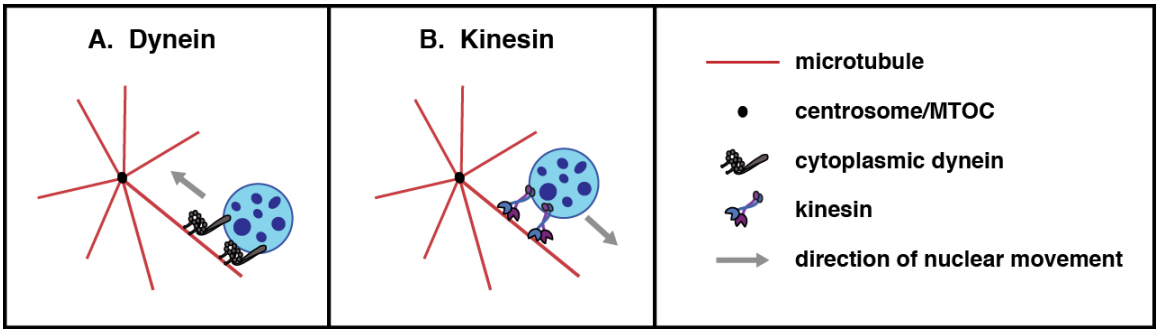

Figure 1.4. Nuclear-bound motors drive nuclear movement along microtubules. (A) Cytoplasmic dynein transports the nucleus toward the centrosome. (B) Kinesin motors move nuclei away from the centrosome toward the plus-end of the microtubule.

While the two general mechanisms of nuclear movement and positioning can be described independently, MTOC-dependent nuclear movement is often linked to microtubule-tracking mechanisms. This is because the centrosome, unlike the spindle pole body in yeast, is not embedded in the nuclear envelope and its tight association to the nucleus is often maintained by mechanisms analogous to the microtubule-tracking mechanisms described above. This was first described in experiments in sea urchin

embryos in which the centrosomes and nuclei were forcibly separated by centrifuging the embryos in the presence of low dose colcemid to depolymerize the microtubules. Upon inactivation of the drug, the nuclei were observed to track along newly polymerized microtubules and resume a tight association with the centrosome (Aronson, 1971). Similar nuclear tracking has been observed in proliferating cells following reformation of the nuclear envelope at the end of mitosis (Murray et al., 1996).

Given that microtubule minus ends are embedded in the centrosome, minus-end directed activity of dynein motors from the nuclear surface would result in a pulling force that brings the nucleus and the centrosome toward one another. Consistent with this idea, acute inhibition of dynein in U2OS cells by injection of function-blocking antibodies leads to an almost immediate separation of the nucleus and the centrosomes (Splinter et al., 2010). Interestingly, additional inhibition of kinesin-1 prevents this separation, suggesting that kinesin-1 provides the force that drives the nucleus away from the centrosome (Splinter et al., 2010). In *C. elegans*, dynein interacts with the outer nuclear envelope protein Zyg-12. Dynein pulls the centrosome and nucleus closer together, thereby establishing association of nuclear-bound Zyg-12 with a Zyg-12 splice variant localized to the centrosome, promoting a more stable attachment between the centrosome and the nucleus (Malone et al., 2003). Although Zyg-12 is not conserved, functional homologues of Zyg-12 that connect motors to the nuclear envelope in other organisms have now been identified, as will be further discussed in section V.

Though most microtubule-dependent nuclear positioning involves the mechanisms described above, a number of atypical mechanisms have also been described. For example, during Drosophila oocyte polarization, as the nucleus moves from the posterior to the anterior margin, there is a prominent indentation visible in the rear nuclear surface. This is correlated with the position of the centrosome, which is located behind the nucleus and evidence from live imaging of EB1-GFP comets suggests that growing microtubules push the

nucleus to move it to its anterior position (Zhao et al., 2012). Similar reports of microtubule-pushing on the nucleus has also been described in the follicle epithelial cells of the Drosophila pre-vitellogenic egg primordia, where pushing forces have been described to rapidly rotate or 'wriggle' the nucleus as it slowly migrates to the basal membrane (Szikora et al., 2013).

Nuclear Movements Mediated by the Actin and Intermediate Filament Networks

The actin cytoskeleton also contributes to nuclear positioning through roles in nuclear anchoring as well as by active movement of nuclei. An example of actin-based restraint of nuclear movement occurs during the rapid movement of cytoplasm from nurse cells into the oocyte during Drosophila oogenesis. The nuclei in the nurse cells are held in place by striated actin bundles extending from plasma membrane to form a cage around the nuclei (Guild et al., 1997; Gutzeit, 1986). Loss of these bundles occurs following mutation of the actin bundling proteins, villin and fascin, and results in unanchored nuclei becoming lodged in the ring canals where they prevent the flow of cytoplasm into the oocyte (Robinson and Cooley, 1997). Another example of nuclear anchoring by actin occurs in the four large syncytial hypodermal cells that cover the body of the adult *C. elegans* worm. Normally, nuclei are evenly spaced and retain this positioning as the underlying muscles contract and relax. However, mutations or displacement of the outer nuclear membrane protein ANC-1 cause a nuclear anchorage defect in which nuclei float freely in the cytoplasm and often aggregate together (Hedgecock and Thomson, 1982; Starr and Han, 2002). ANC-1 is an actin binding protein and over-expression of the actin binding domain results in a dominant negative nuclear anchorage defect, suggesting that association of the actin cytoskeleton with ANC-1 on the nuclear envelope anchors nuclei in these hypodermal cells (Starr and Han, 2002).

There are also a growing number of examples in which nuclei are actively moved through the cell by actin-dependent mechanisms. For example, in the developing CNS, contraction of the acto-myosin network at the rear of the nucleus in migrating neurons has been shown to aide the movement of the nucleus forward, working in conjunction with the microtubule/dynein-dependent process described above (Bellion et al., 2005; Schaar and McConnell, 2005; Tsai et al., 2007). Studies of nuclear positioning during polarization of cells in culture such as fibroblasts and astrocytes have shown that the actin cytoskeleton plays an active role in the rearward movement of the nucleus (Dupin et al., 2011; Gomes et al., 2005; Luxton et al., 2010). In these cells, rearward-flowing cables of actin move across the dorsal surface of the nuclear envelope, perpendicular to the direction of cell migration (Luxton et al., 2010). Because these transmembrane actin-associated nuclear (TAN) lines are attached to proteins on the outer nuclear envelope, this rearward actin flow induces nuclear movement. Movement of these TAN lines is likely driven by myosin II activity, as inhibition of the motor prevents actin flow and nuclear movement (Gomes et al., 2005). Another network of highly organized, dynamic, contractile actin filament bundles containing phosphorylated myosin have also been identified to span the dorsal surface of the nucleus in a number of cell types (Khatau et al., 2009). This perinuclear actin cap is aligned with the direction of migration, and links the nucleus to a subset of focal adhesions that are important for the mechano-sensing of matrix stiffness at the cell periphery (Kim et al., 2013). While this structure regulates nuclear shape, its role in nuclear movement is still unclear (Khatau et al., 2009). It is also not clear whether TAN lines and the perinuclear actin cap co-exist in the same cell.

While the role of the intermediate filament network in nuclear positioning is not as apparent as that of the actin and microtubule-networks, there is evidence in a number of systems to suggest that these filaments can also influence nuclear movement and anchorage. For example, MFT-16 fibroblasts, which lack vimentin, display excessive dynein-

dependent nuclear rotation, whereas restoration of the intermediate filament returns rotation levels to that of wild-type MFT-6 cells (Gerashchenko et al., 2009). Additionally there is evidence that during astrocyte cell migration, the force of actin retrograde flow at the leading edge is relayed to the nucleus by subsequent flow and asymmetric accumulation of intermediate filaments (nestin, vimentin, and GFAP) in front of the nucleus (Dupin et al., 2011). Finally, the cytolinker protein plectin, which interacts with the outer nuclear envelope protein nesprin-3, also interacts with intermediate filaments, suggesting a mechanical connection between the intermediate filament network and the nuclear envelope (Wilhelmsen et al., 2005).

In summary, nuclear positioning is an active process in which different components of the cytoskeleton act cooperatively to move and maintain the location of a nucleus. The relative contribution of the cytoskeletal elements and associated motors varies depending on cell type and can change during cell differentiation and migration. However, regardless of the specific mechanism, it is clear that all of these processes are highly dependent on interactions between the nuclear envelope and the cytoskeletal networks. As discussed in the next section, we have made significant progress over the last fifteen years in uncovering the molecular details of these interactions, which in turn has greatly aided our understanding of the mechanisms of nuclear positioning.

V. Linking the Nucleus to the Cytoskeleton

The Nuclear Envelope and the Nuclear Lamina

The nuclear envelope is a specialized extension of the endoplasmic reticulum (ER) that surrounds and compartmentalizes the genome in eukaryotic cells. It also serves to provide physical rigidity to the nucleus, plays an important role in chromatin organization and

permits only selective traffic of proteins and RNA into and out of the nucleus by passage through nuclear pore complexes (Hetzer, 2010). It is composed of two concentric bilayer membranes, the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), that are contiguous at the nuclear pore complexes. The ONM is continuous with the rough ER so that the space between the inner and outer membranes (the perinuclear space) is directly connected with the lumen of the ER (Watson, 1955). Despite the lipid continuity between the nuclear envelope and the ER, both the ONM and INM contain proteins that are not typically enriched in the ER (Schirmer and Gerace, 2005). These include the proteins of the nuclear pore complexes, the LINC (linkers of the nucleoskeleton and cytoskeleton) complex proteins that will be discussed in greater detail below, as well as a number of additional inner nuclear membrane proteins, including lamin B receptor (LBR), lamina-associated polypeptide (LAP), emerin, and MAN1, that interact with chromatin and/or the nuclear lamina (Hetzer, 2010).

The nuclear lamina underlies the inner nuclear membrane and is a meshwork of type-V intermediate filaments composed of A- and B-type lamins. Unlike other intermediate filaments, all lamins contain a nuclear localization signal and are transported into the nucleus (Lehner et al., 1986; Loewinger and McKeon, 1988; Monteiro et al., 1994). Mature B-type lamins retain a farnesyl group through which they can associate directly with the INM (for review see (Dechat et al., 2010)). While B-type lamins are ubiquitously expressed and essential for cell viability, A-type lamins are developmentally regulated, with expression in most tissues appearing only after birth (Lehner et al., 1987; Rober et al., 1989).

A major role for the nuclear lamina is to maintain nuclear shape and provide structural support. The lamina acts as a molecular shock absorber, thought to be especially critical function in tissues that are exposed to mechanical forces such as muscle (Cohen et al., 2008; Dahl et al., 2004; Panorchan et al., 2004). Additionally, nuclear lamins interact with INM proteins to maintain their localization to the NE (Foisner and Gerace, 1993; Lin et

al., 2000; Worman et al., 1988) and decrease lateral diffusion within the membrane (Ellenberg et al., 1997; Ostlund et al., 1999). There is also growing evidence that the lamina plays important in regulating chromatin position and organization, transcription and gene expression, DNA replication and possibly DNA repair (for review see (Dechat et al., 2010)). Given all these structural and regulatory roles, it is not surprising that defects in the lamina result in a myriad of human diseases, such as progeria, muscular dystrophy, cardiomyopathy and lipodystrophy (Bonne and Quijano-Roy, 2013).

The nuclear envelope and the nuclear lamina also play an important role in nuclear positioning. With the identification of SUN-KASH proteins that form bridges across the nuclear envelope came the understanding that the cytoskeleton is mechanically linked to the nucleoskeleton. Force generation by filaments and motors in the cytoplasm are transmitted across the nuclear membrane to the structural components inside the nucleus (Lombardi et al., 2011), providing the coupling needed for effective nuclear movement and anchoring.

The LINC Complex

LINC complexes (LInkers of the Nucleoskeleton and the Cytoskeleton) are composed of outer nuclear membrane KASH (Klarsicht, ANC-1 and Syne Homology) proteins and inner nuclear membrane SUN (Sad1 and UNC-84) proteins. These proteins interact in the perinuclear space to form a bridge across the nuclear envelope. LINC complexes have been found in fungi, animals and plants and connect centrosomes, actin filaments, intermediate filaments and microtubules to the surface of the nucleus (Figure 1.5).

Identification of the first SUN domain protein, UNC-84 (Malone et al., 1999), resulted from studies of *C. elegans* mutants with uncoordinated motility caused by defects in nuclear migration (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). UNC-84 localized to the nuclear envelope and the C-terminal ~175 residues showed similarity to the *S. pombe* spindle pole body protein Sad1 and to two predicted mammalian proteins which were then

named SUN1 and SUN2 (Malone et al., 1999). The conserved region was termed the SUN domain and further studies have now shown it is highly conserved across evolution. SUN domain proteins include Mps3 in *S. cerevisiae*, UNC-84 and SUN1/MTF-1 in *C. elegans*, Klaroid and Giacomo in *D. melanogaster*, and 5 mammalian proteins SUN1, SUN2, SUN3, SPAG4 and SPAG4L (Crisp et al., 2006; Jaspersen et al., 2002; Kracklauer et al., 2007; Malone et al., 1999; Shao et al., 1999).

All SUN domain proteins are conserved type-II inner nuclear membrane proteins and contain at least one transmembrane domain and a C-terminal SUN domain that localizes inside the lumen of the nuclear envelope (Starr and Fridolfsson, 2010). The recent crystal structures of human SUN2 reveals a homotrimer that resembles a three-lobed clover made of SUN domains sitting on a triple coiled-coil stalk which is suggested to span the 50 nm space between the INM and ONM (Sosa et al., 2012; Zhou et al., 2012). The structures also revealed that each SUN trimer binds three KASH peptides in the grooves between the adjacent SUN domains and that a disulfide bond between the SUN and KASH domains may further stabilize the complex (Sosa et al., 2012).

In contrast to the C-terminal SUN domain, the nucleoplasmic domains of SUN proteins are not conserved, however many interact with lamins or chromatin. While these interactions are crucial for retention of certain SUN proteins to the INM, others don't rely solely on lamins for localization. For instance, mammalian SUN1 and SUN2 both interact directly with A- and B-type lamins (Crisp et al., 2006; Haque et al., 2006), however only SUN2 mislocalizes significantly to the ER in fibroblasts lacking A-type lamins, suggesting SUN1 has additional mechanisms for INM retention (Crisp et al., 2006; Haque et al., 2006; Hasan et al., 2006). In *S. pombe*, which lacks lamins, Sad1 interacts with centromeres and heterochromatin through the heterochromatin-binding protein Ima1 (King et al., 2008). Additional work is still needed to better understand how the SUN proteins are targeted to the INM and how they connect to the nucleoskeleton.

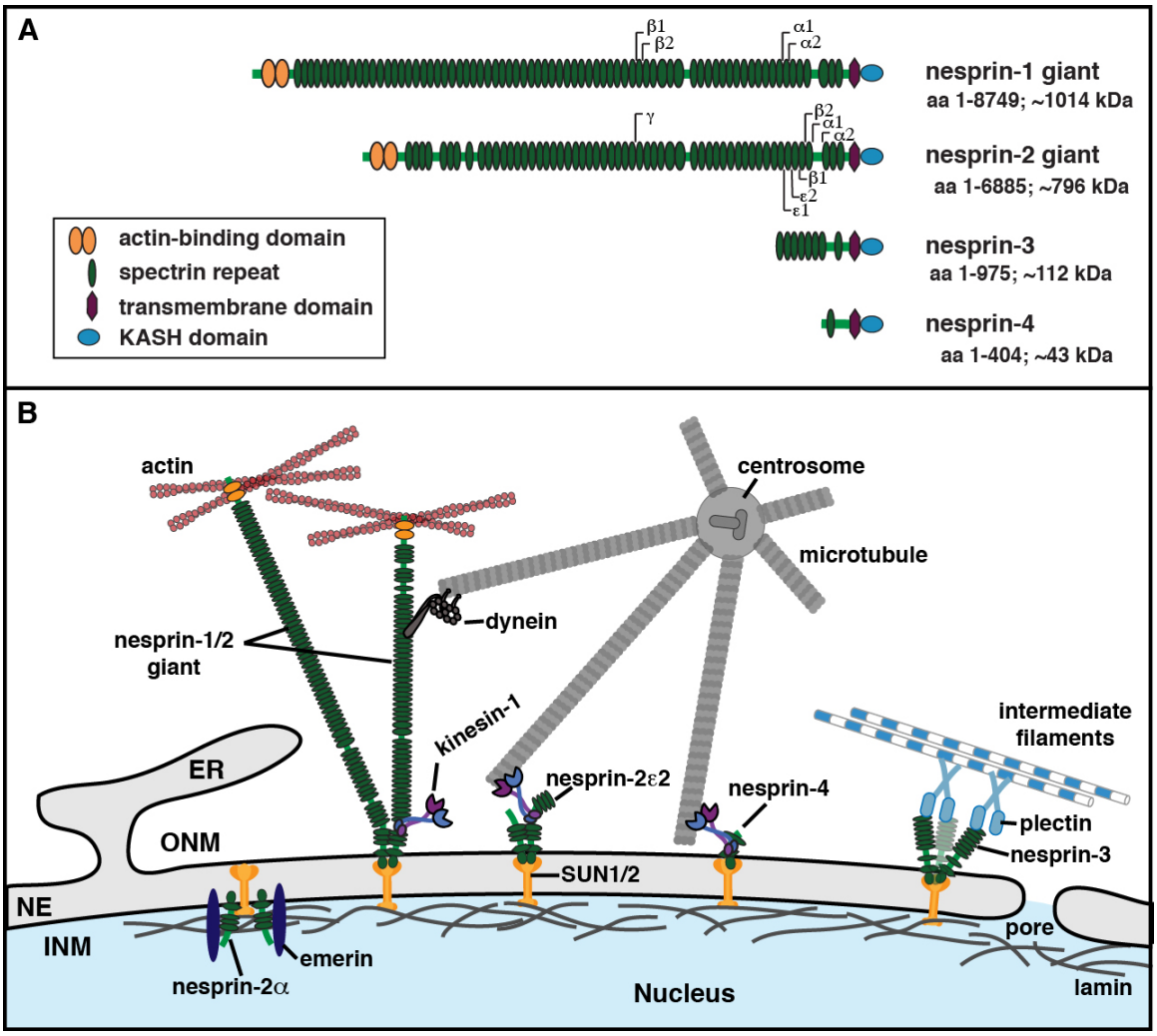

Figure 1.5. LINC complexes.

(A) Domain structure of the major nesprin isoforms in humans. Nesprin-1 and nesprin-2 are alternatively spliced to produce a number of KASH-domain containing isoforms, as well as KASH-less isoforms (not shown). (B) Illustration of LINC complexes spanning the nuclear envelope and interacting with the cytoskeleton and the nucleoskeleton. SUN proteins on the inner nuclear membrane (INM) interact with both the nuclear lamina and the KASH domain of nesprins. Nesprin-1 and -2 giant interact directly with the actin cytoskeleton, nesprin-3 binds intermediate filaments indirectly via the cytolinker plectin, and nesprin-1, and -2 as well as nesprin-4 associate with microtubule motors, including cytoplasmic dynein and kinesin-1.

The KASH family of proteins constitutes the other half of the protein bridge across the nuclear envelope (Figure 1.5). The founding members of this family include the *D. melanogaster* Klarsicht, *C. elegans* ANC-1 and mammalian Syne/Nesprin-1 and -2 proteins. Molecular cloning of ANC-1 resulted in the identification of a conserved KASH domain in each of these proteins (Starr and Han, 2002). KASH domains consist of a predicted transmembrane region followed by a stretch of ~35 amino acids at the very C-terminus of the protein. This domain is necessary and sufficient for targeting the proteins to the nuclear envelope where it interacts in the perinuclear space with the SUN domain proteins as described above (reviewed in (Starr and Fridolfsson, 2010)). While most KASH proteins reside on the outer nuclear membrane, there is evidence that some of the shorter mammalian splice isoforms of nesprin-1 and -2 can also localize to the INM (Mislow et al., 2002a; Mislow et al., 2002b; Morris and Randles, 2010; Zhang et al., 2005).

The N-terminal domains of KASH proteins, which are variable in size and domain structure, extend into the cytoplasm where they interact with a variety of cytoskeletal elements. The largest KASH proteins, *C. elegans* ANC-1, the *Drosophila* protein MSP-300, and the giant isoforms of mammalian nesprin-1 and -2 all contain a pair of calponin homology (CH) domains at the N-terminus which mediate their interaction with the actin cytoskeleton (Starr and Han, 2002; Volk, 1992; Zhen et al., 2002). MSP-300 and nesprins contain large central domains composed of spectrin repeats, similar to those found in other CH-domain containing actin binding proteins such as α-actinin and dystrophin (Rosenberg-Hasson et al., 1996; Zhang et al., 2001). These repeats are ~100 residues and fold to form highly coiled, 5 nm long triple helical bundles (Yan et al., 1993). ANC-1 also contains a large central region consisting of repetitive helical domains with short coiled-coil domains, which may function analogously to the spectrin repeats (Starr and Han, 2003). Evidence from experiments with nesprin-2 constructs lacking the central domain, suggests that the role of the extended central domain may be to physically separate the actin-binding domain from

the nuclear envelope (Zhen et al., 2002). An alternative hypothesis is that these giant nesprins form a spectrin-like filamentous lattice-like basket around the nucleus that provides support for the ONM (Schneider et al., 2008). Support for this model is based on evidence that the spectrin repeats of nesprins dimerize (Mislow et al., 2002a) and that the CH domains of nesprins -1 and -2 can also interact with nesprin-3 (Lu et al., 2012). Most of the available evidence indicates that the actin-binding KASH proteins serve to anchor nuclei in place in cells (Starr and Han, 2002; Zhang et al., 2007). However, nesprin-2 giant connects TAN lines to the nuclear envelope, suggesting that KASH proteins may also be important in actin-based mechanisms of nuclear movement as well (Luxton et al., 2010).

A number of KASH proteins have also been shown to interact indirectly with the microtubule network through microtubule motor proteins. For example, the *C. elegans* protein UNC-83, binds the kinesin-1 motor through an interaction with kinesin light chain-2 (Meyerzon et al., 2009), and also interacts with two separate dynein-regulating complexes (BICD1 and NUD2) to recruit the minus-end directed motor to the nuclear envelope. Together these motors drive nuclear migration in embryonic hypodermal precursor cells (Fridolfsson et al., 2010; Fridolfsson and Starr, 2010). In the fly, dynein is thought to interact with the KASH protein Klarsicht to facilitate nuclear migration in developing photoreceptor cells (Mosley-Bishop et al., 1999; Welte et al., 1998). In the mouse, nesprins in complex with either SUN1 or SUN2 are necessary for brain and retinal development by mediating both interkinetic nuclear migration as well as nucleokinesis during neuronal cell migration in the retina and the cerebral cortex. In these studies, co-immunoprecipitation data suggested both nesprin-1 and -2 bind dynein/dynactin, and that nesprin-2 can also bind kinesin-1 (Yu et al., 2011; Zhang et al., 2009). In a later study, the interaction between kinesin-1 and nesprin-2 was further defined, and shown to involve binding of the TPR domain of kinesin light chain to a region of the nesprin-2 cytoplasmic domain close to the transmembrane domain (Schneider et al., 2011). This region is present in all but the shortest splice KASH-domain-

containing isoforms of nesprin-2 (Duong et al., 2014), but it has not been examined whether nesprin-2 giant can bind actin and kinesin-1 simultaneously.

Mammalian nesprin-4 also binds kinesin light chain to bring kinesin-1 to the nuclear envelope (Roux et al., 2009). Unlike nesprin-1 and -2, which are widely expressed (Zhang et al., 2001), the tissue distribution of the much smaller nesprin-4 is mainly restricted to secretory epithelial cells and sensory epithelial cells of the inner ear (Horn et al., 2013; Roux et al., 2009). In cultured cells, introduction of nesprin-4 to the nucleus increases the distance between the nucleus and the centrosome, presumably because kinesin-1 motors move to the plus-end of centrosomal microtubules, driving the structures apart (Roux et al., 2009). In support of this hypothesis, the loss of nesprin-4 from hair cells in the cochlea results in abnormal positioning of the nuclei at the apical surface of the cell, close to the centrosome, instead of its typical localization at the basal surface of the epithelial cell (Horn et al., 2013).

Mammalian nesprin-3 interacts with the cytolinker protein plectin to connect intermediate filaments to the nuclear envelope (Ketema et al., 2007; Wilhelmsen et al., 2005). While plectin is primarily an intermediate filament binding protein, it also contains a functional actin binding domain (Andra et al., 1998) and has been shown to interact with the microtubule cytoskeleton (Koszka et al., 1985; Svitkina et al., 1996). However, the actin-binding domain of plectin mediates its association with nesprin-3α (Wilhelmsen et al., 2005), which precludes its binding to the actin network (Postel et al., 2011). In contrast, plectin binds MAP2 through its plakin domain (Valencia et al., 2013), suggesting that nesprin-3/plectin may also help link the nuclear envelope to the MT cytoskeleton independently of microtubule motors. Additionally, nesprin-3α binds the CH-domains of the neuronal and muscular isoforms of BPAG1 (bullous pemphigoid antigen 1) and well as MCAF (microtubule-actin crosslinking factor), which harbor microtubule-binding domains, again suggesting possible links between nesprin-3 and microtubules (Ketema and Sonnenberg, 2011).

KASH proteins have also been shown to connect the nucleus to the MTOC. As discussed in section IV, the *C. elegans* KASH protein Zyg-12 mediates attachment of the centrosome to the nucleus, a strategy that involves dynein and dynein-independent activities of Zyg-12 (Malone et al., 2003). Additionally, in *S. pombe,* the KASH proteins Kms1 and 2 in complex with the SUN protein Sad1 creates a physical connection between spindle pole bodies and centromeric chromatin (King et al., 2008; Miki et al., 2004).

The importance of the LINC complexes in nuclear positioning and nuclear function are highlighted by a growing number of human diseases caused by mutations in the proteins that make up these complexes. For instance, mutations in nesprin-1 have been found in patients with autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) (Zhang et al., 2007a), dilated cardiomyopathy (Puckelwartz et al., 2010), autosomal recessive cerebellar ataxia (Gros-Louis et al., 2007), autism (Yu et al., 2013), and autosomal recessive arthrogryposis (arthrogryposis multiplex congenita) (Attali et al., 2009). Mutations in nesprin-2 have been linked to Emery-Dreifuss muscular dystrophy (Zhang et al., 2007a). Furthermore, mutations in both nesprin-1 and nesprin-2 have been identified in patients with a number of types of cancer, including breast, lung, head and neck, pancreatic, ovarian, and colorectal, although it is still unclear what role nesprins are playing in these diseases (Cartwright and Karakesisoglou, 2014). Mutations resulting in truncated nesprin-4 proteins, which do not localize properly to the nuclear envelope, cause progressive high-frequency hearing loss (Horn et al., 2013). Finally, while mutations in SUN1 and SUN2 have not yet been identified as the cause of any human diseases, recent findings suggest that heterozygous mutations in SUN1 may enhance the disease phenotype of muscular dystrophy patients also bearing mutations in emerin or the lamina associated protein LAP2alpha (Li et al., 2014).

Additional Nucleus-Cytoskeleton Connections

While SUN-KASH complexes are the major bridges connecting the nucleus and the cytoskeleton, there is also evidence that the nuclear pore complex (NPC) proteins can mediate interactions as well. For example, dynein/dynactin and kinesin-1 bind the motor adaptor protein Bicaudal2 (BICD2) (Grigoriev et al., 2007; Hoogenraad et al., 2001), which in turn interacts with the nuclear pore complex protein RanBP2 (also known as NUP358) (Splinter et al., 2010). RanBP2 is a large protein that forms extended fibers at the cytoplasmic side of the NPC and acts as a docking factor in nucleocytoplasmic transport (Walde et al., 2012). The association between BICD2 and RanBP2 occurs during the G2 phase of the cell cycle, recruiting dynein motors to the nucleus to pull the centrosome and the nucleus together, with antagonistic action by recruited kinesin-1 motors. These complexes serve to prevent centrosome detachment from the nucleus during entry into mitosis (Splinter et al., 2010). The NPC protein Nup133 is also necessary for efficient anchoring of dynein/dynactin to the nuclear envelope in prophase, through a complex involving CENP-F and NudE/NudEL (Bolhy et al., 2011). This interaction is distinct from the RanBP2-BICD2 pathway and inhibition of either pathway leads to defects in centrosome-nucleus association. Indeed, evidence suggests these two pathways act sequentially to recruit dynein/dynactin in G2 to the NPC during the dynein-dependent apical migration of nuclei toward the apically located centrosome during interkinetic nuclear migration in radial glial progenitor cells in the vertebrate neocortex. Inhibition of the BICD2 pathway arrests nuclei early in migration whereas inhibition of the Nup133 pathway acts later in apical migration, however, in either case, nuclei are arrested in a premitotic state, suggesting migration is necessary for mitotic entry (Hu et al., 2013).

VI. Nuclear Positioning in Skeletal Muscle Cells

Overview

Perhaps one of the most remarkable examples of nuclear positioning in vertebrates occurs in skeletal muscle fibers. Muscle fibers are multinucleated cells formed by fusion of post-mitotic mononucleated myocytes (Capers, 1960; Cooper and Konigsberg, 1961; Lash et al., 1957; Stockdale and Holtzer, 1961). During embryogenesis, mononucleated myoblasts undergo rounds of proliferation to produce daughter myocytes that fuse to initially form multinucleated myotubes (Engel and Franzini-Armstrong, 1994), which in humans, begins around 10-13 weeks of gestation depending on the specific muscle being formed (Romero et al., 2013). As the myotube matures into an adult muscle fiber, the centrally-located nuclei move to the periphery of the cell and become anchored under the muscle cell plasma membrane, or sarcolemma. By the 20th week of development, the muscle nuclei have elongated and are evenly distributed along the length of each fiber and by ~24-26 weeks of human gestation, most fibers have peripheral nuclei (Engel and Franzini-Armstrong, 1994; Romero et al., 2013). Additionally, a subset of ~3-8 nuclei are found under the post-synaptic membrane at the neuromuscular junction (Kelly and Zacks, 1969; Nakai, 1969). These nuclei are not only larger and rounder than extrasynaptic nuclei, but they are also transcriptionally specialized for maintenance of the synapse (Brosamle and Kuffler, 1996; Cardasis, 1979; Jevsek et al., 2006; Merlie and Sanes, 1985; Sanes et al., 1991; Sanes and Lichtman, 1999).

During myogenesis in vertebrates, fusions of myoblasts with myoblasts or myoblasts with myotubes occurs asynchronously and it is unpredictable as to where along the length of a myotube a myoblast will fuse (Harris et al., 1989). The fusion process is most easily visualized in movies of myocytes in culture, where mononucleated cells can be seen to migrate, align end-to-end, and subsequently fuse. Myotubes in culture tend to elongate

through end-on fusion with myocytes or through fusion to other myotubes (Capers, 1960; Fear, 1977). Despite observations of cell fusion during myogenesis as early as the 1950's, the mechanistic details are still under active investigation (Abmayr and Pavlath, 2012). However, fusion creates myotubes that can contain hundreds of centrally localized nuclei. As the myotube matures into a muscle fiber, it grows in size from a mean diameter of ~7μm to 17-23 μm at birth in humans and the nuclei move to the periphery of the cell where they lie adjacent to the sarcolemma (Romero et al., 2013)(Figure 1.6).

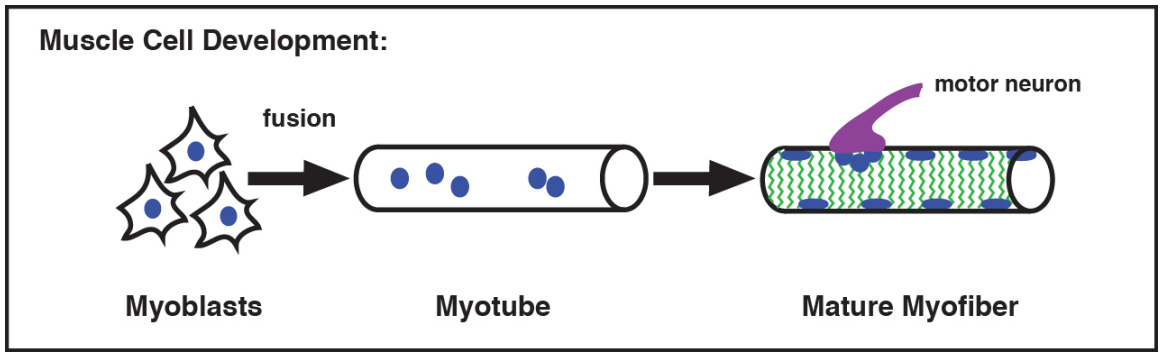

Figure 1.6. Nuclear position during the stages of muscle cell development. Mononucleated myoblasts fuse to form myotubes. The nuclei in these immature cells are mobile. As the myotubes mature into myofibers, the nuclei move to the periphery of the cell where they are anchored beneath the sarcolemma. Nuclei are regularly spaced along the myotube length to maximize internuclear distance. A noticeable exception is the cluster of 3-8 nuclei located under the neuromuscular junction.

A thorough analysis of nuclear position in the mature myofiber through microinjection of labeled DNA indicates that the distribution of nuclei is optimized to achieve the greatest average distance between nuclei. This finding was irrespective of fiber size or fiber type. This distribution resembles computer simulations based on algorithms in which the nuclei repel one another during positioning, and this optimized distribution has been hypothesized

to minimize transport distances between nuclei (Bruusgaard et al., 2003). In support of this hypothesis are data showing that while cytoplasmic and sarcolemmal proteins can spread widely throughout a myotube, Golgi proteins and contractile proteins do not range far from their nucleus of origin, suggesting that mRNA does not diffuse throughout the muscle fiber. These findings have led to the idea that muscle fibers are composed of a mosaic of domains that are largely controlled by the activity of one myonucleus (Hall and Ralston, 1989; Pavlath et al., 1989; Ralston and Hall, 1989).

While the peripheral nuclei in a mature, healthy fiber do not display appreciable mobility (Bruusgaard et al., 2003), following damage to the muscle fiber, nuclei can be once again found in center of the fiber (Lash et al., 1957; Rich and Lichtman, 1989; Schmalbruch, 1976). These centrally located nuclei are thus considered to be a marker of muscle repair. As such, elevated numbers of central nuclei in muscle biopsies are a hallmark of muscle disorders including Duchenne muscular dystrophy, Emery-Dreifuss muscular dystrophy, and the centronuclear myopathies, and it is believed that this is a consequence of the continual myofiber repair associated with these disorders (Dubowitz and Sewry, 2007). During regeneration, activated satellite cells fuse with the damaged fiber and the newly incorporated nucleus moves to the center of the cell (Yin et al., 2013). It is unclear whether the central position of this nucleus is simply a consequence of the repair process, or whether it serves an essential role in the regeneration process. However, under normal conditions, the myonuclei are returned to the periphery of the fiber (Lash et al., 1957; Li et al., 2011; Rich and Lichtman, 1989; Schmalbruch, 1976; Terada et al., 2009), such that regenerated muscles in a healthy individual are morphologically and functionally indistinguishable from undamaged muscles (Yin et al., 2013).

Together, these observations strongly suggest that nuclear positioning in skeletal muscle cells is an active and regulated process during both the development and regeneration of the muscle fiber. This is further supported by findings that mutations in

proteins important in nuclear positioning are being found in patients with muscle disorders (Mejat et al., 2009; Mejat and Misteli, 2010; Zhang et al., 2007a).

Early Observations of Myonuclear Movement

Indeed, early observations of myogenesis of chick muscle cells in culture suggested that nuclei in myotubes are highly mobile. Upon fusion, the nucleus from a mononucleated cell was reported to "rapidly incorporate into the host sarcoplasm and subsequently move about among the other nuclei" (Capers, 1960). Nuclei were described to display "rotation, rocking and linear excursion through the cytoplasm producing transitory associations of nuclei in various configurations" (Cooper and Konigsberg, 1961). Interestingly, nuclear rotation often occurred in more than one plane, in either the clockwise or counterclockwise direction and changes in the direction of rotation were observed (Capers, 1960). Nuclei often showed dramatic deformation as they rotated, changing shape from circular to oval to dumbbell-like and sometimes this deformation resulted in the production of folds in their membranes. Importantly, time-lapse phase-contrast imaging of nuclear deformation, especially the formation and resolution of nuclear folds, provided strong support against the model of amitotic nuclear division that had been proposed as a mechanism for muscle multinucleation (Capers, 1960; Cooper and Konigsberg, 1961; Weed, 1936).

Though this work was clearly important for advancing our understanding of muscle development, these intriguing observations of nuclear translocation and rotation did not receive further attention until 1987 when Englander and Rubin investigated the movement of nuclei in myotubes in relation to the formation of acetylcholine receptor (AChR) clusters on the sarcolemma (Englander and Rubin, 1987). Working both with chick and rat myotubes, these authors reported that ~85% of nuclei moved at a rate of least 3-4μm per hour, with an average rate of 13.5 μm/hour. They also noted nuclear rotation and that nuclei were able to move around or pass other nuclei. However, when myotubes were treated with extract from

Torpedo electric organ to cause clustering of AChRs, they noted that nuclei within one nuclear diameter (~8μm) of a cluster were essentially immobile. They observed nuclei stop under AChR clusters and upon cluster dispersal, once immobile nuclei resumed migration. Importantly, these authors were the first to determine that nuclear translocation and rotation were dependent on the microtubule cytoskeleton, as treatment with the microtubule-depolymerizing drug colchicine stopped nuclear movement. In contrast, blocking actin polymerization with cytochalasin D did not alter nuclear translocation or rotation (Englander and Rubin, 1987).

The microtubule cytoskeleton in muscle

During myogenesis, the microtubule cytoskeleton is reorganized from the radial, centrosomal array found in mononucleated myoblasts to a parallel linear array oriented along the long axis of myotubes (Tassin et al., 1985a; Warren, 1974)(Figure 1.7). This reorganization of microtubules is believed to underlie the changes in cell elongation associated with myogenesis as well as to allow for the proper organization of the developing myofibril cytoskeleton (Bischoff and Holtzer, 1968; Croop and Holtzer, 1975; Holtzer et al., 1985; Pizon et al., 2005; Saitoh et al., 1988; Warren, 1968). Work by Tassin et al. (1985) in human muscle cultures was the first to show redistribution of centrosomal material to the nuclear envelope shortly after myoblast fusion. Additionally, through treatment of the myotubes with nocodazole followed by wash-out, the authors clearly show re-growth of microtubules from the nuclear periphery (Tassin et al., 1985). Although their work pre-dated identification of the individual centrosomal proteins, subsequent studies have localized pericentrin, pericentriolar material protein 1 (PCM-1), γ-tubulin, and ninein to the nuclear envelope as well as to cytoplasmic foci in myotubes (Bugnard et al., 2005; Musa et al., 2003; Ralston et al., 2001; Srsen et al., 2009). Contrary to early reports, recent work indicates that redistribution of centrosomal proteins is gradual and begins prior to cell fusion in

differentiated myoblasts (Srsen et al., 2009; Zaal et al., 2011). Localization of centrosomal components to the nuclear periphery has also been described in mature cardiac myocytes (Kronebusch and Singer, 1987) as well as in isolated mouse skeletal muscle fibers (Ralston et al., 2001). More recent work suggests that microtubule nucleation from the myotube nucleus occurs ~5 times/100 sec on average, and while Golgi complex elements on the nuclear envelope are occasional sources of nucleation, most comets are nucleated from nuclear areas devoid of Golgi markers (Zaal et al., 2011).

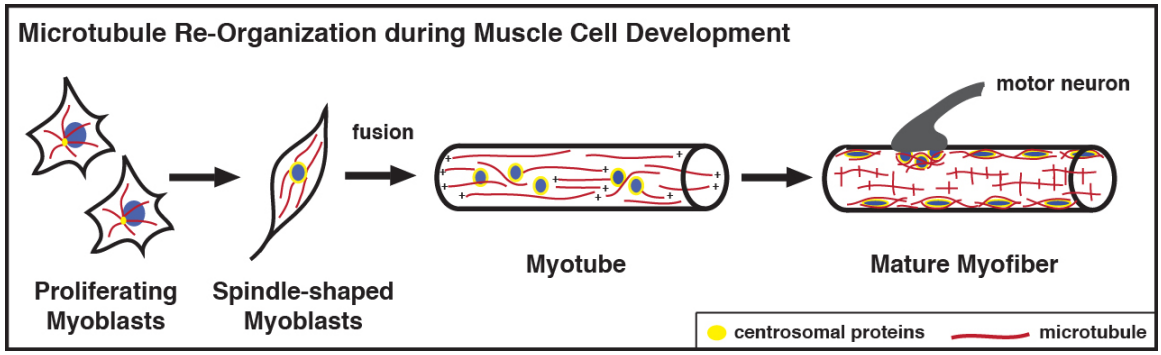

Figure 1.7. Microtubule re-organization during muscle cell development.
Microtubules form a radial in proliferating myoblasts. In differentiating myoblasts, centrosomal proteins redistribute to the nuclear envelope and microtubules re-organize to form an elongated array. In myotubes, microtubules are nucleated from the nuclear surface and form a bidirectional parallel array, with plus-ends prominent at myotube poles. Microtubules surround nuclei in mature fibers, including a dense meshwork around the subsynaptic nuclei. Microtubules are also form intricate lattice under the sarcolemma and between myofibrils. Dynamic microtubules are observed at all stages of differentiation.

The expression of fluorescently-tagged microtubule plus-end binding proteins has enhanced our understanding of microtubule organization and dynamics in myotubes. Observation of GFP-EB1 comets with confocal microscopy in mouse C57 myotubes revealed

that microtubule growth trajectories are mostly anti-parallel and aligned with the major axis of the cell (Pizon et al., 2005). Rates of microtubule growth were fairly homogenous within a given cell, but varied between myotubes (average speed = 21 ± 6.7 μm/min, range 14 – 29 μm/min). Additionally, the pole-to-pole direction of comets was not always equal, but could vary according to focal position within a myotube (Pizon et al., 2005). In stark contrast to bidirectional microtubule dynamics in the middle of the cell, the microtubules are uniformly polarized out toward the cell cortex at the ends of the myotube (Zhang et al., 2009a). My own work with GFP-EB3 in C2C12 myotubes reveals this same pattern of microtubule dynamics, however, we also note dynamic microtubules polymerizing between adjacent nuclei as well as within invaginations in the nuclear surface (Wilson and Holzbaur, 2012)(Also see Chapter 2).

The re-organization of the microtubule cytoskeleton during fusion and differentiation of myotubes is also accompanied by post-translational modifications of the microtubules (Gundersen et al., 1989). While microtubules in proliferating myoblasts are mainly tyrosinated, as the cells differentiate and elongate prior to fusion, there is an increase in the levels of detyrosinated tubulin, concomitant with an increase in microtubule stability. Microtubules in multinucleated myotubes retain high levels of Glu tubulin and at later stages of myogenesis, increases in the levels of acetylated tubulin are also noted (Gundersen et al., 1989). Interestingly, loss of EB1 from myoblasts prevents the normal increase in Glu-tubulin levels (Zhang et al., 2009a), and when induced to differentiate, myoblasts lacking EB1 or EB3 are unable to elongate or fuse to form myotubes (Straube and Merdes, 2007; Zhang et al., 2009a). Furthermore, work in cultured muscle cells and mouse embryonic tissue has also shown that levels of muscle-specific variants of the microtubule associated protein, MAP4 (mMAP4), are also upregulated early during myogenesis (Mangan and Olmsted, 1996). Loss of mMAP4 does not affect myoblast fusion, but does significantly compromise myotube formation and differentiation, causing short myotubes with disorganized

microtubules and myofibrils (Mangan and Olmsted, 1996). However, over-expression of mMAP4 is not sufficient to induce the reordering of the microtubule array that occurs during myogenesis (Casey et al., 2003). While these studies indicate that microtubule stabilization is essential for myogenesis, additional work is needed to further clarify how microtubule modifications and MAPs influence microtubule stabilization and reorganization in myotube formation.

Adult muscle fibers have a more intricate microtubule lattice that is subsarcolemmal, perinuclear and found within the intermyofibrillar spaces (Boudriau et al., 1993; Ralston et al., 1999). The intermyofibrillar microtubules are mostly longitudinal but some oblique and transverse staining can also be observed (Boudriau et al., 1993). Microtubules are more abundant under the sarcolemma, and patterns of microtubules vary more clearly between fiber types. In soleus muscles (primarily type I fibers), microtubules form dense interlacing bundles around the nuclei and in long longitudinal lines between nuclei, whereas in the fast type IIB fibers of the tensor fascia latae (TFL) and extensor digitorum longus (EDL), the microtubules appear more organized. These microtubules form a more orthogonal pattern, individual microtubules are more easily noted and microtubule asters, suggesting sites of nucleation, are more obvious (Ralston et al., 1999; Ralston et al., 2001). In all fiber types microtubules are found in close association with nuclei, this is especially evident for the cluster of nuclei at the post-synaptic membrane. Additionally, prominent asters of microtubules are often evident at the poles of TFL and EDL fibers, consistent with higher pericentrin content at the nuclear poles (Bruusgaard et al., 2006; Ralston et al., 1999; Ralston et al., 2001). Changes in fiber activity can modulate the content and organization of microtubules, such that denervation of muscle fibers decreases the density of microtubule staining throughout the fibers, and stimulating fast twitch EDL fibers for 2 weeks with a 20Hz slow-twitch protocol increases the density and disorganization of subsarcolemmal microtubules (Ralston et al., 1999; Ralston et al., 2001).

Although most of our understanding of microtubule structure in adult muscle fibers is based on static immunofluorescent images from isolated fibers, very recent *in vivo* imaging of flexor digitorum brevis muscle fibers provides insight into the microtubule dynamics that support this network (Oddoux et al., 2013). Live imaging of EB3-GFP and mCherry-tubulin imaging reveals constant nucleation and growth on top of a durable microtubule frame, and superresolution microscopy suggests most tracks contain 2-4 microtubules. The authors suggest that what appears to be a static network may actually be entirely composed of dynamic microtubules, which may be better suited to managing the compressive forces associated with contraction. Additionally, they show that microtubules are nucleated at both nuclear sites as well as discrete, immobile Golgi elements that appear to be positioned along z-lines (Oddoux et al., 2013). While new microtubules grow along existing bundles, it is still unclear how these bundles obtain their orthogonal organization. However, it has been shown that microtubules interact directly with dystrophin (Prins et al., 2009) and that muscle fibers from the dystrophin-deficient mdx mouse exhibit microtubule disorganization (Percival et al., 2007). Similarly, the membrane adaptor protein ankyrin B has also been shown to bind microtubules (Bennett and Davis, 1981; Davis and Bennett, 1984) and loss of ankyrin B from muscle cells results in disruption of the subsarcolemmal microtubule organization (Ayalon et al., 2008), suggesting that dynamic microtubules may use dystrophin and ankyrin on the sarcolemma as a guide for microtubule organization.

Myonuclear positioning in Mature Muscle Fibers

Unlike nuclei in developing myotubes, nuclei in mature muscle fibers are immobile over the course of several days of observation suggesting they become anchored at the sarcolemma (Bruusgaard et al., 2003). However, changes in activity or age of the fibers can alter myonuclear distribution and shape (Bruusgaard et al., 2006; Ralston et al., 2006; Ralston et al., 1999). Denervation increases the proportion of clustered nuclei ~4-fold

(Ralston et al., 1999), and denervation combined with a chronic stimulation protocol results in marked association of EDL nuclei with newly generated blood vessels (Ralston et al., 2006). These chronic protocols also result in significant remodeling of the fibers and the muscle as a whole, suggesting that changes in nuclear positioning may be secondary to cytoskeletal changes that occur over the course of days and weeks. While these protocols alter microtubule organization as described above, they also alter organization of the desmin intermediate filament network. In denervated rat muscles, desmin redistributes from a mostly transverse staining pattern to a longitudinal and disorganized pattern with aggregation of unpolymerized desmin around nuclei (Ralston et al., 2006). Additionally, desmin-null muscle fibers have aberrant nuclear positioning, with large aggregates of nuclei interspersed with areas of very few or no nuclei. However, these nuclei are still found at the periphery of the fiber (Ralston et al., 2006). These data suggest that both microtubules and desmin are necessary for proper distribution and anchoring of nuclei in mature muscle fibers. Additionally, it has been suggested that the subsarcolemmal actin network associated with AChR clusters immobilizes nuclei at the developing postsynaptic membrane (Englander and Rubin, 1987).

Anchoring of the nuclei by the cytoskeleton in mature fibers implies that there must be coupling between the nuclear envelope and the cytoskeletal elements. Indeed there is substantial evidence that the LINC complex proteins are mediating this interaction in muscle, as they do in other cell types. In fact the first mammalian nesprin proteins were identified in a yeast two-hybrid screen for proteins that interact with the muscle-specific tyrosine kinase (MuSK), an essential component of the postsynaptic membrane (Apel et al., 2000). Immunostaining for nesprin-1 (Syne-1) was present on all adult mouse muscle nuclei, but synaptic nuclei were noticeably brighter. The authors noted that enrichment occurred gradually over the course of the first postnatal month, suggesting that nesprin-1 was not required for aggregation of nuclei at the synapse or transcriptional specialization of nuclei,

but for maintenance of these characteristics. Searching sequence databases, the authors also identified nesprin-2 (Syne-2) based on similarity to nesprin-1 and show by RT-PCR that nesprin-2 is also expressed in skeletal and cardiac muscle (Apel et al., 2000).

Subsequent work has confirmed the localization of various isoforms of both nesprin-1 and nesprin-2 to the nuclear envelope in skeletal muscle cells, including both the Giant isoforms and the short isoforms that interact with lamin A/C and emerin (Mislow et al., 2002b; Zhang et al., 2005; Zhang et al., 2001). Evidence also suggests that isoforms of nesprin-2 are abundant in the cytoplasm and associate with the sarcomere of the adult muscle fiber, though the significance of this localization is still unclear (Zhang et al., 2005). The SUN domain containing SUN1 and SUN2 proteins are also found on the nuclear envelope in mammalian skeletal muscle (Crisp et al., 2006; Ding et al., 2007; Lei et al., 2009). While SUN1 and SUN2 are unchanged in expression or localization throughout muscle development, evidence from human muscle culture and muscle biopsies suggest nesprins switch isoforms as they mature. Nesprin-1 predominates in myoblasts, myotubes and regenerating immature fibers, while nesprin-2 is more abundant in mature fibers except on synaptic nuclei (Apel et al., 2000; Randles et al., 2010). Additionally, there appears to be a switch toward higher expression of the smaller nesprin-1 and nesprin-2 isoforms in mature fibers compared to the giant isoforms (Randles et al., 2010; Zhang et al., 2005). Nesprin-3 is also expressed in skeletal muscle cells (Wilhelmsen et al., 2005), though its localization and expression pattern during development in mammalian muscle has not yet been examined.

The necessity of the LINC complex in nuclear positioning in adult skeletal muscle has been demonstrated with a number of transgenic mouse lines. In an initial study, muscle-specific over-expression of the KASH domain of nesprin-1, which partially displaced endogenous nesprin-1 (and likely nesprin-2) from the envelope, led to an ~60% decrease in numbers of synaptic nuclei. No changes were observed in the location, size, or shape of extrasynaptic nuclei, despite the presence of the dominant negative KASH domain (Grady et

al., 2005). These findings were recapitulated using a nesprin-2 KASH dominant negative construct (Zhang et al., 2007b). Furthermore, deletion of the nesprin-1 KASH domain, which effectively removed all isoforms of nesprin-1 from the nuclear envelope, resulted in complete loss of synaptic nuclei as well as abnormal clustering of non-synaptic nuclei, suggesting that nesprin-1 is essential for proper nuclear spacing and anchoring in muscle. Conversely, deletion of nesprin-2 KASH, did not alter the positioning of either synaptic or non-synaptic nuclei. However, nesprin-1 and nesprin-2 KASH double knock-out mice were unable to breathe and died shortly after birth, likely due to defects in the central nervous system (Zhang et al., 2007b). Together these data suggest that nesprin-1 and -2 both play roles in myonuclear anchorage, but that nesprin-1 plays a more prominent or specialized role.

Additional studies examining the loss of nesprin-1 from the nuclear envelope report significant increases in the numbers of centrally-located nuclei, failure of nuclei to remain attached to the myofibers following glycerol-mediated removal of the sarcolemma and decreased strain transmission during controlled fiber stretching. These alterations in nuclear positioning and mechanics are associated with increased perinatal lethality, small size, loss of coordination, kyphoscoliosis, and reduced exercise capacity (Puckelwartz et al., 2009; Zhang et al., 2010).

Loss of SUN1 and SUN2 from the nuclear envelope in myofibers also results in altered nuclear positioning. *SUN1*$^{-/-}$ mice only exhibit a modest decrease in synaptic nuclei, consistent with the continued presence of nesprin-1 on the nuclear envelope, however, SUN1/2 double-knockout mice do not survive, and show obvious loss of synaptic nuclei as well as aggregation of non-synaptic nuclei (Lei et al., 2009). Nesprin-1 was absent from the nuclear envelope in these mice, again indicating the importance of the nesprins in myonuclear positioning.

While the specific identity of the nesprin splice isoforms involved in anchoring nuclei have not yet been established, no defects were reported in skeletal muscle in mice lacking

the c-terminal calponin-homology domain of nesprin-2 giant (Luke et al., 2008). This suggests that actin-binding by nesprin-2 giant may not be necessary for anchoring nuclei in muscle fibers, consistent with decreased expression of the giant isoform in mature fibers (Randles et al., 2010). However, more analysis of the myonuclei in these mice as well as transgenic mice lacking the nesprin-1 actin-binding domain is necessary before ruling out a role for the calponin-homology domains in myonuclear anchoring. Finally, zebrafish lacking nesprin-3 do not have altered myonuclear positioning in 3-day old embryos, nor do the fish display motility defects as embryos or adults, suggesting that nesprin-3 is dispensable for nuclear positioning in fish muscle fibers (Postel et al., 2011). However, it is still unclear whether nesprin-3 plays a role in nuclear positioning in mammalian muscle cells.

As introduced in Section V, recent genetic analyses have identified mutations in nesprin-1 and -2 in patients with Emery-Dreifuss muscular dystrophy (Zhang et al., 2007a). These mutations map to cytoplasmic regions in the C-terminal portion of the giant isoforms; these regions are also found in almost all of the shorter KASH-containing isoforms. Immunohistochemistry in patient muscle tissue, indicates that localization of mutant nesprins to the nuclear envelope is reduced, as is staining for emerin (Zhang et al., 2007a). This is consistent with data indicating that emerin mutations (which cause x-linked EDMD) disrupt binding of emerin to nesprin-1 and nesprin-2 and are associated with loss of nesprin-1 and -2 from the nuclear envelope (Wheeler et al., 2007; Zhang et al., 2007a). While it has not been examined whether the muscle fibers from patients with nesprin or emerin mutations show impaired nuclear positioning, EDMD2 patients with mutations that impair lamin processing and localization, have reduced levels of SUN1 on the nuclear envelope, and show obvious clustering of myonuclei in muscle fibers (Mattioli et al., 2011).

These mouse and human studies clearly reveal the necessity of the LINC complex for proper nuclear positioning in muscle, however, the defects in positioning are described almost exclusively as defects in nuclear anchoring, without much acknowledgement that

mispositioning of nuclei may also be due to defects in nuclear movement during myogenesis. With this acknowledgment comes the realization that there are large gaps in our understanding of nuclear movement and anchoring in developing and adult muscle cells. For example, by what mechanism do the microtubules support nuclear translocation and rotation in developing cells – are microtubule motors involved? Are nesprins involved? How do nuclei move from the center of the myotube to the periphery of the cell and how do they stop moving? What cytoskeletal elements and which nesprin isoforms are interacting in mature cells to anchor nuclei? What is special about these connections at the post-synaptic membrane that makes them more susceptible to nuclear mispositioning? Why do nesprins switch isoforms as muscle cells mature? Are some isoforms responsible for nuclear movement while others anchor nuclei?

Emerging roles for microtubule motors in myonuclear movement

In the last few years, significant first steps have been made toward answering these questions. In particular, the question of how microtubules control nuclear movement has received a good deal of attention, and from work both in cultured muscle cells and *in vivo*, it is now apparent that the microtubule motor proteins, cytoplasmic dynein and kinesin-1, play essential roles in myonuclear movement. Moreover, growing evidence also suggests that nesprins also play an important role in motor-dependent nuclear movements.

In work by Metzger et al. (2012), a mutagenesis screen revealed a myonuclear positioning mutant in the Drosophila embryo. The nuclei in these mutant flies failed to migrate normally through the developing muscle cells, resulting in a centralized cluster of nuclei. These flies were found to carry a nonsense mutation in the gene encoding the microtubule associated protein, ensconsin (also known as MAP7 or E-MAP-115 in vertebrates). Depletion of MAP7 in cultured C2C12 and primary mouse myotubes phenocopied the fly and resulted in significant nuclear aggregation. When no obvious

alteration in the microtubule network was observed, a yeast two-hybrid screen was conducted to look for proteins that interact with MAP7, which identified kinesin heavy chain. Flies with a kinesin-null mutation, or a motor-dead mutation, as well as mouse myotubes depleted of Kif5B, all showed significant nuclear aggregation defects. Moreover, the authors saw no positioning defect when the dominant negative KASH domain of nesprin-2 was expressed in C2C12 myotubes, but did note rescue of nuclear alignment with expression of a Kif5B-MAP7 chimera protein, leading them to propose a model in which Kif5B interacts with MAP7 to cross-link and slide anti-parallel microtubules. In their model, the microtubule minus-ends are still attached to the nuclear envelope, such that microtubule sliding would push nuclei apart (Metzger et al., 2012), similar to how the Eg5 kinesin motor facilitates spindle elongation during mitosis (Ferenz et al., 2010). However this proposed mechanism does not account for the nuclear rotation and deformation previously observed in cultured myotubes (Capers, 1960; Englander and Rubin, 1987).

My own work in C2C12 myotubes also identifies an essential role for Kif5B in myonuclear movement and positioning. As is discussed in Chapter 2 of this thesis, in cells depleted of Kif5B, rates of nuclear translocation were reduced, nuclear rotation was essentially abolished, and nuclei aggregated dramatically at the myotube center (Wilson and Holzbaur, 2012). Immunocytochemistry indicates localization of both kinesin light chain and kinesin heavy chain on the nuclear envelope and contrary to Metzger et al., we noted a tendency for the nuclei to aggregate when decorated with the dominant negative EGFP-nesprin-2-KASH construct. Additionally, in binding assays, nesprin-2 pulled down both KLC and Kif5B from myotube lysates, suggesting that nesprins localize Kif5B to the nuclear envelope where the motors act to drive nuclear translocation and rotation along the microtubule network. Importantly, this model does permit for the observed nuclear rotation and deformation that occurs during nuclear movement (Wilson and Holzbaur, 2012)(Also see Chapter 2).

The role of Kif5B in myonuclear positioning was even further substantiated by a conditional knock-out of Kif5B from skeletal muscle in the mouse (Wang et al., 2013a). These mutant mice survive only one postnatal day at most because they have difficulty breathing and are unable to eat. They have severe limb muscle dystrophy and display no limb movement. Myofibers are abnormally short in the Kif5B-deficient muscles, and central aggregation of nuclei and mitochondria is prominent. Additionally, the muscles have disorganized myofibril and intermediate filament assembly and localization. Although these fiber defects may be secondary to nuclear aggregation, Kif5B was found to interact directly with desmin and transport it to the myotube ends, as well as transport nestin, α-sarcomeric actin, and non-muscle myosin IIB through indirect means, suggesting that Kif5B plays essential roles in many aspects of muscle cell development (Wang et al., 2013a). Notably, in myotube cultures from these mice and their wild-type littermates, these authors confirm that Kif5B localizes to the nuclear envelope and show evidence that both the light chain binding domain and the autoinhibitory IAK motif are indispensible for this localization (Wang et al., 2013b). These data further support a model in which kinesin-1 motors drive nuclear dynamics from the nuclear surface, similar to movement of nuclei in *C. elegans* hypodermal precursor cells (Meyerzon et al., 2009).

There is growing evidence to suggest that dynein also plays an important role in myonuclear movements in developing muscle cells. Immunostaining for components of the dynein/dynactin complex localizes the motor to the nuclear envelope in C2C12 myotubes (Cadot et al., 2012; Wilson and Holzbaur, 2012). Consistent with this finding, we find that depletion of DHC with siRNA in cultured myotubes reduces nuclear translocation and rotation, and results in localized nuclear aggregation, although this effect was not as pronounced as in Kif5B-deficient myotubes (Wilson and Holzbaur, 2012)(Also see Chapter 2). Observations of end-on myoblast to myotube fusion, also revealed that depletion of DHC, DIC or $p150^{Glued}$ reduced the rate at which a new nucleus moved into the center of a

myotube (Cadot et al., 2012). These latter observations, combined with the localization of components of the dynactin complex on the nuclear envelope, led to a proposed model in which nuclear-based dynein motors pull on microtubules emanating from an adjacent nucleus, thereby pulling the nuclei toward one another (Cadot et al., 2012). While this model does not account for nuclear rotation and does not explain why loss of dynein from the myotube results in aggregation of nuclei, these observations may be more readily explained if the microtubules that dynein is pulling on are not necessarily still attached to the nuclei. In our proposed model, nuclear-based dynein would serve to assist nuclear-based kinesin motors to maneuver the nuclei along the bipolar microtubule network, providing more flexibility in navigating around cellular obstacles (Wilson and Holzbaur, 2012).

Further work in *Drosophila* embryo muscles suggests that both dynein and kinesin-1 localize to the nuclear envelope, however, there is spatial segregation of motors such that Kif5B is located on the side closest to the myotube end (leading edge), whereas dynein is on the opposite surface (lagging edge) (Folker et al., 2014). Furthermore, these authors find prominent localization of dynein at the cell cortex at the muscle poles (Folker et al., 2012; Folker et al., 2014). They propose a model in which cortical dynein pulls on microtubules emanating from the nuclear surface to pull nuclei toward muscle poles. Simultaneously, nuclear-based kinesin motors “stretch” the leading edge of the nucleus toward the pole and release of dynein from microtubules at the rear of the nucleus relieves this tension as the nucleus is pulled to the muscle pole. Although they do note rare rotations of nuclei, or changes in direction, they argue that the nucleus always reorients such that the leading edge is maintained. The nuclei in kinesin and dynein mutant embryos changed direction more frequently and have trouble maintaining a leading edge (Folker et al., 2014). It is unclear how the motors are associating with the nucleus in the muscle cells of the fly embryo.

In summary, the available data provide substantial evidence that kinesin-1 and cytoplasmic dynein are necessary for microtubule-dependent nuclear movement and

positioning in developing muscle cells. However, the mechanisms by which each motor exerts its role is less clear. More work is necessary to directly test the models proposed in these recent studies. To this end, the experiments presented in Chapter 3 of this dissertation are aimed at testing the hypothesis that kinesin-1 is necessary on the nuclear envelope for proper nuclear distribution in developing myotubes.

CHAPTER 2: Opposing Microtubule Motors Drive Robust Nuclear Dynamics in Developing Muscle Cells

This chapter is adapted from:

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I. Summary

Dynamic interactions with the cytoskeleton drive the movement and positioning of nuclei in many cell types. During muscle cell development, myoblasts fuse to form syncytial myofibers with nuclei positioned regularly along the length of the cell. Nuclear translocation in developing myotubes requires microtubules, but the mechanisms involved have not been elucidated. We find that as nuclei actively translocate through the cell, they rotate in three-dimensions. The nuclear envelope, nucleoli, and chromocenters within the nucleus rotate together as a unit. Both translocation and rotation require an intact microtubule cytoskeleton, which forms a dynamic bipolar network around nuclei. The plus- and minus-end directed microtubule motor proteins, kinesin-1 and dynein, localize to the nuclear envelope in myotubes. Kinesin-1 localization is mediated at least in part by interaction with klarsicht/ANC-1/Syne homology (KASH) proteins. Depletion of kinesin-1 abolishes nuclear rotation and significantly inhibits nuclear translocation, resulting in the abnormal aggregation of nuclei at the midline of the myotube. Dynein depletion also inhibits nuclear dynamics, but to a lesser extent, leading to altered spacing between adjacent nuclei. Thus, oppositely directed motors acting from the surface of the nucleus drive nuclear motility in myotubes. The variable dynamics observed for individual nuclei within a single myotube likely result from the stochastic activity of competing motors interacting with a complex bipolar microtubule cytoskeleton that is also continuously remodeled as the nuclei move. The three-dimensional rotation of myotube nuclei may facilitate their motility through the complex and crowded cellular environment of the developing muscle cell, allowing for proper myonuclear positioning.

II. Introduction

Nuclear movement and positioning in many systems is controlled by connections between the nuclear envelope and the cytoskeleton (reviewed in (Fridolfsson and Starr, 2010)). Microtubule-dependent nuclear movement is particularly important for cells as they migrate and differentiate during development. For example, nuclear movement is tightly coupled to neuronal migration during brain development and mutations that disrupt this movement result in severe developmental defects such as lissencephaly (reviewed in (Kuijpers and Hoogenraad, 2011)). In neurons, nuclear migration requires the minus-end directed microtubule motor protein, dynein. Dynein walks the nucleus toward the centrosome along a microtubule cage that surrounds the nucleus (Shu et al., 2004; Tsai et al., 2007; Xie et al., 2003). In contrast, as nuclei migrate through hypodermal precursor cells in the developing C. *elegans* embryo, kinesin-1 is the predominant motor moving the nucleus toward the plus-end of a polarized parallel non-centrosomal bundle of microtubules, with dynein driving small backsteps along this network (Fridolfsson and Starr, 2010). In these examples, both the polarity of the microtubule network and the type of motors present on the nuclear surface determine the overall direction of nuclear translocation.

Proper nuclear positioning is also critical in skeletal muscle cells. Mammalian skeletal muscle fibers are large multinucleated cells formed by the fusion of hundreds of post-mitotic mononucleated myocytes. Adult muscle fibers can extend many centimeters in length and, except for a cluster of specialized nuclei at the neuromuscular junction, the nuclei are found at the periphery of the cell, evenly spaced along the long-axis of the fiber (Bruusgaard et al., 2003; Kummer et al., 2004). This positioning is thought to ensure sufficient transcriptional capacity as well as to minimize transport distances between the nuclei and the cytoplasm in these extraordinarily long cells (Bruusgaard et al., 2003). Abnormally clustered nuclei have been found in patients with autosomal dominant Emery-

Dreifuss muscular dystrophy (Mattioli et al., 2011), suggesting that correct nuclear positioning may be required for proper muscle function.

Nuclei in developing chick myotubes have been observed to translocate along the long axis of the cell (Capers, 1960; Cooper and Konigsberg, 1961; Cooper, 1958). Although this translocation was shown to be dependent on an intact microtubule cytoskeleton (Englander and Rubin, 1987), the mechanisms that drive this translocation have not yet been explored.

Early studies in primary myotubes suggested that nuclei may rotate as they translocate (Capers, 1960; Cooper and Konigsberg, 1961; Cooper, 1958). Nuclear rotation has been explored in cultured fibroblasts, where nuclei rotate in two dimensions, within the plane of substrate attachment (Ji et al., 2007; Levy and Holzbaur, 2008). This rotation occurs more frequently in migrating cells and is driven by dynein motors (Levy and Holzbaur, 2008). The function of rotation in migrating fibroblasts is still unclear, but has been suggested to be important in maintaining nuclear centrality. Given the length and complexity of the myotube, it is possible that nuclear dynamics during development, including both translocation and rotation, are essential for proper distribution of nuclei in the mature muscle fibers; however, this has not yet been examined.

In this study, we use live cell microscopy to examine the dynamics of nuclear movement in developing C2C12 myotubes, a well-established model system that faithfully replicates most features of early myogenesis and myofibril assembly, with cytoskeletal organization and dynamics closely resembling that of developing myotubes *in vivo* (reviewed in (Sanger et al., 2010)). We find that nuclei translocate within myotubes and also display robust three-dimensional rotation. Kinesin-1 and dynein both localize to the nuclear surface, likely mediated at least in part through interactions with the klarsicht/ANC-1/Syne homology (KASH) proteins. While both motors contribute to nuclear dynamics, kinesin-1 is the more dominant motor in this system. Loss of either kinesin or dynein causes abnormal

aggregation and inappropriate dispersal of nuclei in myotubes, indicating that normal nuclear dynamics are essential for the proper distribution of nuclei in developing muscle cells.

III. Results

Nuclei both translocate and rotate in three dimensions in developing myotubes

Myotubes are formed as myoblasts fuse to generate multinucleated syncytial cells. As additional myoblasts fuse, the length of the cell increases concomitant with an increase in the number of nuclei per myotube (Fig. 2.1). Most myotubes display a relatively even distribution of nuclei throughout the length of the cell (Fig. 2.1A), with the average distance between nuclei in myotubes 7 days post differentiation (D7) found to be 22.7±0.95 μm (mean ± s.e.m.; *n* = 630 nuclei in 66 myotubes).

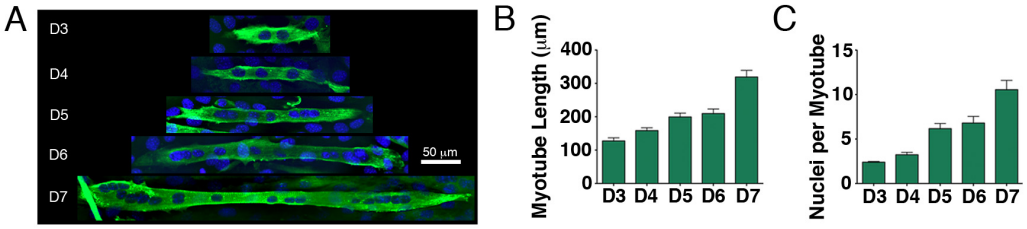

Figure 2.1. Nuclei distribute throughout the cell during myotube differentiation. (A) Representative images of myotubes differentiated for 3-7 days *in vitro* (D3-D7). Nuclei were stained with Hoechst dye; α-actinin is in green. Images are maximum projections of confocal z-series. (B,C) Myotube length and number of nuclei per myotube are shown (mean ± s.e.m.; *n* > 30 myotubes).

We imaged C2C12 myotubes with time-lapse phase microscopy and found that nuclei display robust and complex dynamics. Nuclei translocate along the length of the developing cell (Fig. 2.2A, Video 1) at an average rate of 11.7±7.8 μm/hr (0.2±0.13 μm/min; mean ± s.d.), similar to initial observations of nuclear translocation in primary myotubes (Englander and Rubin, 1987). Average velocities remained relatively constant in myotubes

differentiated for 4-7 days in culture. We focused primarily on myotubes differentiated 7 days in order to study dynamics in cells that had undergone substantial myofibrillogenesis; past this time point myotubes begin to form branched networks and contract too strongly for accurate analysis.

Most nuclei in myotubes are mobile and exhibit complex dynamics, characterized by episodic, rather than continuous, movement. Translocation velocities of individual nuclei can vary greatly over the course of >3 hours of imaging (Fig. 2.2A, Video 1). Nuclei migrate in either direction along the long-axis of the cell; individual nuclei will occasionally change direction. Nuclei within the same myotube can move individually, or in groups, and nuclei can pass one another as they translocate (Fig. 2.2A, arrowhead), suggesting that the dynamics of each nucleus is influenced, but not dependent, on those nearby.

Remarkably, nuclei also rotate in three-dimensions, as assessed by monitoring the movement of nucleoli in time-lapse phase imaging (Fig. 2.2A, arrow; Video 1). To monitor this rotation more precisely, we labeled the DNA with a live-cell Hoechst dye, and used confocal microscopy to track the positions of the brightly stained chromocenters in focal stacks over time (Fig. 2.2B; Video 2). Within an individual nucleus, the positions of chromocenters remain fixed relative to one another over the time course of observation (15 min), allowing accurate tracking of their trajectories in XYZ space.

Nuclei rotate clockwise or counterclockwise about the axes perpendicular to the long axis of the myotube. Occasionally individual nuclei change their direction of rotation (Video 1). The extent and rate of rotation varies considerably, with 50-60% of nuclei rotating more than 2°/min, some reaching velocities as high as 14.4˚/min (Fig. 2.2B, bottom panel). Isolated nuclei, as well as nuclei moving in concert with others, can rotate; the rate and direction of rotation for individual nuclei appear to be independent of the dynamics of neighboring nuclei. Rotation is usually accompanied by translocation. The ability of nuclei to pass one another closely correlates with nuclear rotation. Analysis of time series from 75

myotubes indicates that in 29 pass events, all involved rotation of at least one nucleus, and in 93% of these events, both nuclei rotated. The observed rotation during passing is often accompanied by deformation of nuclear shape (Fig. 2.2A; Video 1).

When nuclei rotate in two-dimensions in fibroblasts, nucleoli within the interior of the nucleus rotate with the nuclear lamina and nuclear envelope as an intact structure (Ji et al., 2007; Levy and Holzbaur, 2008). To determine whether this is also true for nuclei rotating in three-dimensions, we transfected myotubes with RFP-laminA to label the nuclear lamina, co-labeled the DNA with Hoechst dye to observe chromocenters and examined nuclear rotation using time-lapse confocal microscopy. Comparison of chromocenter movements with fiduciary marks in the RFP-laminA labeling show that the nuclear lamina is rotating along with the chromatin (Fig. 2.2C; Video 3). Expression of a GFP-tagged emerin (GFP-emerin) construct reveals that the inner membrane of the nucleus also rotates in tandem with the Hoechst-labeled chromocenters.

To determine whether structures associated with the outer nuclear membrane also move in conjunction with the envelope, we expressed either a DsRed-Centrin-2 construct or mCherry-GPP130 and observed the dynamics of the labeled nuclei. We found that the centrosomal protein centrin-2 localizes in puncta both in the cytoplasm and at the outer surface of the nucleus, as described for other centrosomal proteins in myotubes (Bugnard et al., 2005; Srsen et al., 2009). DsRed-Centrin-2 likely identifies sites of microtubule nucleation, known to occur from the surface of the nuclei in these cells (Bugnard et al., 2005; Tassin et al., 1985b; Zaal et al., 2011). Tracking shows that individual centrin-2 puncta rotate with the nucleus (Fig. 2.2C; Video 3). We also found that the Golgi, labeled with mCherry-tagged Golgi matrix protein, GPP130, (Linstedt et al., 1997), rotates with the nucleus (Fig. 2.2C; Video 3). Together, these experiments reveal that the nuclear interior and nuclear envelope rotate together as a unit in three-dimensions, along with associated organelles including the Golgi.

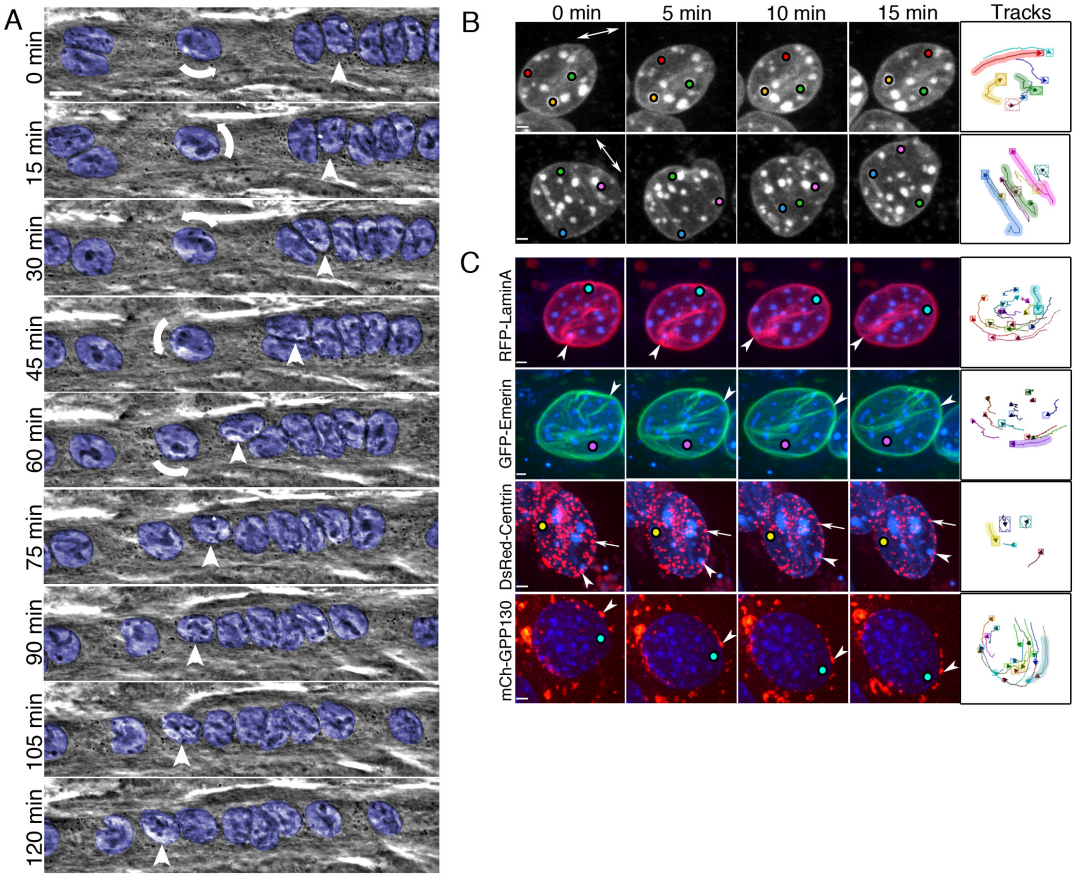

Figure 2.2. Nuclei actively rotate and translocate during myotube differentiation.

Figure 2.2. Nuclei actively rotate and translocate during myotube differentiation. (A) Phase images from a time-lapse sequence of nuclear migration in a D7 myotube (Video 1). The long-axis of the myotube runs in the horizontal direction; all visible nuclei in the myotube have been pseudo-colored blue. The arrow highlights the counter-clockwise rotation of a nucleus, visible in phase by watching prominent nucleoli; the arrowhead follows the path of a nucleus as it deforms and passes an adjacent nucleus. Scale bar = 10 μm. (B) Representative examples of nuclear rotation (Video 2). DNA was labeled with Hoechst dye and maximum projections of confocal z-stacks are shown over time. Bidirectional arrow indicates the long-axis of the myotube and colored dots label bright chromocenters to aid visualization of rotation. (C) Nuclei in myotubes expressing fluorescent constructs to label the nuclear lamina (LaminA), inner nuclear membrane (emerin), outer nuclear membrane (centrin-2) or Golgi (GPP130) are shown (Video 3). Colored dots label bright chromocenters in each nucleus; fiduciary marks of nuclear envelope-associated structures are demarcated with arrowheads and arrows. Chromocenters were tracked in XYZ over time. The right panels in B & C highlight representative tracks of individual chromocenters during rotation of the nuclei. Shaded tracks correspond to the chromocenters labeled with colored dots in the time series. Scale bar = 2 μm.

As myotubes develop in culture, we noted a dramatic change in the shape of nuclei in myoblasts compared to those in myotubes (Fig. 2.3). While nuclei in mononucleated myoblasts resemble the disc-shaped structures seen in fibroblasts, in myotubes the nuclei are more spherical (Fig. 2.3A,B). This change in shape correlates with changes in nuclear rotation. The flattened nuclei in myoblasts occasionally exhibit two-dimensional rotation in the plane of the substratum, which resembles the nuclear rotation described in fibroblasts (Ji et al., 2007; Levy and Holzbaur, 2008), and is distinct from the three-dimensional rotation observed in more fully developed myotubes.

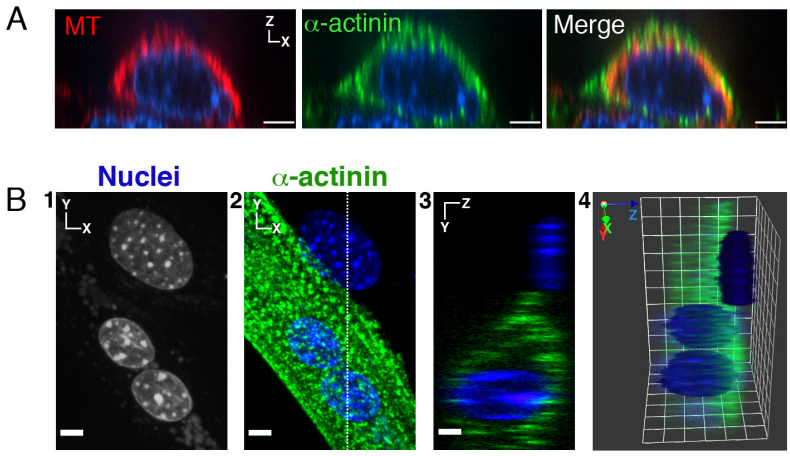

Figure 2.3. Nuclei are spherical in myotubes.

(A) Image of myotube in cross-section stained with antibodies to tubulin (red) and α-actinin (green). Nuclei are labeled with Hoechst dye (blue). Microtubules in myotubes run parallel to the developing myofibril network, forming a tube around the centrally-located nuclei (one Y plane in XZ, scale = 5 μm). (B) The nuclei in myotubes adopt a spherical shape whereas the nuclei in myoblasts are wider and flatter. Cells were transfected with YFP-α-actinin to show development of myofibrils in myotubes and nuclei are stained with Hoechst dye. Panels 1 & 2 show maximum projections of a confocal z-series of a nucleus in a myoblast (top) and two

nuclei in a neighboring myotube. Panel 2 includes the YFP-α-actinin signal to denote the myotube. The dotted line indicates the location of the single YZ plane shown in panel 3. In this cross-section, the difference in the height of the nuclei in the myoblast and myotube can be appreciated. Panel 4 is a three-dimensional rendering of these nuclei to again emphasize the spherical vs. flattened shape of the myotube and myoblast nuclei, respectively. For panels 1-3, scale = 5 μm.

Microtubules are necessary for both nuclear translocation and rotation in myotubes

During myogenesis, the microtubule cytoskeleton is reorganized from the radial, centrosomal array found in mononucleated myoblasts to a linear array oriented along the long-axis of multinucleated myotubes (Pizon et al., 2005; Warren, 1974). In cross-section, both the microtubules and the myofibrils surround the centrally located nuclei (Fig. 2.3A), with the microtubule array located more toward the interior, consistent with studies showing that nascent myofibrils form initially at the cell cortex and move inward (reviewed in (Sanger et al., 2010)).

To explore the polarity and dynamics of the microtubule array, we labeled the plus-tips of microtubules with a fluorescently-tagged end-binding protein (GFP-EB3) that tracks with the growing end of the microtubule (Akhmanova and Steinmetz, 2008). At the ends of the myotube, microtubules are generally oriented with their plus-ends toward the cortex (Fig. 2.4A, 2 and 2', 2C; Video 4). In contrast, in the center of the cell, we observe an anti-parallel microtubule organization along the long axis of the cell (Fig. 2.4A, 1 and 1', 2.2B; Video 4), also described by (Pizon et al., 2005; Zhang et al., 2009a). Most cells showed a small

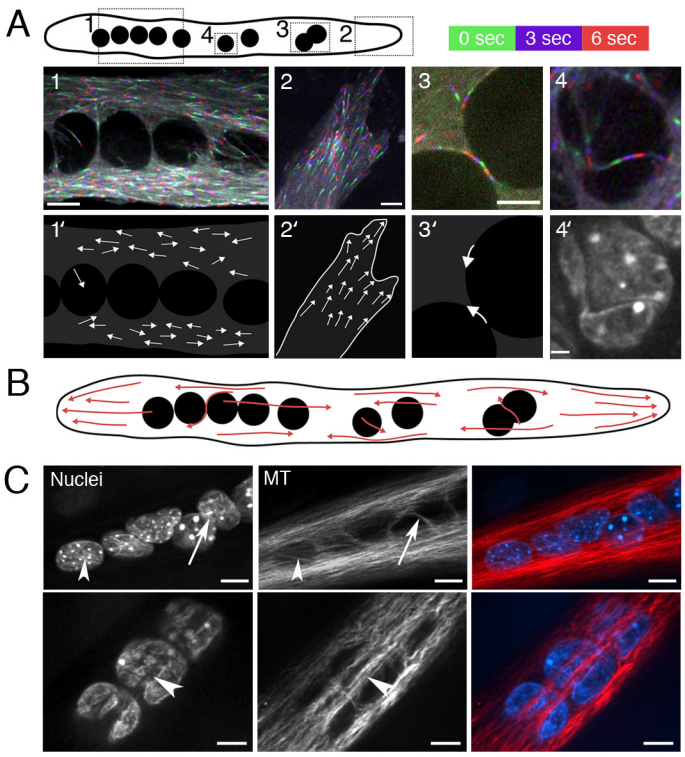

Figure 2.4. The microtubule cytoskeleton forms a dynamic network around nuclei in myotubes.

(A) Myotubes were transfected with GFP-EB3 to label the growing plus-ends of microtubules (Videos 4 and 5). Images were obtained every 3 seconds and three sequential frames were pseudo-colored with green, purple and red, respectively, and overlaid (1-4) to show the direction of microtubule growth. Scale bars = 10 μm in panel 1 and 5 μm in panels 2 & 3. 1′-3′ depict the direction of representative microtubule growth. 4′ shows the invagination in the nuclear surface. The DNA was labeled with Hoechst dye, single Z-plane in XY, scale bar = 2 μm. (B) Model of microtubule orientation within a myotube. (C) Microtubules lie between adjacent nuclei (arrow) and within invaginations (arrowheads) on the nuclear surface. Cells were stained for tubulin (red) and nuclei were labeled with Hoechst dye (blue). Single Z-plane in XY, scale bars = 10 μm.

overall bias in microtubule growth toward one end of the myotube (58.6±2.4 vs. 41.4±2.4 (mean ± s.e.m.) for dominant vs. non-dominant direction, respectively). Often there were areas within a myotube that exhibited highly polarized growth, raising the possibility that local differences in microtubule polarity may influence microtubule-dependent nuclear movement.

Microtubules are often found in close apposition to the nucleus. Microtubules lie between adjacent nuclei (Fig. 2.4C, top panel, arrow) as well as within invaginations, or grooves, on the nuclear surface (Fig. 2.4C, arrowheads). The deeper, wider grooves contain both microtubules (Fig. 2.4C, bottom panel) and myofibrils (not shown), whereas only microtubules are found in smaller invaginations (Fig. 2.4C, top panel, arrowhead). Microtubules in close contact with the nuclear surface are dynamic. Microtubules can polymerize along the curve of the nuclear surface (Fig. 2.4A, 3 and 3' top arrow; Video 5), between two nuclei (bottom arrow), and within invaginations (Fig. 2.4A, 4 and 4'; Video 5). Together, these observations indicate that the microtubule network is in close association with the nuclei in myotubes, forming a complex and constantly changing network around each nucleus.

Microtubule depolymerization effectively inhibits nuclear translocation in primary myotubes, whereas the inhibition of actin dynamics had no effect (Englander and Rubin, 1987). In C2C12 cells, we found that treatment of myotubes with concentrations of nocodazole that depolymerize the microtubule network (Fig. 2.5A) significantly reduced the rate of nuclear translocation when compared to the DMSO-treated control myotubes (0.082±0.009 μm/min vs. 0.181±0.032 μm/min, respectively; mean ± s.e.m., $p < 0.001$) (Fig. 2.5B,D). Additionally, nuclear rotation was abolished in myotubes treated with 10 μg/ml nocodazole (Fig. 2.5C,E; Video 6). Thus, microtubules are necessary for both nuclear translocation and rotation.

While an intact microtubule network is necessary for nuclear dynamics, nuclei are still able to translocate and rotate in myotubes treated with a lower dose of nocodazole (100

ng/ml) (Video 7). This concentration inhibits microtubule growth (Video 7, central panel), but does not eliminate the microtubule network, suggesting that dynamic microtubules are not essential for nuclear translocation or rotation.

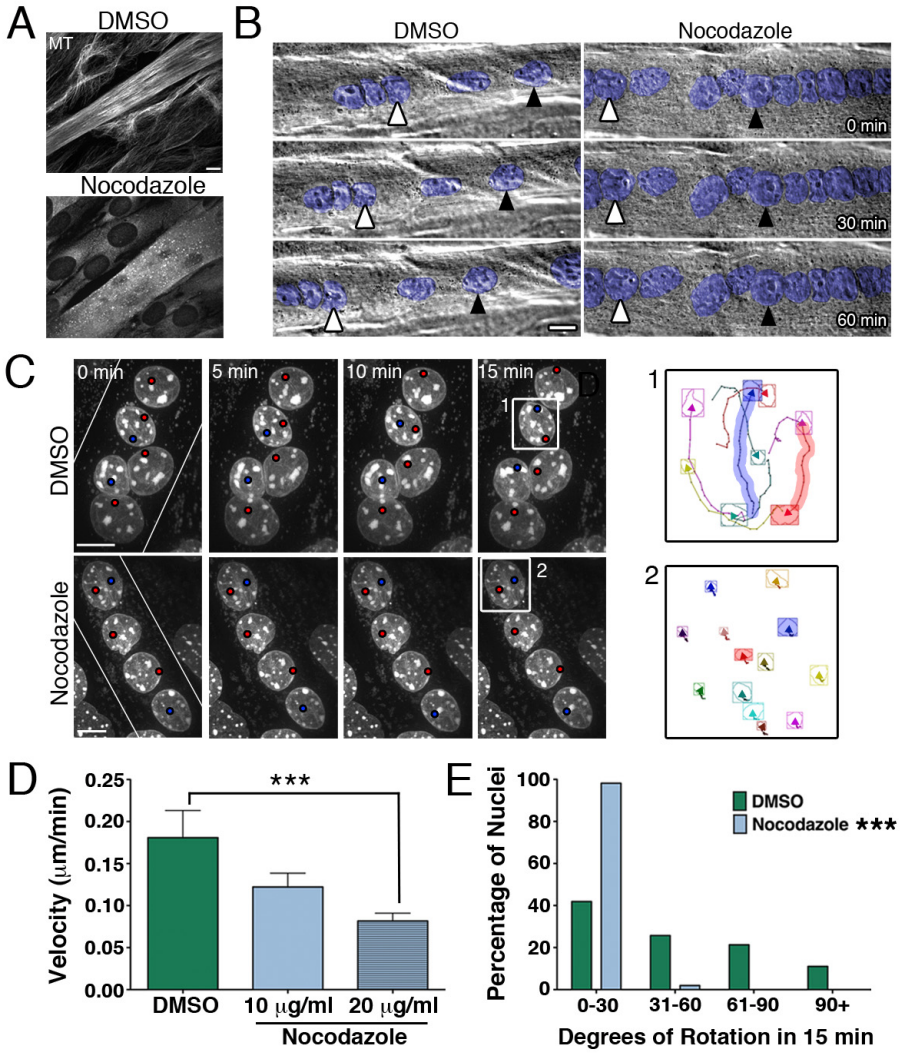

Figure 2.5. The microtubule cytoskeleton is required for both nuclear translocation and rotation.

Figure 2.5. The microtubule cytoskeleton is required for both nuclear translocation and rotation.

(A) Representative images of myotubes and surrounding myoblasts treated with DMSO or nocodazole (10 μg/ml) for 30 min at 37°C. Cells were stained with antibodies to tubulin; scale bar = 20 μm. (B) Phase images from time-lapse sequences of nuclear migration in myotubes treated with DMSO (left) or 10 μg/ml nocodazole (right). The long-axis of the myotube run in the horizontal direction and all visible nuclei in myotubes have been pseudo-colored blue. Arrowheads follow the migration of representative nuclei. Scale bar = 10 μm. (C) Representative examples of nuclear rotation in myotubes treated with DMSO (top panel) or 10 μg/ml nocodazole (bottom panel) (Video 6). DNA was labeled with Hoechst dye and maximum projections of confocal z-stacks are shown over time. White lines depict the orientation of the myotube and colored dots label bright chromocenters to aid visualization. Right panel highlights representative chromocenter tracks during rotation of the boxed nuclei on the left. Shaded tracks correspond to the chromocenters labeled with colored dots in the time series. Scale bars = 10 μm. (D) Mean velocity of nuclear translocation following treatment of myotubes with DMSO or nocodazole (mean ± s.e.m.; ***, $p < 0.001$, ANOVA; *n*>30 nuclei in 5-7 myotubes). (E) Quantification of the degree of nuclear rotation in treated myotubes (***, $p < 0.001$, χ-square test, shown are pooled data for *n*>100 nuclei in 20-22 myotubes).

Kinesin-1 drives nuclear translocation and rotation in myotubes

In other systems displaying microtubule motor-dependent nuclear translocation and/or rotation such as migrating neurons and fibroblasts, the motors act from the surface of the nucleus (Levy and Holzbaur, 2008; Zhang et al., 2009b). To determine whether the kinesin-1 motor localized to the nuclear envelope, we performed immunofluorescence on D7 C2C12 myotubes. Antibodies directed against kinesin light chains 1 and 2 (KLC1/2) and kinesin heavy chain (KHC, mAb 1614) abundantly decorate the nuclear surface (representative images are shown in Fig. 2.6). A similar pattern was also found when myotubes were stained with a second pan-KHC antibody (SUK4 mAb, not shown). We also found that transfection of myotubes with a GFP-tagged KIF5C tail domain construct, which binds to KHC partner proteins, including KLC1/2 (Bi et al., 1997; Konishi and Setou, 2009), led to uniform decoration of the nucleus envelope (Fig. 2.6).

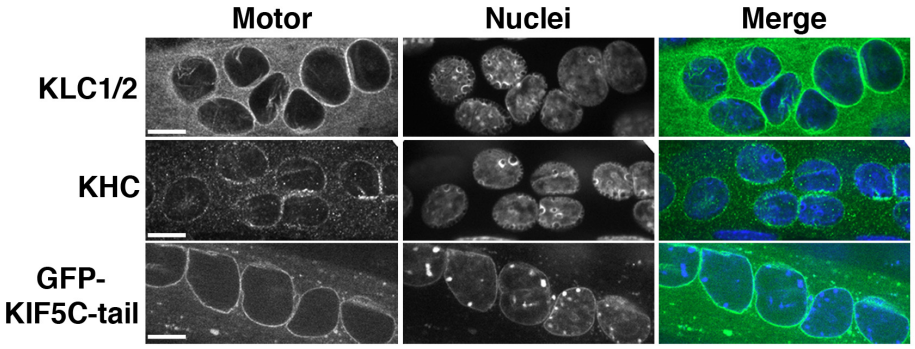

Figure 2.6. Kinesin-1 localizes to the nuclear envelope in myotubes.
Myotubes were stained for subunits of the plus-end directed microtubule motor kinesin-1 (kinesin light chains 1/2 (KLC1/2); kinesin heavy chain (KHC)). Also shown is a myotube transfected with EGFP-KIF5C tail, the domain of KHC that interacts with KLC1/2. Myotubes were fixed with methanol for KLC1/2 and KHC. DNA was labeled with Hoechst dye. Scale = 10 μm.

KASH proteins mediate links between the nucleus and the cytoskeleton in many systems (reviewed in (Starr and Fridolfsson, 2010)), and the KASH protein nesprin-2 has recently been shown to bind to KLC (Schneider et al., 2011). To examine this interaction in myotubes, we performed GST-pulldown assays from C2C12 myotube lysate with the minimal KLC binding domain of nesprin-2, residues 6348-6552 and observed specific binding of KLC1, KLC2 and KHC to the nesprin-2 construct (Fig. 2.7A). This result suggests that nesprin-2 mediates, at least in part, the localization of kinesin-1 to the nuclear envelope in myotubes.

To further test this possibility, we expressed the KASH domain of nesprin-2α (EGFP-nesprin-2α-KASH) in myotubes and assessed its effect on nuclear rotation. This domain localizes specifically to the nuclear envelope in myotubes (Fig. 2.7B) and acts as a dominant negative by displacing the endogenous nesprins from the nuclear membrane (Grady et al., 2005; Zhen et al., 2002). We found that expression of EGFP-nesprin-2α-KASH partially displaced kinesin-1 from the nuclear envelope in myotubes (Fig. 2.7C). Although this displacement was not complete, we found that EGFP-nesprin-2α-KASH positive nuclei rotated less than those in control cells (Fig. 2.7D). Furthermore, expression of the dominant negative construct induced aggregation of nuclei along the myotube, with a decreased mean distance between adjacent nuclei (13.0±0.9 μm vs. 19.3±0.9 μm in EGFP-nesprin-2α-KASH positive myotubes as compared to control myotubes expressing EGFP, respectively, mean ± s.e.m., $p<0.0001$; mean myotube length was not different)(Fig. 2.7E).

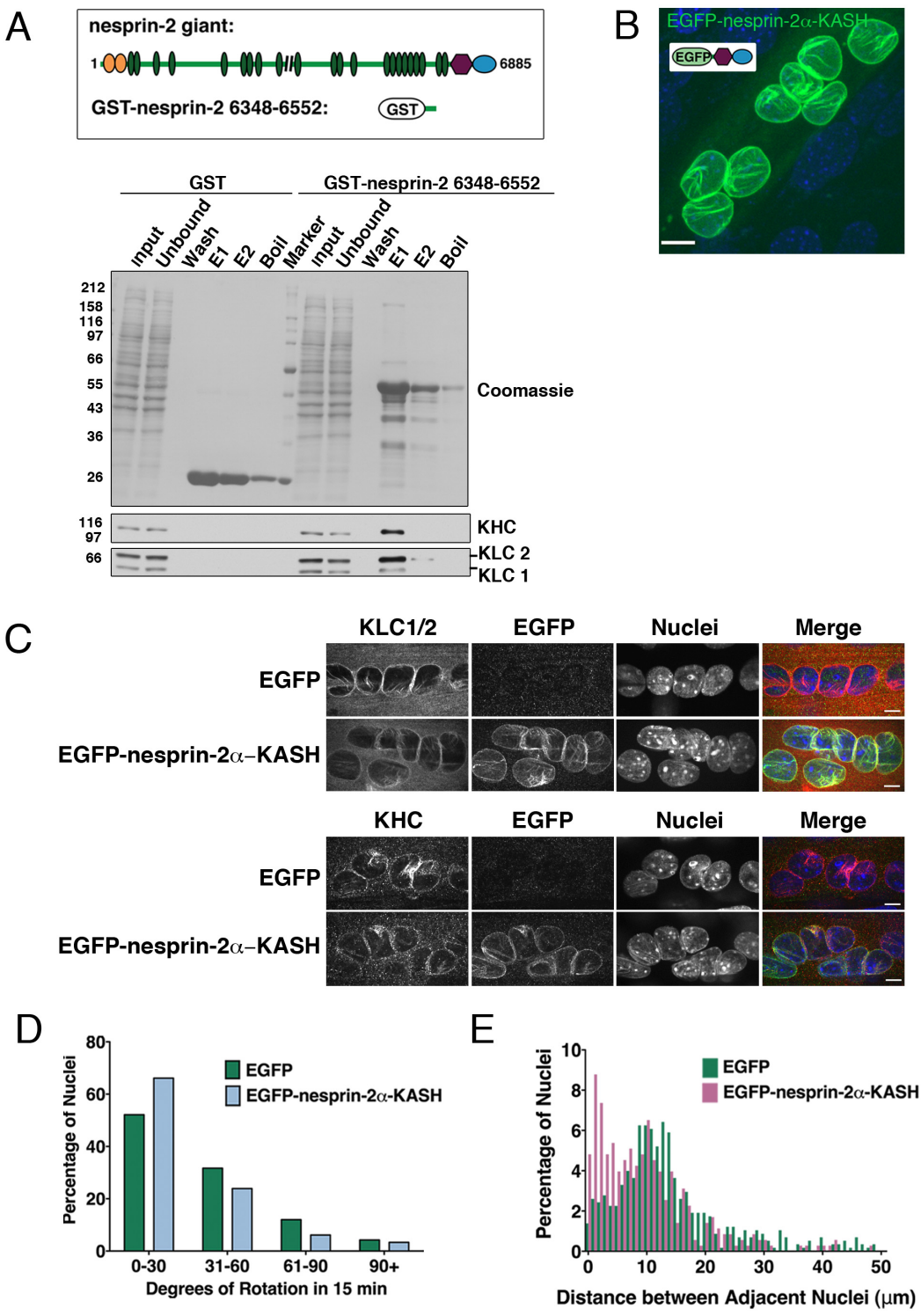

Figure 2.7. Kinesin-1 localizes to the nuclear envelope in myotubes and binds to the LINC protein, nesprin-2.

Figure 2.7. Kinesin-1 localizes to the nuclear envelope in myotubes and binds to the LINC protein, nesprin-2.

(A) Schematic of nesprin-2^{giant} and GST-nesprin-$2^{6348\text{-}6552}$ (orange, green, purple and blue shapes represent the actin-binding domain, spectrin repeats, transmembrane domain and KASH domain, respectively). Pull-down assays with GST-nesprin$2^{6348\text{-}6552}$ or GST beads incubated with C2C12 myotube lysate. Bound protein was eluted first with 80mM glutathione (2 rounds: E1,E2); beads were then boiled in denaturing buffer (Boil). Blots were probed with antibodies to KLC1/2 (63-90 mAb) and KHC (SUK4 mAb); blot shown is representative of three independent replicates. (B) Representative image of a myotube expressing EGFP-nesprin-2α-KASH (maximum projection of a confocal z-series; DNA was labeled with Hoechst dye, scale bar = 10 μm). (C) Myotubes were transfected with either EGFP or EGFP-nesprin-2α-KASH and stained for subunits of the plus-end directed microtubule motor kinesin-1 (kinesin light chains 1/2 (KLC1/2); kinesin heavy chain (KHC)) and for EGFP. Myotubes were fixed with methanol, DNA was labeled with Hoechst dye. Scale = 10 μm. (D) Quantification of the degree of nuclear rotation in myotubes treated with EGFP or EGFP-nesprin2α-KASH (χ-square test; p = 0.18; shown are pooled data for *n*>140 nuclei from 19-21 myotubes). (E) Frequency distributions of the distance between adjacent nuclei in myotubes treated with EGFP- nesprin-2α-KASH (1 μm bin width; less than 8% of the data lies above 50 μm so distribution is truncated at 50 μm for clarity, *n* > 382 nuclei in >29 myotubes).

In order to more directly determine whether kinesin-1 drives translocation and/or rotation in myotubes, we reduced the expression of the motor with siRNA and evaluated the resulting effects on nuclear dynamics. Myotubes were transfected with siRNA against KIF5B, which is the only kinesin-1 isoform expressed in skeletal muscle (Kanai et al., 2000), on day 4 of differentiation and nuclear dynamics were assessed 72 hours later in D7 myotubes. We were able to consistently reduce the levels of KIF5B to ~20% percent of expression levels in mock siRNA-treated myotubes (Fig. 2.8A).

The mean nuclear translocation velocity in KIF5B deficient cells was reduced to 48.7 ± 3.5% (mean ± SEM) of mock-treated myotubes (Fig. 2.8B). Although we did note a small decrease in the mean rate of translocation in the KIF5B SCR control myotubes, the dynamics of nuclear movement in KIF5B-deficient myotubes were distinctly different than those in either the mock or KIF5B SCR control myotubes. Nuclei tended to pile on top of one another and squeeze together as a unit in the KIF5B-deficient myotubes rather than exhibiting persistent, independent translocation.

We found that depletion of KIF5B also led to a striking reduction in nuclear rotation (Fig. 2.8C,D and Video 8), similar to the effect of nocodazole (Fig. 2.5E). In a parallel experiment, we found that inhibiting kinesin-1 using a dominant negative GFP-KIF5C tail-domain construct, which acts to displace endogenous motors from cargos (Bi et al., 1997; Konishi and Setou, 2009), also induced a decrease in nuclear rotation (Fig. 2.8E). Together, these observations suggest that KIF5B drives nuclear dynamics by acting from the surface of the nucleus. Consistent with this hypothesis, we found that KHC is no longer localized to the nucleus in cells treated with siRNA-treated cells, although some residual signal for KLC1/2 persists (Fig. 2.8F). However, it is also possible that loss of the motor alters microtubule organization in myotubes, which may in turn impact on the dynamics of the nuclear movement. In >50% of KIF5B-deficient myotubes, the microtubule network was not different from that seen in control cells (Fig. 2.8G (SCR and siRNA panel 1); 58% of

myotubes, *n* = 50). In 42% of KIF5B-depleted cells, microtubules tended to form a more complex network in the center of the myotube, but only in the vicinity of nuclei. In these myotubes, microtubules appeared to wrap more frequently around the nuclei, with the direction of GFP-EB3 comets relative to the long-axis of the myotube more variable (Fig. 2.8G (siRNA panel 2); Video 9). However, in regions devoid of nuclei such as myotube ends, microtubule organization and polarity were indistinguishable from control myotubes (Fig. 2.8H; Video 9). We also noted KIF5B-deficient myotubes can develop organized myofibrils, as judged by YFP-α-actinin localization in D7 C2C12 cells (Fig. 2.8G, siRNA panel 1 inset). Kinesin-1-depleted myotubes were observed to contract spontaneously, again suggesting that these cells develop functional sarcomeres despite the mis-organization of the nuclei.

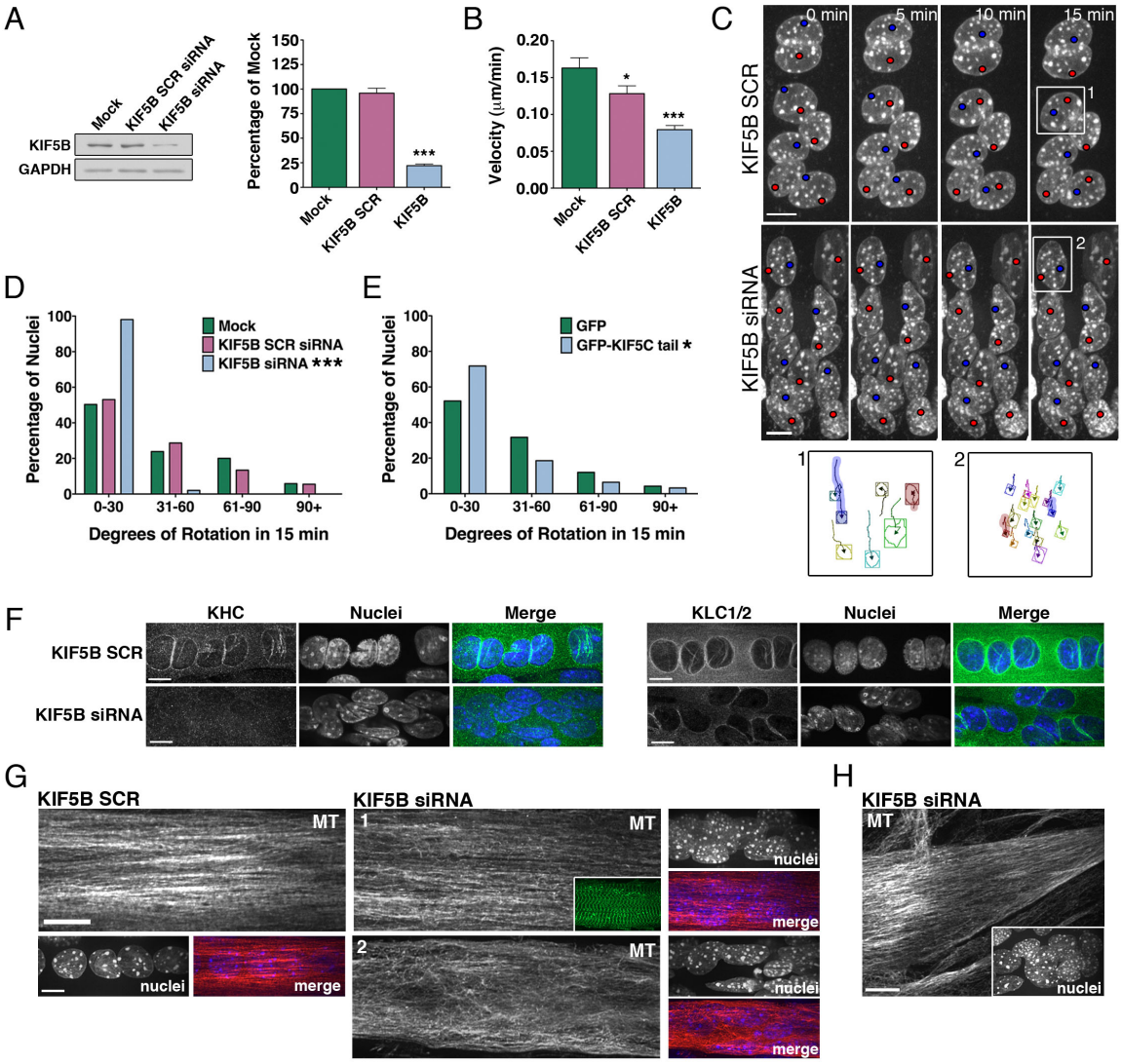

Figure 2.8. KIF5B acts from the nuclear surface to drive nuclear translocation and rotation in myotubes.

Figure 2.8. KIF5B acts from the nuclear surface to drive nuclear translocation and rotation in myotubes.

Myotubes were treated with siRNA to KIF5B or with scrambled control oligos (SCR). (A) Representative immunoblots and quantification for KIF5B in myotubes treated with siRNA and SCR siRNA for KIF5B; GAPDH serves as a loading control (mean ± s.e.m.; ANOVA, *n* = 3 independent replicates). (B) Mock- or siRNA-treated myotubes were imaged with phase microscopy for 60 min and absolute velocity was quantified (mean ± s.e.m.; ANOVA; *n*>50 nuclei in 10-12 myotubes). (C) Representative examples of nuclear rotation in myotubes treated with KIF5B SCR siRNA or KIF5B siRNA (Video 8). DNA was labeled with Hoechst dye and maximum projections of confocal z-stacks are shown over time on the left. Red and blue dots label chromocenters to aid visualization. Right panel highlights representative chromocenter tracks during rotation of the boxed nuclei on the left. Shaded tracks correspond to the chromocenters labeled with colored dots in the time series. Scale bars = 10 μm. (D, E) Quantification of the degree of nuclear rotation in myotubes treated with siRNA (D) or GFP-KIF5C tail dominant negative construct to disrupt KIF5B localization/function (E) (χ-square test, shown are pooled data for *n*>120 nuclei from 20-27 myotubes). (F) Treated myotubes were stained for subunits of the plus-end directed microtubule motor kinesin-1 (kinesin heavy chain (KHC); kinesin light chains 1/2 (KLC1/2)). DNA was labeled with Hoechst dye. Scale = 10 μm. (G & H) Treated myotubes were stained for tubulin; DNA was labeled with Hoechst dye. Representative images from the central region of the myotubes are shown in G (inset in siRNA panel 1 shows α-actinin staining). Representative image from the end of a KIF5B siRNA treated myotube is shown in H; inset shows the aggregation of nuclei from the center region of this myotube, which is to the left of the region of the myotube shown. Scale = 10 μm. For all panels, ∗, $p < 0.05$, ∗∗, $p < 0.01$, ∗∗∗, $p < 0.001$ vs. Mock or GFP.

Dynein also contributes to nuclear translocation and rotation

Antibodies to both dynein and dynactin reveal that the minus end-directed microtubule motor complex is also enriched at the nuclear periphery of myotubes, in addition to a strong cytosolic localization of the motor consistent with a broad range of cellular functions (Fig. 2.9). The localization of dynein and dynactin on the envelope was more irregular than that observed for KHC and KLC1/2 (Fig. 2.6). This may reflect a limitation of epitope accessibility, or may suggest that the dynein complex is less homogenously distributed across the nuclear surface than the kinesin-1 motor.

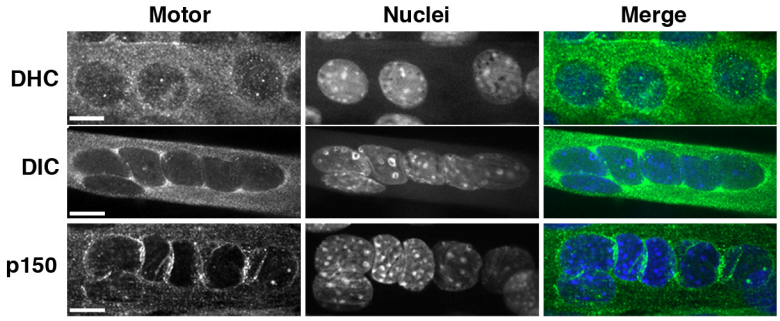

Figure 2.9. Dynein localizes to the nuclear envelope in myotubes.

Myotubes were stained for components of the minus-end directed dynein/dynactin complex (dynein heavy chain (DHC); dynein intermediate chain (DIC); $p150^{Glued}$ subunit of dynactin). Myotubes were fixed with methanol for p150, and with 1:1 acetone-methanol for DHC and DIC. DNA was labeled with Hoechst dye. Scale = 10 μm.

To test the role of dynein in nuclear dynamics, we used siRNA to deplete the motor. We were able to consistently reduce the levels of DHC to ~20% percent of expression levels in mock siRNA-treated myotubes after 72 hours of knock-down (Fig. 2.10A). DHC siRNA concomitantly reduces levels of DIC, suggesting that the dynein complex is destabilized, as has been observed in other cell types (Caviston et al., 2007; Levy and Holzbaur, 2008). Immunofluorescence analysis of myotubes treated with DHC siRNA demonstrate a depletion of DHC and DIC throughout the cell (Fig. 2.10B). Although western blot analysis indicates that overall expression levels of $p150^{Glued}$ were not reduced in cells treated with DHC siRNA (not shown), immunostaining for $p150^{Glued}$ suggests that dynactin may also be depleted from the nuclear envelope in dynein-depleted myotubes (Fig. 2.10B).

Knockdown of DHC reduced the average rate of nuclear translocation and rotation (Fig. 2.10C,D). However, the effect of dynein depletion on nuclear dynamics was not as striking as observed for the kinesin-1 motor. Mean nuclear translocation velocity in dynein-deficient cells was reduced to 64.7% of mock-treated myotubes (Fig. 2.10D), with 25% of the nuclei continuing to rotate more than 30° in 15 min, as compared to only 2% of nuclei in KIF5B-deficient myotubes (Figs. 2.10E, 2.8D). These data suggest that while both dynein and KIF5B contribute to nuclear dynamics, the plus-end directed KIF5B is the more dominant motor.

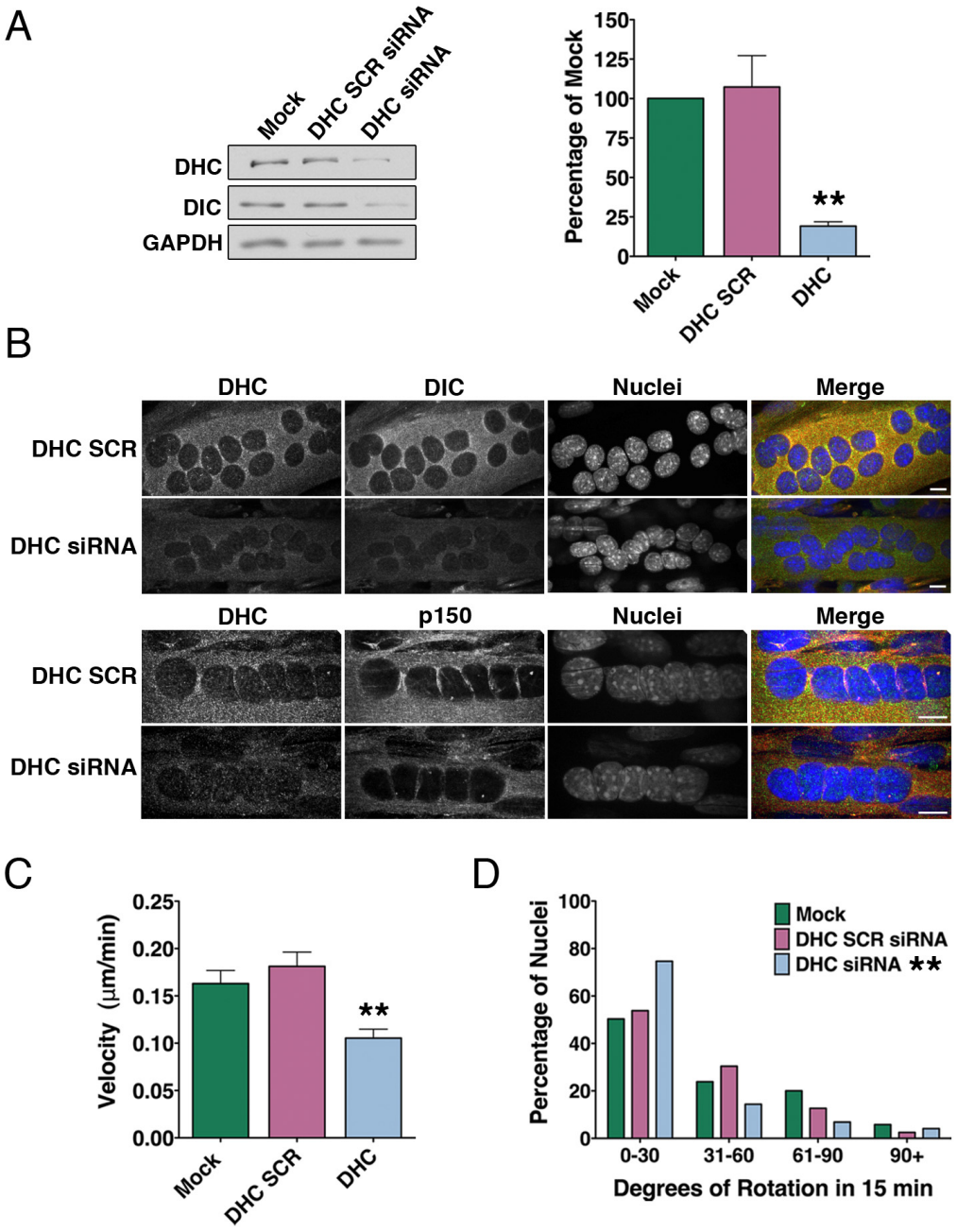

Figure 2.10. Dynein drives nuclear translocation and rotation.

(A-D) Myotubes were treated with siRNA to DHC, or with scrambled control oligos (SCR).

(A) Representative immunoblots and quantification for dynein heavy chain (DHC) and dynein intermediate chain (DIC) in myotubes treated with siRNA for DHC; GAPDH serves as a

loading control (mean ± s.e.m.; ANOVA, *n* = 3 independent replicates). (B) Myotubes treated with DHC siRNA or DHC SCR siRNA were stained for DHC, DIC, & $p150^{Glued}$. DNA was labeled with Hoechst dye. Scale = 10μm. (C) Mock- or siRNA-treated myotubes were imaged with phase microscopy for 60 min and absolute velocity was quantified (mean ± s.e.m.; ANOVA; *n*>50 nuclei in 10-12 myotubes). (D) Quantification of the degree of nuclear rotation in myotubes treated with DHC siRNA. Data for siRNA is compared to the same Mock data as in Fig. 2.8D (χ-square test, shown are pooled data for *n*>120 nuclei from 20-27 myotubes). For all panels, **, $p < 0.01$.

Motor-dependent nuclear dynamics are necessary for proper nuclear distribution in myotubes

After seven days of differentiation, myotubes display a relatively even distribution of nuclei along the length of control cells (Fig. 2.1A; Fig. 2.11A,B). However, we noted that the nuclei in myotubes deficient for KIF5B tended to densely aggregate at the midline of the cell (Fig. 2.11A,B). This aggregation was also observed in myotubes expressing the GFP-KIF5C tail construct. In myotubes treated with siRNA for DHC, we observed some nuclear aggregation, but these aggregates were not enriched toward the myotube center as seen with kinesin-1 disruption. Instead, clusters of nuclei were observed along the length of the myotube in dynein-depleted cells (Fig. 2.11A,B).

Mean myotube length was not different between control and treated myotubes, nor did we detect a difference in the number of nuclei per myotube. However, the distance between nuclei was noticeably reduced when either kinesin or dynein function was disrupted (Fig. 2.11C,D). The mean distance between adjacent nuclei was significantly smaller in KIF5B-depleted or dynein-depleted myotubes (Fig. 2.11D), and disruption of kinesin function

using a dominant negative approach (expression of GFP-KIF5C tail) also significantly reduced the average distance between nuclei (Fig. 2.11D). These data indicate that proper nuclear dynamics are necessary to prevent aggregation of nuclei. Live cell data suggest that nuclei need to rotate in order to pass one another. As nuclei in myotubes treated with siRNA to KIF5B or dynein do not rotate normally (Figs. 2.8D, 2.10D), reduced rotation may be sufficient to induce nuclear aggregation.

To further compare the effects of motor depletion or inhibition on nuclear localization, we plotted the position of nuclei as a function of distance along the myotube length (Fig. 2.11E). Nuclei in KIF5B-deficient myotubes clustered near the midline as compared to control myotubes. This same pattern was seen for cells treated with GFP-KIF5C tail. Conversely, despite their increased propensity to aggregate, the nuclei in dynein-deficient myotubes were found along the full myotube length, similar to the distribution of nuclei in the control cells (Fig. 2.11E). This suggests, that in addition to driving rotation and preventing aggregation, KIF5B is also necessary for proper translocation of the nuclei out toward the ends of these elongated cells.

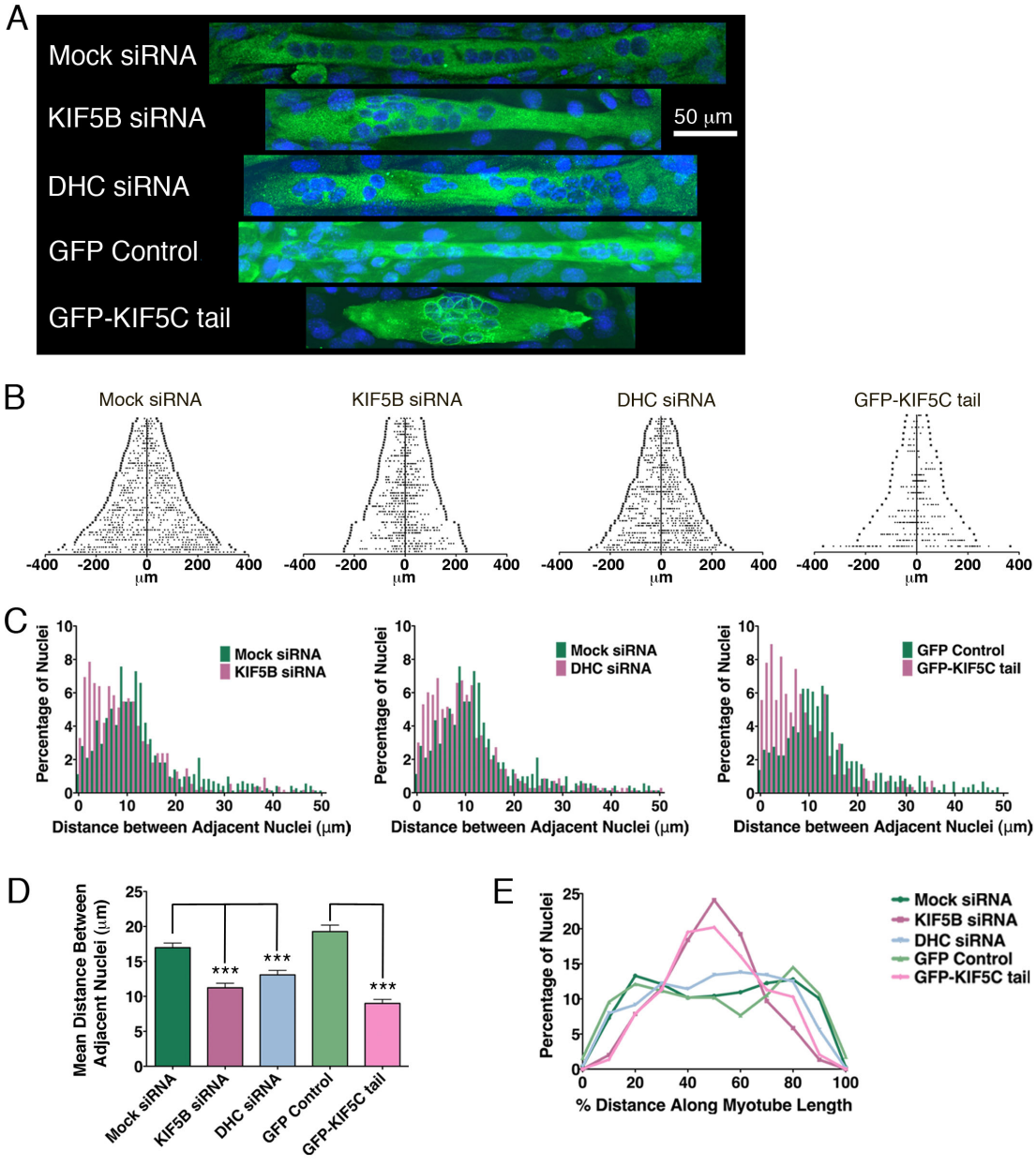

Figure 2.11. Motor-dependent nuclear dynamics are necessary for proper distribution of nuclei in myotubes.

Figure 2.11. Motor-dependent nuclear dynamics are necessary for proper distribution of nuclei in myotubes.

Myotubes were transfected with either siRNA + GFP, GFP or GFP-KIF5C tail. (A) Representative images of treated myotubes. Nuclei are stained with Hoechst dye, images represent maximum projections of confocal z-series. (B) Nuclear distribution in myotubes. Each line on the y-axis represents an individual myotube, organized according to length (*n* = 42-55 siRNA myotubes; 23 myotubes GFP-KIF5C tail). The ends of the myotube are marked with a square; circles represent individual nuclei. (C) Frequency distributions of the distance between adjacent nuclei in myotubes treated with siRNA or GFP-KIF5C tail (1 μm bin width; less than 8% of the data lies above 50μm so distributions are truncated at 50 μm for clarity). (D) Mean distance between adjacent nuclei (mean ± s.e.m.; ANOVA, ***, $p < 0.001$). (E) Histogram depicts the position of nuclei as a percent of the distance along the myotube length (bin width = 10%).

IV. Discussion

Nuclei in developing muscle cells are very mobile (Capers, 1960). We propose that this mobility is necessary to achieve an even distribution of nuclei along the length of mature myofibers. Consistent with this hypothesis, we find that the microtubule motor proteins kinesin-1 and dynein are required for normal nuclear dynamics in developing myotubes. Depletion or inhibition of these motors leads to nuclear aggregation and abnormal nuclear distribution. The motor proteins localize to the nuclear envelope, likely mediated at least in part by interactions with the KASH proteins of the LINC complex, and drive both nuclear translocation and rotation along the microtubule network.

The nuclear dynamics we observe in C2C12 myotubes are consistent with initial observations in primary chick myotubes (Capers, 1960; Cooper and Konigsberg, 1961; Cooper, 1958; Englander and Rubin, 1987). We find that nuclei translocate along the long-axis of the myotube, rotate in all dimensions and can even pass one another. Both primary and C2C12 myotubes exhibit similar developmental patterns to what has been described *in vivo*, including microtubule arrangement and myofibril assembly (Sanger et al., 2010; Warren, 1974). Thus, the nuclear dynamics described here likely inform on the dynamics that occur during vertebrate myotube development.

These nuclear dynamics require an intact microtubule network (Fig. 2.5). Nuclear rotation was abolished by microtubule depolymerization. While translocation was inhibited in nocodazole-treated cells, some residual nuclear movement suggests that there could be additional forces involved, such as contractions of the myofibril network. Myofibrils are often in close contact with nuclei and their contractions might also influence nuclear movement (Video 10). To investigate this possibility, we inhibited contraction with N-benzyl-ptoluene sulphonamide (BTS), a specific inhibitor of skeletal muscle myosin (Cheung et al., 2002), and found that the nuclei were still able to translocate and rotate (Video 11). Thus, myofibril

contraction is likely to play only a minor role. During our phase imaging of myotube dynamics, we often observed bulk fluid dynamics within the cell that resemble the cytoplasmic streaming described in plants and *Drosophila* oocytes (Serbus et al., 2005; Shimmen, 2007)(Video 1). This flow may also contribute to nuclear dynamics and organelle distribution in myotubes, either directly, or indirectly by influencing the polarity of the microtubule network.

Our observations indicate that kinesin-1 is the primary motor driving nuclear dynamics in myotubes (Fig. 2.8), while dynein contributes to a lesser extent. Though dynein is the primary driver of nuclear movement in migrating neurons (Shu et al., 2004; Tsai et al., 2007; Vallee et al., 2009) and nuclear rotations in fibroblasts (Levy and Holzbaur, 2008), in C. *elegans* hypodermal precursor cells, kinesin-1 activity predominates (Fridolfsson and Starr, 2010). Kinesin has a significantly higher stall force than mammalian dynein (~5-6 pN for kinesin vs. 1.1 pN for dynein; see (Hendricks et al., 2010) and references therein). Thus, within the complex cellular environment of the myotube, which is densely packed with developing myofibrils, kinesin-1 may be a more effective driver of the motility of this large (~10 μm diameter) organelle. The localization of both kinesin and dynein to the nucleus suggests that a population of motors acts from the nuclear surface to drive both nuclear translocation and rotation. Reduced nuclear rotation observed in myotubes expressing either the GFP-KIF5C tail or EGFP-nesprin-2α-KASH dominant negative constructs also argues for nuclear-based motor activity, since the localization of these constructs to the nuclear envelope can displace endogenous motors.

KIF5B binds to the nuclear envelope in myotubes, at least in part, through interactions with nesprins (Fig. 2.7), outer nuclear membrane KASH proteins known to connect the nuclear lamina with the cytoskeleton (Starr and Fridolfsson, 2010). Our data confirm the interaction between kinesin-1 and nesprin-2, mediated by binding to kinesin light chain (Schneider et al., 2011); however, nesprin-1 may also contribute to kinesin recruitment

to the nucleus. Dynein and dynactin have been shown to precipitate with nesprin-1 and -2 from mouse brain lysate (Zhang et al., 2009), although dynein did not bind to the nesprin construct used here. The interaction between dynein and nesprins may be mediated through a different domain, or may be recruited to the nuclear envelope through other binding partners. Mature skeletal muscle fibers and C2C12 myotubes express both nesprin-1 and nesprin-2, with the relative expression of alternatively spliced isoforms modulated over the course of muscle development (Apel et al., 2000; Randles et al., 2010; Zhang et al., 2005; Zhang et al., 2001). Evidence from knock-out mice suggests that nesprin-1 and nesprin-2 are both critical in muscle for nuclear positioning and anchorage (Zhang et al., 2010; Zhang et al., 2007b). Moreover, mutations in both nesprin-1 and nesprin-2 have been found in patients with Emery-Dreifuss muscular dystrophy, suggesting that nuclear positioning and/or anchorage in skeletal muscle is essential for proper muscle function (Zhang et al., 2007a).

We propose that nuclear-bound motors influence nuclear dynamics by exerting force on the local microtubule network. The net sum of all forces would dictate the direction and speed of nuclear rotation and translocation. Kinesin distribution at the nuclear envelope appears patchy, whereas the GFP-KIF5C tail construct decorates the envelope more evenly, suggesting that under normal conditions not all of the kinesin binding sites on the envelope are occupied. A non-uniform distribution of KIF5B on the envelope could create asymmetrical areas of force generation that may lead to nuclear rotation. The dynein motor complex also appears to be non-uniformly distributed, best illustrated by the staining for the dynactin subunit $p150^{Glued}$, which shows enhanced accumulation between adjacent nuclei (Fig. 2.9). An uneven distribution of oppositely directed motors is also likely to lead to force imbalances that further influence nuclear dynamics.

The microtubule network in myotubes forms a dynamic bidirectional array along the long-axis of the cell, surrounding the centrally located nuclei ((Tassin et al., 1985; Warren, 1974) and see Fig. 2.5). However, microtubules are also found between adjacent nuclei and

lie within invaginations on the nuclear surface. Most or all of these microtubules are dynamic and therefore the network surrounding a given nucleus is always changing, which will significantly influence how the motors on the surface of an individual nucleus contribute to nuclear dynamics. Most myotubes exhibited a slight overall bias in microtubule growth directed toward one end of the myotube. We also noticed that in many myotubes, the net direction of nuclear movement was toward one end of the cell. It is possible that a bias in the direction of overall microtubule growth correlates with the net direction of nuclear movement, especially given the recent finding that even subtle biases in microtubule polarity are sufficient to direct cargo transport (Parton et al., 2011). Therefore, it is likely that the nuclear dynamics are influenced both by motors bound to the nuclear envelope, and by dynamics and polarity of the microtubule network (see model, Fig. 2.12).

The distribution of nuclei along the length of C2C12 myotubes was most severely disrupted upon depletion of KIF5B, leading to nuclear aggregation near the midline of the myotube (Fig. 2.11). Similarly, loss of kinesin-1 function in *Drosophila* embryos also caused central aggregation of myonuclei in a recent study (Metzger et al., 2012). This is in line with our observation that loss of KIF5B more substantially inhibited nuclear translocation and rotation. We hypothesize that the rotation of nuclei is essential to fluidize the dynamics of these large organelles. The decreased mobility in KIF5B-deficient myotubes may inhibit nuclei from moving around obstacles in their path, including other nuclei, myofibrils, or even the dense microtubule network surrounding the nucleus. In live cell recordings, one or both of the nuclei involved in passing events rotated as they moved past one another, consistent with this hypothesis. Thus, loss of nuclear rotation in the kinesin-depleted myotubes may be a critical factor leading to nuclear aggregation at the cell center. Loss of dynein also caused aggregation of nuclei, although the nuclei were still able to distribute along the length of the myotube. This suggests that although kinesin may be the primary driver, dynein is also

needed for proper nuclear spacing. Loss of dynein-driven mobility might cause nuclear traffic jams, which appear as localized aggregates at discrete sites along the cell length.

While motor-driven rotation and translocation from the nuclear surface appears to be the dominant driver of movement in this system, the process may have a feed-forward component. Microtubules in the myotube are nucleated in part from the surface of nuclei (Tassin et al., 1985; Zaal et al., 2011), polymerizing in all directions (Tassin et al., 1985). Thus nuclei actively contribute to the development of a bidirectional microtubule array in myotubes. Since loss of kinesin activity would be expected to be most significant at myotube ends where microtubules are polarized with plus-ends oriented outward, as kinesin is depleted, the nuclei begin to aggregate toward the center of the myotube, and microtubule organization in cell areas depleted of nuclei becomes more unipolar and less bipolar (Fig. 2.8H and Video 9). Further, the local microtubule network surrounding the aggregated nuclei becomes increasingly disordered; we observed a direct correlation between degree of nuclear clumping and local disorganization of the microtubule cytoskeleton, consistent with this model (Fig. 2.8G and data not shown). Together, these processes create a progressively more complex cytoskeletal network for the nuclei to navigate through, thereby further reducing nuclear translocation and rotation and further enhancing aggregation over time.

Thus, we propose that the dynamics of the nuclei in this system are stochastic, dictated by the organization and dynamics of the microtubule network surrounding an individual nucleus as well as the complement of motors on its surface. This allows for a fluid distribution of nuclei in the myotube; as differentiation continues, the distributed nuclei will become anchored in place under the sarcolemma in myofibers. It has been proposed that proper nuclear positioning is necessary to ensure sufficient transcriptional capacity and minimize transport distances in the myofiber (Bruusgaard et al., 2003), which likely affects muscle function. Indeed, nesprin-1 null mice, exhibit abnormal distribution of nuclei, which

correlates with significantly decreased exercise capacity (Zhang et al., 2010). Similarly, *Drosophila* mutant larvae expressing mutant MAP7 display nuclear positioning defects during muscle development, which correlates with decreased locomotion (Metzger et al., 2012), again suggesting that normal nuclear distribution is required for normal muscle function.

Nuclear dynamics may also contribute to the ability of skeletal muscle fibers to repair themselves after injury. During fiber repair, myogenic cells fuse with the injured fiber, contributing a new nucleus (Charge and Rudnicki, 2004). These nuclei are positioned at the periphery of the myofiber (Li et al., 2011; Rich and Lichtman, 1989; Terada et al., 2009), and it is likely that both nuclear translocation and rotation are required for this positioning. In diseases like the muscular dystrophies, where mechanically induced muscle damage occurs frequently, nuclear dynamics may be essential for proper fiber repair and subsequent muscle function.

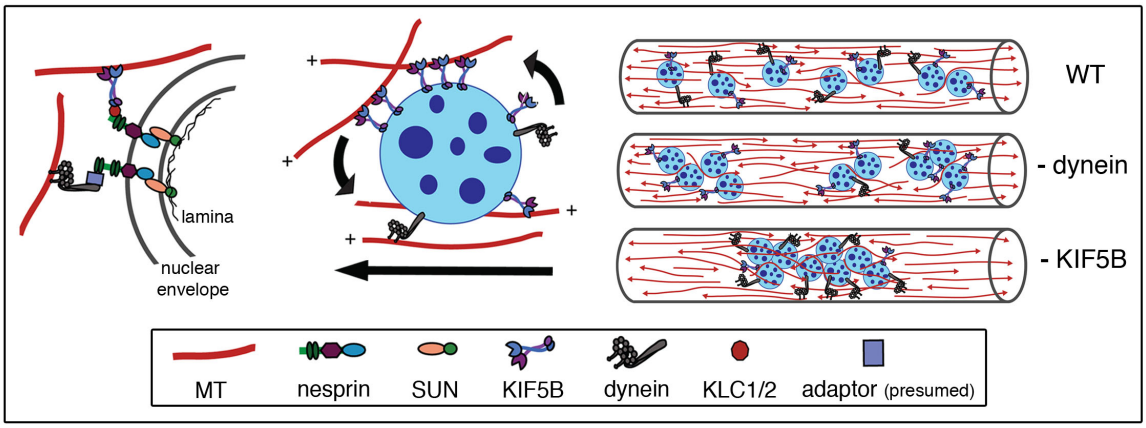

Figure 2.12. Model depicting the roles of opposing kinesin and dynein motors in the nuclear dynamics in developing myotubes.

Motors are likely bound to the nucleus by connections with nesprin-1 or -2. The number and distribution of opposing motors on a nucleus and the polarity of the local microtubule network determine the direction and speed of rotation and translocation. Depletion of either dynein or KIF5B from myotubes causes abnormal aggregation and inappropriate dispersal of nuclei along the length of the myotube.

CHAPTER 3: Nesprins Anchor Kinesin-1 Motors to the Nucleus to Drive Nuclear Distribution in Developing Muscle Cells

A version of this chapter has been submitted for publication as a manuscript entitled:

Wilson, M.H., and Holzbaur, E.L. Nesprins Anchor Kinesin-1 Motors to the Nucleus to Drive Nuclear Distribution in Muscle Cells.

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I. Summary

During skeletal muscle development, nuclei move dynamically through the cell in a microtubule-dependent manner. The microtubule motor protein kinesin-1 drives nuclear dynamics in developing myotubes. Loss of kinesin-1 leads to improperly positioned nuclei in culture and *in vivo*. Two models have been proposed to explain how kinesin-1 functions to move nuclei in myotubes. In the cargo model, kinesin-1 acts directly from the surface of the nucleus, whereas in an alternative model, kinesin-1 moves nuclei indirectly by sliding anti-parallel microtubules. Here, we use two experimental strategies to test the hypothesis that an ensemble of Kif5B motors acts from the nuclear envelope to distribute nuclei throughout the length of syncytial myotubes. First, using an inducible dimerization system, we show that controlled recruitment of truncated, constitutively active kinesin-1 motors to the nuclear envelope is sufficient to prevent the nuclear aggregation resulting from depletion of endogenous kinesin-1. Second, we identify a conserved kinesin light chain (KLC) binding motif in the nuclear envelope protein nesprin-2 and show that recruitment of the motor complex to the nucleus via this LEWD motif is essential for nuclear distribution. Together, our findings demonstrate that the nucleus is a kinesin-1 cargo in myotubes and that nesprins function as nuclear cargo adaptors. The importance of achieving and maintaining proper nuclear position is not restricted to muscle fibers, suggesting that the nesprin-dependent recruitment of kinesin-1 to the nuclear envelope by binding of the conserved LEWD motif to kinesin light chain is a general mechanism for cell-type specific nuclear positioning during development.

II. Introduction

The position of the nucleus in a cell is dynamically controlled by interactions between the nuclear envelope and the cytoskeleton, and defects in nuclear positioning are often associated with cellular dysfunction and human disease, including muscular dystrophy, cardiomyopathy, lissencephaly, and hearing loss (Gundersen and Worman, 2013; Starr and Fridolfsson, 2010). Proper nuclear positioning is especially critical in skeletal muscle cell. Skeletal muscle fibers are large multinucleated cells formed by the fusion of post-mitotic myoblasts. The nuclei in mature muscle fibers are anchored under the sarcolemma at the periphery of the cell, and are positioned to maximize internuclear distances, which is suggested to minimize transport distances between the nuclei and the cytoplasm (Bruusgaard et al., 2003). Abnormal aggregation or mispositioning of nuclei are noted in a number of muscle diseases in humans (Mattioli et al., 2011; Romero, 2010) and correlates with muscle weakness and dysfunction in model organisms (Metzger et al., 2012; Wang et al., 2013a), suggesting that nuclear positioning may be required for proper muscle cell function.

During skeletal muscle development, nuclei move dynamically through the cell in a microtubule-dependent manner (Englander and Rubin, 1987; Wilson and Holzbaur, 2012). These dynamics include both translocation and rotation in three dimensions along the long axis of the cell and both are attenuated by depolymerization of the microtubule network (Capers, 1960; Wilson and Holzbaur, 2012). Recently it has become clear that the microtubule motor proteins kinesin-1 (Kif5B) and cytoplasmic dynein power these nuclear dynamics (Cadot et al., 2012; Folker et al., 2012; Folker et al., 2014; Metzger et al., 2012; Wilson and Holzbaur, 2012). Loss of either the plus-end directed kinesin-1 motor or the minus-end directed dynein motor leads to reduced rates of translocation and rotation, although this effect was more pronounced in myotubes depleted of kinesin-1 (Wilson and

Holzbaur, 2012). Furthermore, loss of Kif5B causes dramatic aggregation of nuclei at the mid-line of the cell in culture mouse muscle cells (Metzger et al., 2012; Wilson and Holzbaur, 2012), as well as *in vivo* in the muscles of both *Drosophila* larvae (Metzger et al., 2012) and mice (Wang et al., 2013a).

While it is clear that Kif5B is essential for nuclear dynamics and positioning, two models have been proposed to explain how Kif5B functions to move nuclei in myotubes. In the model proposed by Metzger et al., Kif5B interacts with the microtubule-associated protein MAP7/ensconsin to slide anti-parallel microtubules, thereby pushing adjacent nuclei apart (Metzger et al., 2012)(Fig. 3.1). Alternatively, we have proposed that Kif5B acts from the nuclear surface, moving the nucleus as a giant cargo along the local microtubule network (Wilson and Holzbaur, 2012)(Fig. 3.1). The major evidence for this latter model is immunohistochemistry showing accumulation of components of endogenous kinesin-1 on the nuclear envelope in cultured C2C12 myotubes (Wilson and Holzbaur, 2012) and more recently in primary mouse myotubes (Wang et al., 2013b).

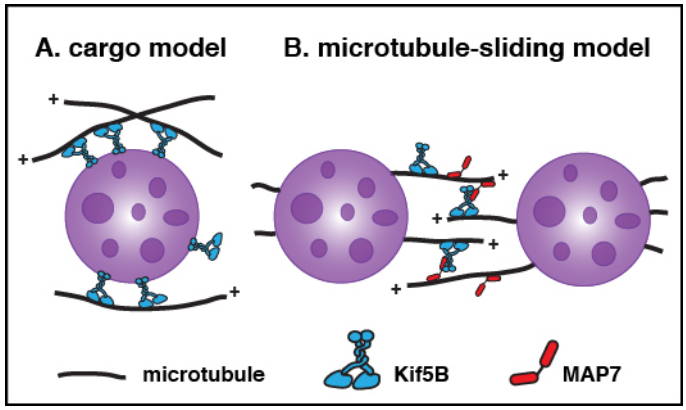

Figure 3.1. Proposed models of Kif5B-dependent nuclear movement in myotubes.

The kinesin-1 complex is a heterotetramer, composed of a dimer of Kif5 kinesin heavy chains (KHC) and two regulatory kinesin light chains (KLC). In a number of cell types, including *C. elegans* hypodermal precursor cells (Meyerzon et al., 2009) and mammalian epithelial cells (Horn et al., 2013; Roux et al., 2009; Schneider et al., 2011), KLC mediates the association of KHC with the nuclear envelope through interactions with members of the KASH (Klarsicht ANC-1 and SYNE Homology) family of proteins. These proteins, including the vertebrate nesprins, interact via their KASH domain in the perinuclear space with SUN (Sad1, UNC-84) proteins that span the inner nuclear membrane and associate with the nuclear lamina (Starr and Fridolfsson, 2010). Immunocytochemistry in mammalian muscle cells shows that KLC is present on the nuclear envelope (Wilson and Holzbaur, 2012), whereas KLC mutants in *Drosophila* do not show defects in nuclear positioning in muscle (Metzger et al., 2012). Therefore, it is still unclear whether KLC interacts with KASH proteins to mediate the binding of Kif5B to the nuclear envelope in muscle.

However, evidence from knock-out mice suggests that nesprin-1 and nesprin-2 are critical in both skeletal muscle and cardiomyocytes for nuclear positioning (Banerjee et al., 2014; Zhang et al., 2010; Zhang et al., 2007b). Moreover, mutations in both nesprin-1 and nesprin-2 have been found in patients with Emery-Dreifuss muscular dystrophy (Zhang et al., 2007a), which is characterized by progressive skeletal muscle weakness and cardiomyopathy. These findings not only suggest that nuclear positioning in muscle cells is essential for proper muscle function, but that an interaction between kinesin-1 and nesprins may facilitate the nuclear dynamics that are necessary for proper nuclear positioning.

In this study, we use two experimental strategies to directly test the cargo model of Kif5B-dependent nuclear positioning in myotubes. First, we show that controlled recruitment of a truncated, constitutively active Kif5B motor to the nuclear envelope is sufficient to prevent the nuclear aggregation resulting from depletion of endogenous Kif5B. We identify a conserved KLC-binding motif in nesprin-2, and show that this interaction is required for the

localization of kinesin-1 motors to the nuclear membrane. Furthermore, we show that specific point mutations in KLC that disrupt the interaction with nesprins are sufficient to induce nuclear aggregation, again showing that kinesin-1 is required on the nuclear envelope for proper distribution of nuclei in myotubes. Together, our findings demonstrate that the nucleus is a kinesin-1 cargo in myotubes and that nesprins function as nuclear cargo adaptors.

III. Results

Recruitment of exogenous Kif5B to the nuclear envelope rescues nuclear distribution in myotubes

Depletion of the Kif5B kinesin heavy chain results in dramatic aggregation of nuclei at the mid-line of C2C12 mouse myotubes (Fig. 3.2A,C). Contrary to the effect of mutant KLC in *Drosophila* muscles (Metzger et al., 2012), nuclear aggregation was also evident in myotubes treated with siRNA to both KLC1 and KLC2 (Fig. 3.2B,C). Immunohistochemistry suggests that both Kif5B and KLC accumulate at the nuclear envelope in myotubes (Wilson and Holzbaur, 2012)(Fig. 3.2D). Here we confirm the localization of the kinesin-1 motor complex on the nuclear envelope in C2C12 myotubes by expression of Myc-Kif5B-Halo and EGFP-KLC2 constructs in live cells (Fig. 3.2E). Further, we noted loss of Myc-Kif5B-Halo signal from the nuclear envelope in myotubes treated with siRNA to KLC1 & 2 (Fig. 3.2E), supporting the hypothesis that KLC mediates the recruitment of KHC to the nucleus.

To test the hypothesis that Kif5B acts from the nuclear surface to drive nuclear movement, we used an inducible cargo trafficking assay (Kapitein et al., 2010a; Kapitein et al., 2010b) to recruit a truncated, constitutively active Kif5B motor to the nuclear envelope (Fig. 3.3A). This system takes advantage of ligand-mediated heterodimerization of FKBP and FRB protein domains by a cell-permeant non-immunosuppressive analog of rapamycin or rapalog (Clackson et al., 1998; Spencer et al., 1993). By targeting the FKBP domain to a cargo of interest and attaching the FRB domain to an active motor construct, ligand treatment can be used to control motor recruitment to the cargo.

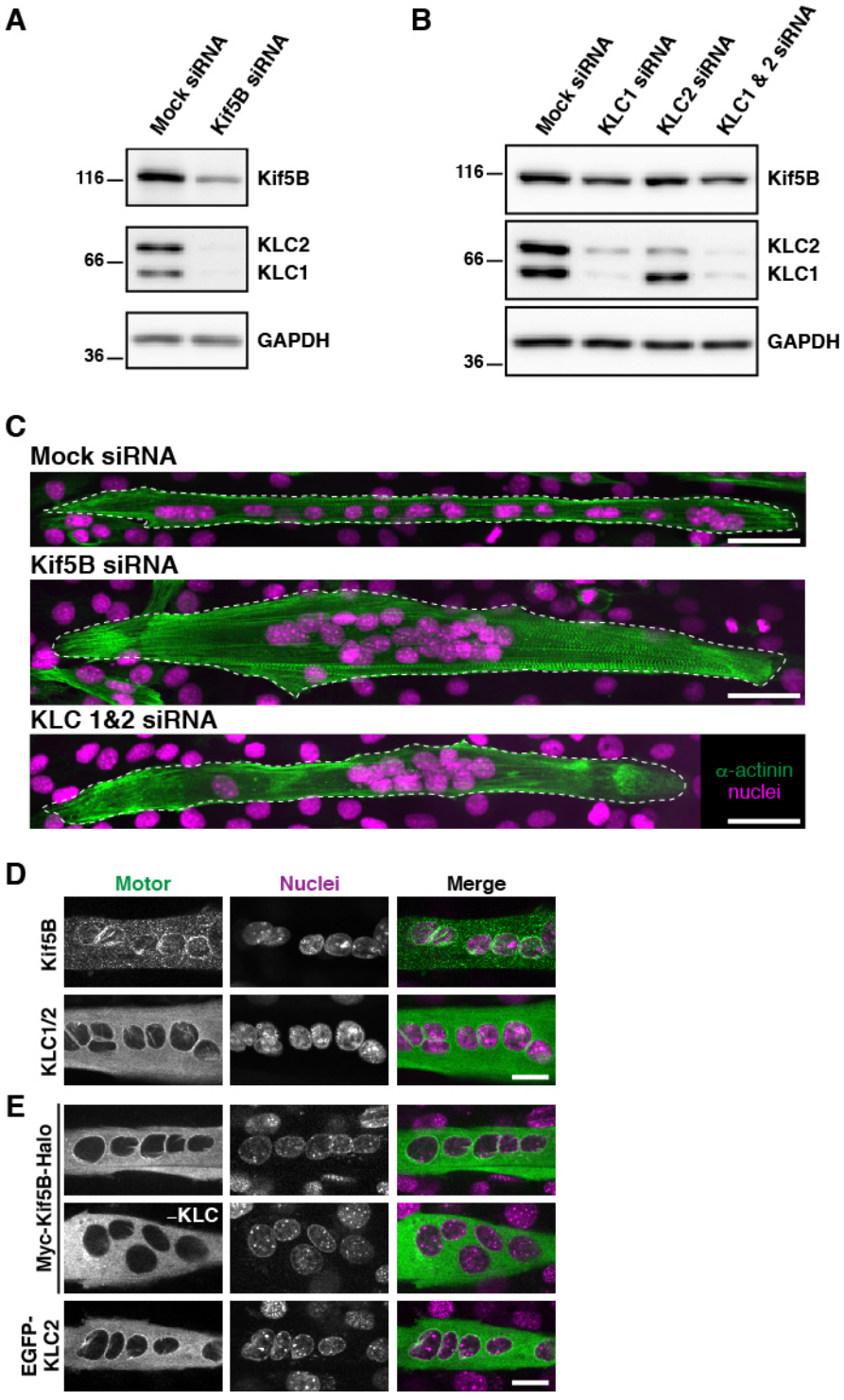

Figure 3.2. Depletion of Kif5B and KLC results in nuclear aggregation.

Figure 3.2. Depletion of Kif5B and KLC results in nuclear aggregation.

(A, B) Representative immunoblots of Kif5B and KLC in myotubes treated with siRNA to Kif5B (A) or KLC1 and KLC2 (B); GAPDH serves as a loading control. Levels of kinesin-1 motor complex components appear to be coordinately regulated, as siRNA to either heavy chain or light chain concomitantly reduces both motor components. (C) Representative images of C2C12 myotubes treated with siRNA to Kif5B, kinesin light chains 1 & 2 or mock transfected. Nuclei were stained with Hoechst dye (magenta); α-actinin is in green. Images are maximum projections of confocal z-sections. Scale bar = 50 μm. (D) Myotubes were fixed with MeOH and immunostained for the Kif5B heavy chain (mAb SUK4) or KLC1 & 2 (mAb 63-90); nuclei are labeled with Hoechst dye. Scale = 20 μm. (**E**) Myotubes were either transfected with Myc-Kif5B-Halo & labeled with Halo-TMR ligand, or transfected with EGFP-KLC2 with or without siRNA to KLC1 & 2. Scale = 20 μm.

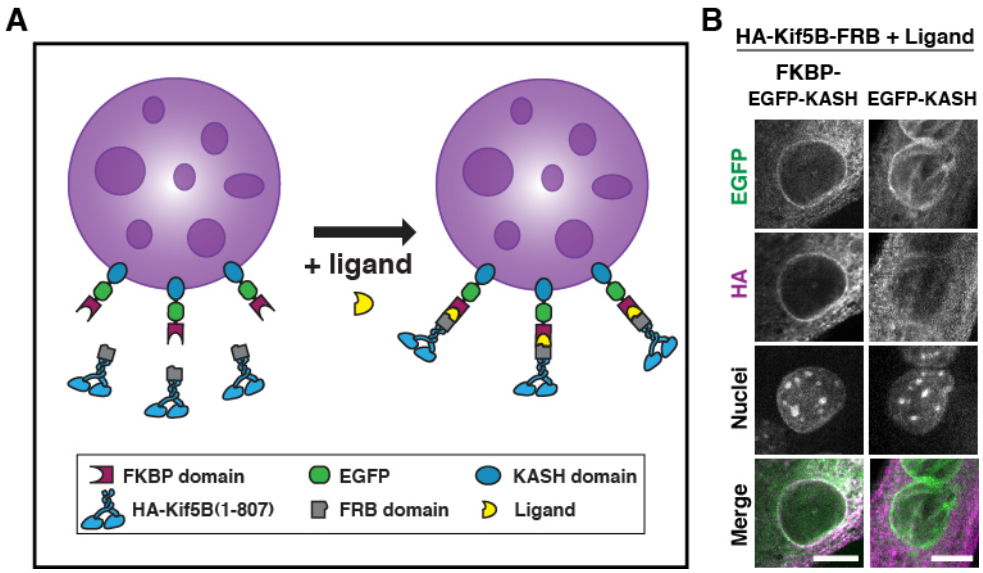

Figure 3.3. Design of the Kif5B Recruitment System.

(A) Depiction of fusion constructs for induced recruitment of truncated Kif5B to the nucleus. FKBP-EGFP-KASH targets the nuclear envelope; HA-Kif5B(1-807)-FRB motor constructs are cytosolic. Following ligand addition, heterodimerization of FKBP-FRB domains anchors constitutively active Kif5B(1-807) to the nuclear envelope. (B) Nuclei in myotubes expressing FKBP-EGFP-KASH successfully recruit HA-Kif5B(1-807)-FRB to the nuclear surface with ligand treatment, whereas nuclei coated with EGFP-KASH do not recruit the motor construct. Ligand was continuous post-transfection at a concentration of 500 nM. Myotubes were fixed ~84 hours post-transfection and immunostained for HA and EGFP. Scale = 10 μm.

We verified the feasibility of this system in C2C12 myotubes by inducing recruitment of HA-Kif5B(1-807)-FRB to FKBP-decorated peroxisomes. Upon ligand addition, these peroxisomes exhibited directed motility toward the plus-ends of microtubules and accumulated at the ends of the myotube, indicating successful ligand-mediated motor recruitment (Fig. 3.4).

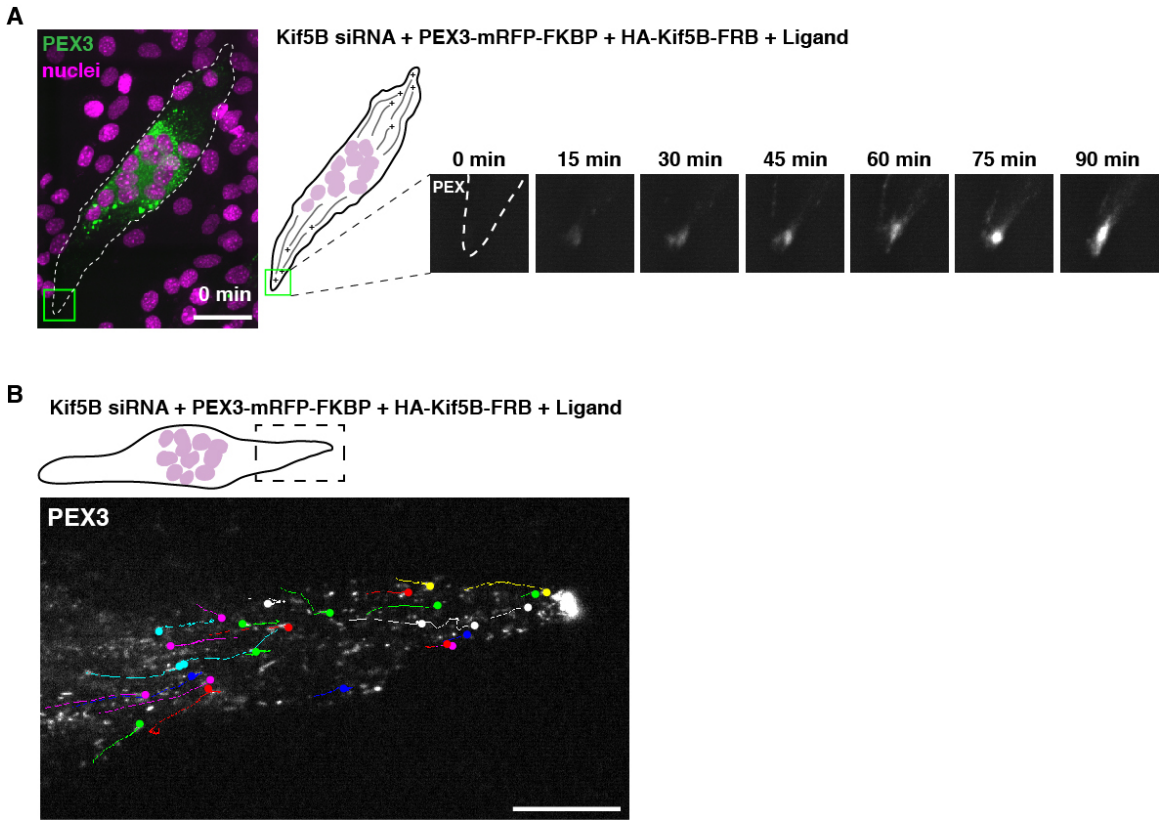

Figure 3.4. Kif5B is recruited successfully to peroxisomes in myotubes.

(A) Myotubes were treated with siRNA to Kif5B to deplete endogenous motors from the cell. Myotubes were also transfected with HA-Kif5B(1-807)-FRB and PEX3-mRFP-FKBP, which targets peroxisomes. Upon ligand addition (1 μM) labeled peroxisomes exhibit directed motility toward the plus-ends of microtubules and begin to accumulate at the ends of the myotube, indicating successful ligand-mediated motor recruitment. Scale = 50 μm. (B)

Movement of individual PEX3-mRFP-FKBP decorated peroxisomes was imaged at higher magnification and time resolution (20 frames per minute) following 60 minutes of ligand treatment. Peroxisomes were tracked manually and tracks are overlaid on the last frame (colored dots). Tracks shown reflect ~90 seconds of imaging. Note that the majority of peroxisomes move toward the end of the myotube where they accumulate. Scale = 20 μm.

Next, we adapted the system for controlled recruitment of the HA-Kif5B(1-807)-FRB construct to the nuclear envelope. We fused the FKBP domain to the C-terminal transmembrane and KASH domains of the nuclear envelope protein nesprin-2 (FKBP-EGFP-KASH)(Fig. 3.3B). Using this strategy, we found that FKBP-EGFP-KASH and a control construct lacking the FKBP domain (EGFP-KASH) both localized to the nuclear envelope in myotubes. However, with ligand treatment, only nuclei decorated with FKBP-EGFP-KASH successfully recruited HA-Kif5B-FRB to their surface (Fig. 3.3B).

To test the hypothesis that kinesin acts from the nuclear surface to properly distribute nuclei in myotubes, we decreased levels of endogenous Kif5B with siRNA and used our inducible heterodimerization system to selectively restore constitutively active kinesin motors to the nuclear envelope. If Kif5B is required on the nuclear envelope, only nuclei decorated with HA-Kif5B-FRB should escape central aggregation and be properly distributed throughout the myotube.

As expected, nuclei in mock siRNA-treated myotubes were distributed throughout the length of the cell, whereas nuclei in Kif5B siRNA-treated myotubes aggregated at the midline of the cell (Fig. 3.5A,D,F). Extended treatment with the rapalog ligand did not visibly alter the morphology or differentiation of these myotubes (Fig. 3.5A, also see Supplementary Fig. 3.S1). When the HA-Kif5B-FRB was co-expressed with the control EGFP-KASH construct

that lacks the FKBP domain in Kif5B siRNA-treated myotubes, no recruitment of kinesin to the nuclear envelope was noted upon ligand treatment (Fig. 3.5B). Importantly, the nuclei in these myotubes remained aggregated at the cell center (Fig. 3.5B,D,F), indicating that the presence of cytosolic HA-Kif5B-FRB was not sufficient to rescue nuclear distribution.

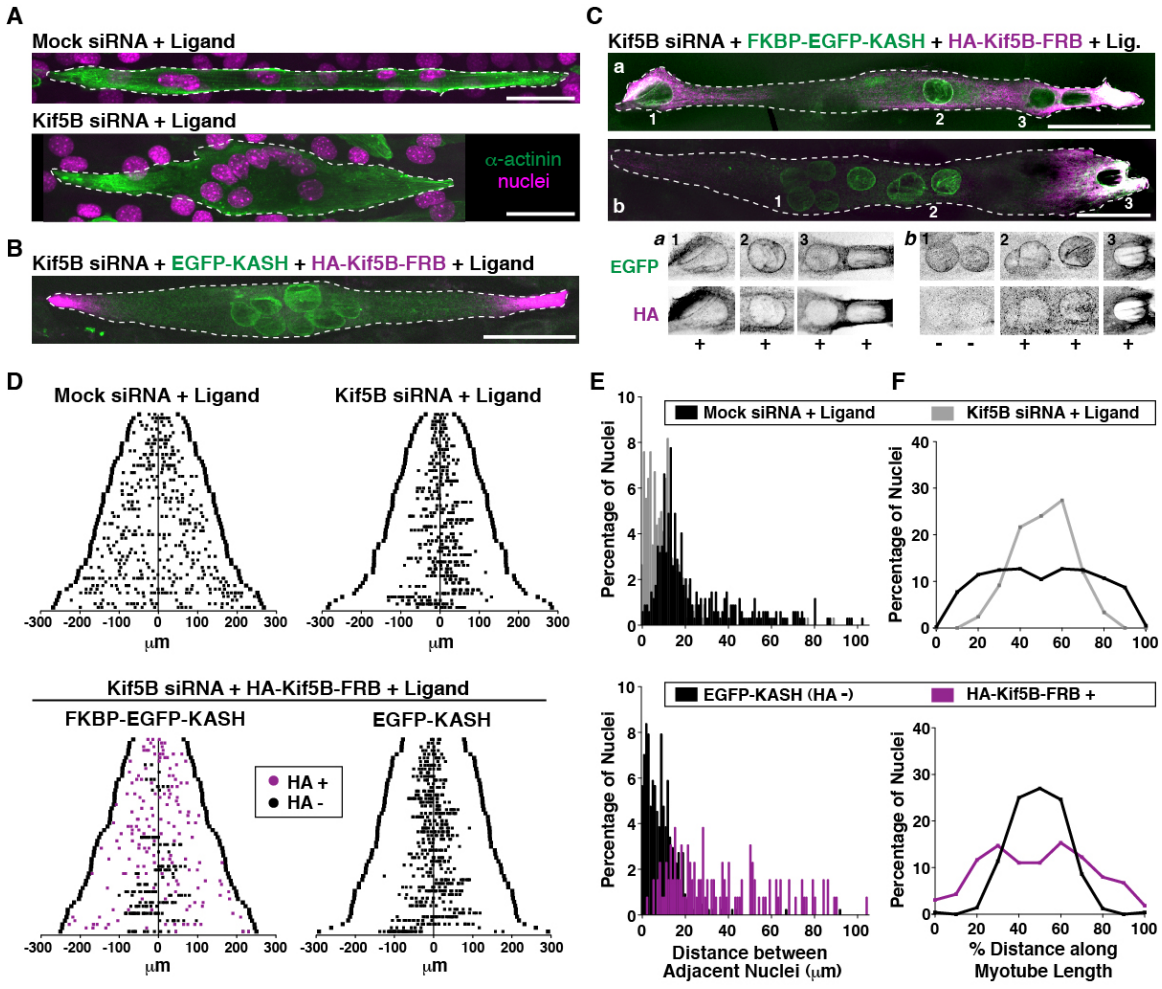

Figure 3.5. Recruitment of exogenous Kif5B to the nuclear envelope rescues nuclear distribution in myotubes.

Figure 3.5. Recruitment of exogenous Kif5B to the nuclear envelope rescues nuclear distribution in myotubes.

(A) Representative images showing normal nuclear distribution in mock-treated cells vs. nuclear aggregation in Kif5B siRNA-treated myotubes that were also treated with ligand. Nuclei were labeled with Hoechst dye (magenta); α-actinin is in green. Images are maximum projections of confocal z-sections. Scale bar = 50 μm. (B) Nuclei in Kif5B siRNA-treated myotubes expressing EGFP-KASH do not recruit HA-Kif5B(1-807)-FRB to the nuclear envelope and aggregate at the cell midline, despite ligand treatment. Fixed myotubes were immunostained for EGFP, HA and with Hoechst (not shown); constitutively-active HA-Kif5B-FRB tends to accumulate near plus-ends of microtubules at myotube ends. Scale bar = 50 μm. (C) Examples of Kif5B siRNA-treated myotubes co-expressing FKBP-EGFP-KASH and HA-Kif5B(1-807)-FRB. With ligand treatment, in some myotubes all nuclei are HA+ (a,*a*), while in others, only a subset of the nuclei recruit the motor (b,*b*); only HA+ nuclei show normal nuclear distribution. Lower panels show inverted grayscale images of the numbered nuclei in the top panels. Plus and minus marks indicate the presence or absence of visible signal for HA-Kif5B(1-807)-FRB on a particular nucleus. Scale bars = 50 μm. (D) Distribution of nuclei in treated myotubes. Each line on the y-axis represents an individual myotube, organized according to length (n = 53-56 myotubes). The ends of the myotube are marked by a dark square; data points represent individual nuclei. HA-Kif5B(1-807)-FRB-decorated nuclei are magenta. (E) Frequency distributions of the distance between adjacent nuclei in treated myotubes (1 μm bin width; less than 2.5% of all the data lies above 105 μm so distributions are truncated for clarity). (F) Histograms depicting nuclear position as a percentage of the distance along the myotube length (bin width = 10%). See Supplementary Figure 3.S1 for additional controls.

In contrast, in Kif5B siRNA-treated myotubes co-expressing HA-Kif5B-FRB and FKBP-EGFP-KASH, treatment with the heterodimerizing ligand induced clear recruitment of the motor to the nuclear envelope (Fig. 3.5C). In over 60% of these myotubes, all nuclei successfully recruited visible levels of HA-Kif5B-FRB (Fig. 3.5Ca & *a*), while in the remaining 40% only some of the nuclei were decorated (Fig. 3.5Cb & *b*). In both populations of myotubes, HA-Kif5B-FRB-positive nuclei were distributed throughout the length of the myotubes (Fig. 3.5C; magenta circles in 3.5D; 3.5F). In contrast, nuclei lacking visible HA staining remained aggregated at the cell center within these same myotubes (black circles in Fig. 3.5D; FKBP-EGFP-KASH plot).

In myotubes deficient for endogenous Kif5B, the distance between adjacent nuclei was significantly smaller than in mock siRNA-treated myotubes (11 ± 13 μm, n = 416 nuclei in 56 myotubes vs. 28 ± 30 μm, 402 nuclei in 54 myotubes, respectively, mean ± s.d., $p<0.001$; Kruskal-Wallis with Dunns post-test; also see the frequency distributions in Fig. 3.5E). The distance between adjacent nuclei was similarly reduced in Kif5B siRNA-treated myotubes co-expressing EGFP-KASH and HA-Kif5B-FRB (10 ± 13 μm, n = 500 nuclei in 53 myotubes; $p<0.001$ vs. mock siRNA). However, the mean distance between adjacent nuclei decorated with HA-Kif5B-FRB was 48 ± 46 μm (n = 180 nuclei in 54 myotubes), which was significantly higher than the distance between nuclei in the mock siRNA-treated myotubes ($P<0.001$). Furthermore, we noticed that HA-Kif5B-FRB-positive nuclei were often at the extreme ends of the myotubes (Fig. 3.5C, D), an area enriched in microtubule plus-ends (Wilson and Holzbaur, 2012). This is reflected in plots of the position of nuclei as a function of distance along the myotube length (Fig. 3.5F, compare mock siRNA to HA-Kif5B-FRB+). These findings are consistent with the cargo-model of Kif5B-dependent nuclear movement in myotubes and suggest that the constitutively active Kif5B construct is even more effective at moving nuclei toward the microtubule plus-ends than the endogenous motor.

A conserved tryptophan-acidic motif in nesprin-2 localizes kinesin light chain to the nuclear envelope

The localization of Kif5B to the nucleus in myotubes is most likely mediated by an interaction between kinesin light chains and nesprins located on the outer nuclear envelope (Wang et al., 2013b; Wilson and Holzbaur, 2012). In vertebrates, the tetratricopeptide repeat (TPR) domain of kinesin light chains has been shown to bind directly to both nesprin-2 (Schneider et al., 2011; Wilson and Holzbaur, 2012) and to nesprin-4 (Roux et al., 2009). In nesprin-2, this binding occurs within a highly conserved, but unstructured, region located between spectrin repeats (SR) 53 and 54 (Schneider et al., 2011)(numbering is according to ref (Simpson and Roberts, 2008) and is based on the human nesprin-2 Giant isoform)(Fig. 3.6A). This region, which has been termed the 'adaptive domain', is predicted to contain a high degree of disordered loops and coils (Zhong et al., 2010). Within this region, there is an almost invariant ~20-residue motif that is predicted to be a 'hot loop' or area of high mobility (Simpson and Roberts, 2008; Zhong et al., 2010). This adaptive domain and invariant motif are also found in nesprin-1 (located between SR71 and 72), though to our knowledge, no binding of KLC to nesprin-1 has yet been reported. In nesprin-4, the kinesin light chain TPR domain has been shown to bind to the region between the SR and transmembrane domain (Roux et al., 2009)(Fig. 3.6A). This region of nesprin-4 does not show obvious similarity to the adaptive domain of nesprin-2.

Within the highly conserved ~20-residue motif in nesprin-1 and nesprin-2, we noticed a 4-residue tryptophan-acidic (W-acidic) motif near the end of this larger motif (Fig. 3.6B). This LEWD motif is similar to binding motifs described in a growing number of other KLC-binding proteins (Aoyama et al., 2009; Dodding et al., 2011; Konecna et al., 2006; Schmidt et al., 2009). A peptide containing the LEWD motif (TN**LEWD**DSAI) from one of these proteins, SKIP (SifA-kinesin interacting protein), was recently crystallized in complex with the TPR domain of KLC2, identifying the binding groove for the LEWD residues (Pernigo et al., 2013).

A search of the KLC binding region of nesprin-4 also revealed the presence of the LEWD motif, though the flanking sequences were not particularly conserved (Fig. 3.6B). Moreover, this LEWD motif is absent from nesprin-3, which binds plectin (Wilhelmsen et al., 2005), and has not been reported to bind KLC.

To test whether the binding of KLC2 to nesprin-2 is mediated by the LEWD motif, we performed co-immunoprecipitation experiments from lysates of COS7 cells co-transfected with HA-KLC2 and the EGFP-tagged fragments of nesprin-2 indicated in Figure 3.6C. While HA-KLC2 co-immunoprecipitated wild-type EGFP-nesprin-2 6348-6552 and 6146-6799 fragments, mutating the WD residues in the nesprin LEWD motif to alanine residues (WD/AA) abolished this binding (Fig. 3.6D). This result strongly suggests that the LEWD motif in nesprin-2 is a functional kinesin light chain-binding motif.

To further verify this interaction, we performed additional co-immunoprecipitation experiments with HA-KLC2 constructs harboring point-mutations in the WD-motif binding groove that have been shown to abrogate binding of KLC2 to cargo (Pernigo et al., 2013). Notably, the R251D mutation in TPR2, and N287L and R312E mutations in TPR3 domains of KLC2 significantly reduced light chain binding to wild-type EGFP-nesprin-2 6146-6799 (Fig. 3.6E, F) and a smaller nesprin-2 construct spanning residues 6348-6552 (Sup. Fig. 3.S2). Furthermore, when we expressed these HA-tagged KLC2 constructs in myotubes depleted of endogenous KLC1 & KLC2, the wild-type HA-KLC2 showed clear accumulation around the nuclei whereas the R251D, N287L and R312E mutations prevented this accumulation (Fig. 3.6G). Quantification of fluorescence intensity along a line spanning the nuclear membrane highlights the lack of recruitment of HA-KLC mutants to the nuclear envelope compared to wild-type (Fig. 3.6H).

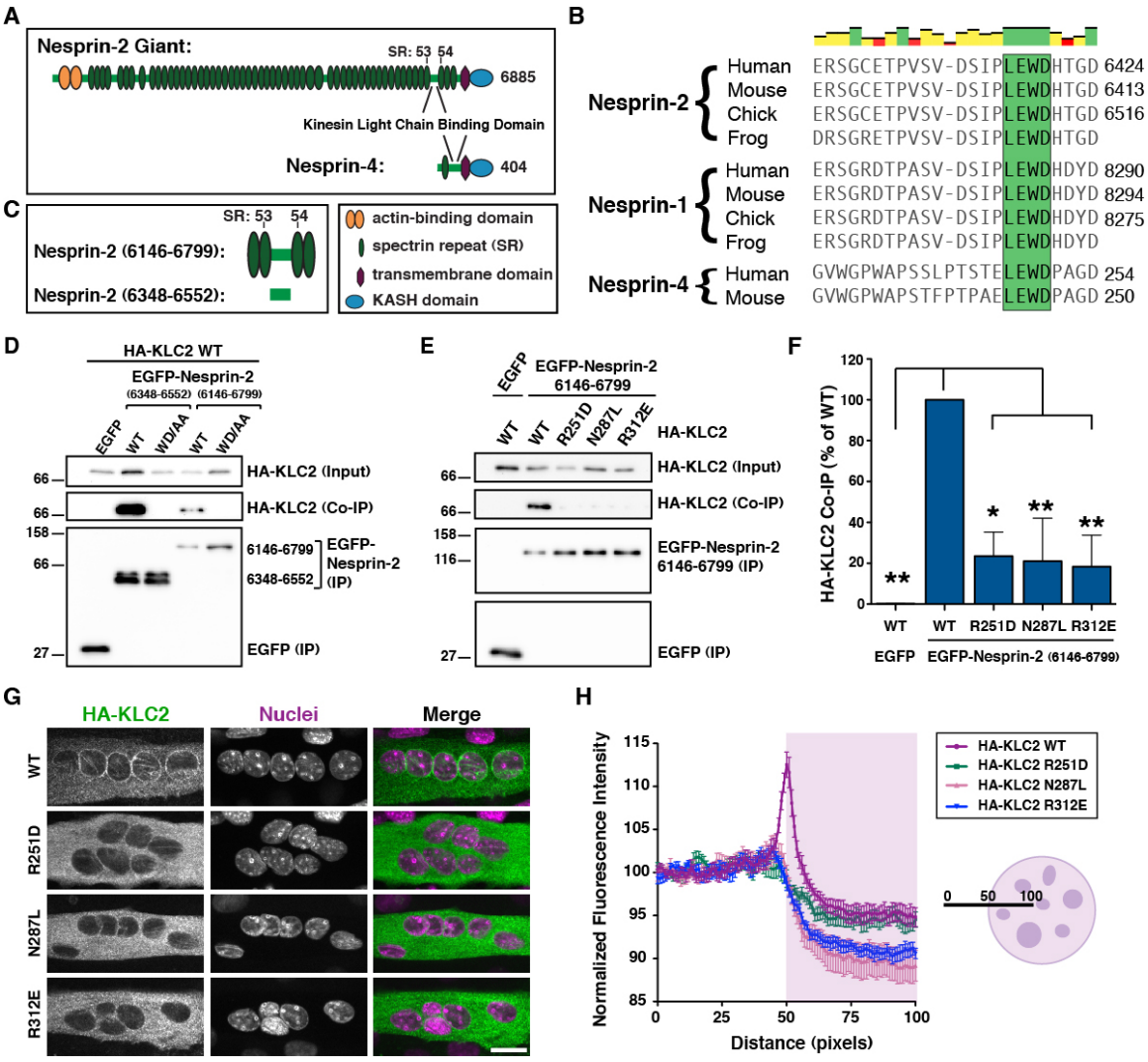

Figure 3.6. A conserved W-acidic motif in nesprin-2 localizes kinesin light chain to the nuclear envelope.

Figure 3.6: A conserved W-acidic motif in nesprin-2 localizes kinesin light chain to the nuclear envelope.

(A) Schematic of nesprin-2 Giant and nesprin-4 highlighting the previously mapped KLC binding domains in each molecule (Roux et al., 2009; Schneider et al., 2011). (B) Alignment of nesprin sequences. Conserved LEWD motif in nesprin-2, -1 and -4 are highlighted, numbering for nesprin-1 and -2 is based on the Giant isoform sequence; Frog sequences are currently incomplete and have been left unnumbered; top panel indicates degree of conservation. (C) Schematic of the EGFP-tagged nesprin-2 fragments used in the following co-immunoprecipitation assays. (D) COS7 cells were transfected with HA-KLC2 and either wild-type EGFP-nesprin-2 fragments or EGFP-nesprin-2 fragments harboring WD/AA mutations in the LEWD motif. IP of EGFP or EGFP-nesprin-2 constructs, followed by immunoblot analysis using both anti-EGFP and anti-HA antibodies indicates that binding of KLC2 is abolished by mutations in the LEWD motif. (E) Similar co-immunoprecipitation experiments were performed using HA-KLC2 constructs with point mutations in the binding groove for the W-acidic motif. (F) Co-immunoprecipitation of HA-KLC2 R251D, N287L and R312E with EGFP-nesprin$^{6146\text{-}6799}$ is significantly reduced (mean ± s.e.m.; from N = 3 independent experiments; ANOVA, **P*<0.05, ***P*<0.01). (G,H) Myotubes were treated with siRNA to both KLC1 and KLC2 and transfected with HA-KLC2 WT or mutant constructs. (G) Fixed myotubes were stained for HA-KLC2. Scale = 20 μm. (**H**) As depicted, quantification of fluorescence intensity along a 100-pixel line centered at the nuclear envelope was performed for the HA-KLC2 signal in these treated myotubes. Fluorescence intensity was normalized to the mean of the first 10 pixels of each line to account for variation in HA-KLC2 expression levels between myotubes (mean ± s.e.m.; N = 10 nuclei per condition from 5 myotubes). See Supplementary Figure 3.S2 for additional co-immunoprecipitation experiments.

Mutations in KLC that disrupt binding to nesprins do not rescue nuclear positioning in myotubes

We have shown that Kif5B is displaced from the nuclear envelope in myotubes treated with siRNA to KLC1 and KLC2 (Fig. 3.2E), and that the R251D, N287L and R312E mutations in the KLC2 TPR domain prevent the localization of KLC to the nuclear envelope (Fig. 3.6G). Therefore, we hypothesized that wild-type but not mutant KLC constructs would rescue the localization of Kif5B to the nuclear surface when expressed in myotubes lacking endogenous KLC1 and KLC2. As expected, in myotubes co-expressing EGFP-KLC2 WT and Myc-Kif5B-Halo in the absence of endogenous KLC, both of the tagged constructs localized to the nuclear envelope (Fig. 3.7A, B). In contrast, Myc-Kif5B-Halo was not recruited to the nucleus in myotubes expressing any one of the three EGFP-KLC2 mutant constructs (Fig. 3.7A, B).

Importantly, the TPR mutations in KLC2 do not disrupt binding of KLC to the Kif5B heavy chain (Supplemental Fig. 3.S3). This suggests that functional motor complexes are still present in these myotubes, even though they are unable to associate with the nuclear envelope. Therefore, if these mutant KLC2 constructs are unable to rescue the nuclear aggregation that results from KLC2 deficiency (Fig. 3.7C), it would again support our hypothesis that nuclear-based Kif5B is necessary for driving nuclear distribution in myotubes.

Indeed, when KLC1 & 2 siRNA-treated myotubes were transfected with HA-KLC2 R251D, N287L or R312E, the nuclei continued to aggregate at the midline of the cell, whereas nuclei in cells expressing HA-KLC2 WT were distributed throughout the length of the myotube (Fig. 3.7D, E, G). While expression of HA-KLC2 WT restored the mean distance between adjacent nuclei to that of mock siRNA-treated myotubes (mock: 27 ± 28μm, n = 389 nuclei in 51 myotubes vs. WT: 26 ± 31μm, n = 414 nuclei in 51 myotubes; mean ± s.d.)(Fig. 4F), the distance between nuclei in the R312E mutant HA-KLC2 myotubes

was not different from that in KLC1 & 2 siRNA-treated myotubes (R312E: 10 ± 13μm, n = 542 nuclei in 54 myotubes) vs. KLC1 & 2 10 ± 11μm (n = 429 nuclei in 51 myotubes; mean ± s.d.; Kruskal-Wallis test)(also see Fig. 3.7F). These findings again indicate that localization of Kif5B to the nuclear envelope is necessary for proper nuclear distribution in developing muscle cells.

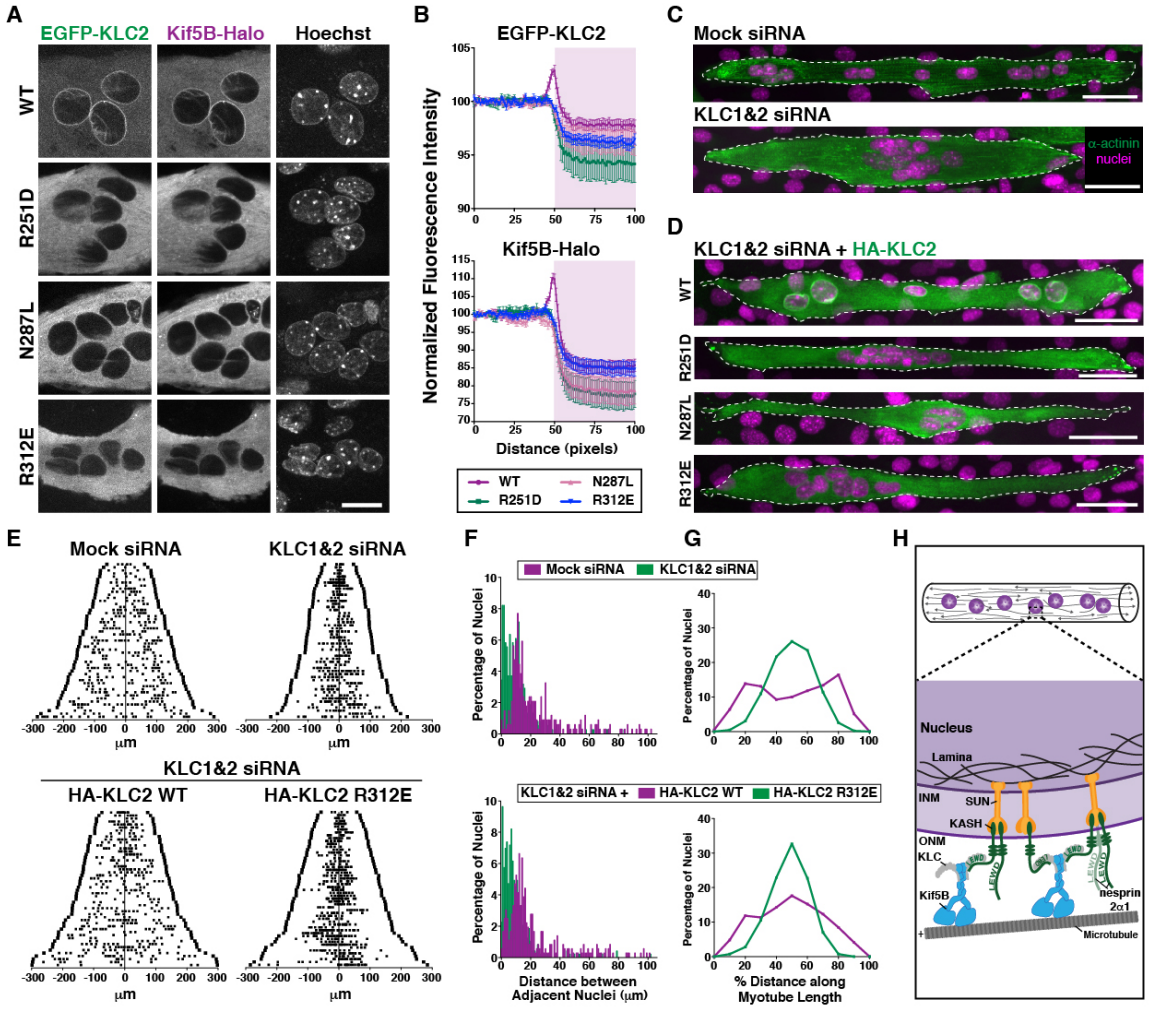

Figure 3.7. Mutations in KLC that disrupt binding to nesprins do not rescue nuclear positioning in myotubes.

(A,B) Myotubes were treated with siRNA to both KLC1 and KLC2 and transfected with EGFP-KLC2 WT, R251D, N287L or R312E and Myc-Kif5B-Halo. (A) The Halo tag was labeled with Halo-TMR ligand before image acquisition in live cells. Scale = 20 μm. (B) Quantification of fluorescence intensity along a 100-pixel line centered at the nuclear envelope was performed for the EGFP-KLC2 and Myc-Kif5B-Halo signals in these treated

myotubes. Fluorescence intensity was normalized to the mean of the first 10 pixels of each line to account for variation in expression levels between myotubes (mean ± s.e.m.; N = 10 nuclei per condition from 5 myotubes). (C) Representative images showing normal nuclear distribution in mock siRNA-treated myotubes compared with nuclear aggregation in KLC1 & 2 siRNA-treated myotubes. Nuclei were labeled with Hoechst dye (magenta); α-actinin is in green. Images are maximum projections of confocal z-sections. Scale bar = 50 μm. (D) Myotubes were treated with siRNA to both KLC1 and KLC2 and transfected with HA-KLC2 WT, R251D, N287L or R312E constructs. Cells were fixed and stained for HA-KLC2 (green), KLC1 (see Figure S4) and nuclei (magenta). Only HA-KLC2 WT rescues the nuclear aggregation caused by loss of endogenous KLC1&2. Scale bar = 50 μm. (E) Distribution of nuclei in treated myotubes. Each line on the y-axis represents an individual myotube, organized according to length (N = 51-54 myotubes). The ends of the myotube are marked by a dark square; data points represent individual nuclei. Only myotubes that lacked immunofluorescent staining for KLC1 were included in the analysis. (F) Frequency distributions of the distance between adjacent nuclei in treated myotubes (1μm bin width; less than 2% of all the data lies above 105 μm so distributions are truncated for clarity). (G) Histograms depicting nuclear position as a percentage of the distance along the myotube length (bin width = 10%). (H) Model illustrating that Kif5B motors are anchored at the nuclear envelope through an interaction between the TPR domain of KLC with the LEWD motif in nesprin-2 (only the short nesprin-2α1 isoform is depicted for clarity). Kif5B motors exert force on the local microtubule network to drive nuclear dynamics, allowing for proper distribution of nuclei throughout the developing myotube. Also see Figure 3.S3 for supplemental data.

IV. Discussion

Using two different experimental strategies, we provide evidence supporting the cargo-model of Kif5B-dependent nuclear movement in developing myotubes. First, using an inducible dimerization system to specifically control the localization of active Kif5B motor constructs to the nuclear envelope, we find that only nuclei decorated with the HA-Kif5B-FRB motor construct escape the nuclear aggregation resulting from depletion of the endogenous motor. In fact, the mean distance between these HA-Kif5B-FRB-positive nuclei was greater than the distance between nuclei in mock-treated myotubes and HA-Kif5B-FRB-positive nuclei were often found at the very extreme ends of the myotubes (Fig. 3.5C, D), which are enriched in microtubule plus-ends (Wilson and Holzbaur, 2012). We speculate that this exaggerated distribution of HA-Kif5B-FRB-decorated nuclei relative to mock siRNA-treated myotubes reflects the constitutive activity of the truncated motor construct compared with the endogenous motor, which can exist in an autoinhibited state and may be subject to regulation while bound to the nuclear envelope (Verhey and Hammond, 2009).

We also show that depletion of KLC from the myotube not only displaces Kif5B from the nuclear envelope, but also results in nuclear aggregation that is indistinguishable from that caused by Kif5B depletion. This nuclear positioning defect can be rescued by expression of EGFP-KLC2 wild-type, which effectively recruits Kif5B to the nuclear envelope. However, point mutations in the TPR domain of KLC2 that prevent binding to nesprin-2 do not rescue the localization of Kif5B to the nuclear envelope and do not rescue nuclear distribution. The results from both of these approaches strongly argue that Kif5B must be localized to the nuclear envelope in order to distribute nuclei throughout the length of the myotube, thereby supporting the cargo-model of kinesin-based nuclear movement.

Other Kif5B cargoes/adaptors in the myotube may contain W-acidic motifs and require the KLC TPR binding groove for Kif5B recruitment. However, many muscle-specific

myofibril components and intermediate filaments, such as α-sarcomeric actin and desmin, bind directly to the Kif5B tail domain (Wang et al., 2013a), and would not be expected to be disrupted by the KLC mutations. In contrast, these experiments argue against the microtubule-sliding model for nuclear movement. In both experimental paradigms, Kif5B was present in the cytoplasm of the myotubes. Although truncated, HA-Kif5B(1-807)-FRB includes the putative MAP7 binding domain (residues 327-536) (Metzger et al., 2012) and should still be able to cross-link and slide microtubules, and we have verified that mutations in the KLC TPR domain do not affect the binding of MAP7 to Kif5B (Sup. Fig. 3.S3D). Though we cannot rule out a role for Kif5B-dependent microtubule sliding in myotube development, we have shown that this mechanism on its own is insufficient to drive nuclear positioning. However, it is possible that MAP7 may participate in relieving kinesin-1 autoinhibition and activating the motor (Barlan et al., 2013), which would account for the nuclear positioning defect noted in MAP7 mutant flies (Metzger et al., 2012).

Our results reveal that the W-acidic LEWD motif in the adaptive domain of nesprin-2 mediates binding of KLC2 to the nuclear envelope. We also expect that KLC1 binds the nesprin LEWD motif, since the TPR residues that mediate the binding of the W-acidic motif are conserved in KLC1 (Pernigo et al., 2013). Moreover, given the high sequence similarity between nesprin-1 and nesprin-2, it is very likely that KLC2 also binds the LEWD in nesprin 1. Both nesprin-1 and nesprin-2 have a number of verified and suggested splice-isoforms with varied tissue distribution, but the LEWD motif is present in all but the shortest of the KASH-domain containing isoforms (Rajgor and Shanahan, 2013). mRNA for nesprin-2 giant and nesprin 2α1 account for ~85% of nesprin-1 & -2 mRNA expressed in adult skeletal muscle (Duong et al., 2014), while nesprin-1 has been reported to be more abundant than nesprin-2 in developing muscle cells (Randles et al., 2010). KLC is likely capable of binding all LEWD-containing nesprins, but accessibility of this site may be regulated *in vivo*. In contrast, the shorter nesprin-1 & -2 isoforms can be found on the inner nuclear membrane in

association with emerin and lamin A/C (Mislow et al., 2002; Wheeler et al., 2007), which likely precludes their binding to kinesin. Finally, it is tempting to speculate that the LEWD motif in nesprin-4, which is predominantly found in secretory epithelial cells (Roux et al., 2009) and the hair cells of the inner ear (Horn et al., 2013), mediates its known interaction with KLC.

Given that the localization of Kif5B to the nuclear envelope is required for proper nuclear positioning, we propose that interaction of nuclear-based motors with the local microtubule network drives the nuclear dynamics observed in myotubes, including both translocation and rotation in three-dimensions (Wilson and Holzbaur, 2012). For smaller cargoes, teams of kinesin-1 motors do not necessarily enhance velocity or travel distance of the organelle (Jamison et al., 2010; Shubeita et al., 2008). However the large ensemble of Kif5B motors recruited to the nucleus by nesprins may be needed to produce the force necessary to move such a large organelle. The dynamics of individual nuclei are variable, with stochastic changes in translational and rotational velocities and direction (Wilson and Holzbaur, 2012). This variability likely derives from the organization of the microtubule cytoskeleton surrounding the nucleus, which is highly dynamic (Wilson and Holzbaur, 2012), as well as the distribution and activity of the Kif5B on the envelope. Furthermore, dynein is also located on the nuclear envelope in myotubes (Cadot et al., 2012; Wilson and Holzbaur, 2012) and would be expected to oppose the activity of kinesin-1, which may allow for more flexibility in moving such a large cargo through the complex cytoskeletal network in the developing myotube, similar to observations in C. elegans hypodermal cells (Fridolfsson and Starr, 2010).

Here, we used two independent experimental strategies to show that the ~10 μm-wide nucleus can be considered a cargo for kinesin-1 in myotubes. By binding kinesin light chain via the LEWD motif, nesprins act as nuclear cargo adaptors for Kif5B, localizing the motor to the nuclear surface (Fig. 3.7H). Achieving and maintaining proper nuclear

positioning is not restricted to muscle fibers, but is critical in many diverse cell types, with aberrant positioning linked to dysfunction and disease (Gundersen and Worman, 2013). Given that the LEWD motif is present in many nesprin-1 and -2 isoforms, as well as in nesprin-4, our results suggest that nesprins mediate the localization of kinesin-1 to the nuclear envelope in a wide variety of cells. Therefore it will be important to learn how recruitment and activity of kinesin-1 on the nuclear envelope is regulated in both developing and mature cells to allow for cell-type specific nuclear positioning.

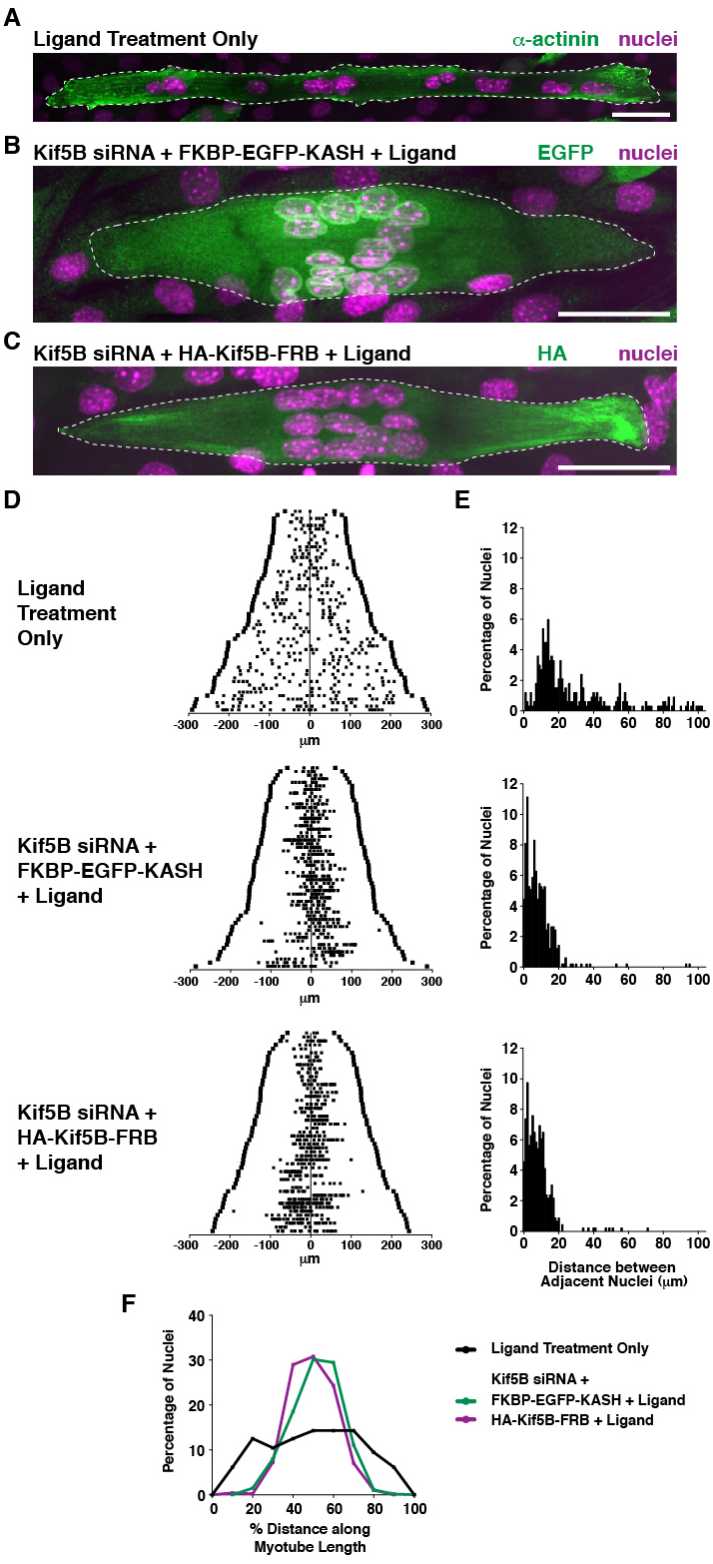

Figure 3.S1. Additional controls associated with Figure 3.5.

Figure 3.S1. Additional controls associated with Figure 3.5.

(A) Representative image showing normal nuclear distribution and myofibril development in a ligand-treated C2C12 myotube. Nuclei are labeled with Hoechst dye (magenta); α-actinin is in green. (B) Nuclei aggregate at the midline of Kif5B siRNA + ligand treated myotubes expressing FKBP-EGFP-KASH. Nuclei are labeled with Hoechst dye (magenta); EGFP is shown in green. (C) Nuclei aggregate at the midline of Kif5B siRNA + ligand-treated myotubes expressing HA-Kif5B(1-807)-FRB. Nuclei are labeled with Hoechst dye (magenta); HA-Kif5B-FRB is in green. Images in A, B & C are maximum projections of confocal z-sections. Scale bar = 50 μm. (D) Distribution of nuclei in treated myotubes. Each line on the y-axis represents an individual myotube, organized according to length (N = 50-57 myotubes). The ends of the myotube are marked by a dark square; data points represent individual nuclei. (E) Frequency distributions of the distance between adjacent nuclei in treated myotubes (1 μm bin width; less than 1.5% of the data lies above 105 μm so distributions are truncated for clarity). (F) Histogram depicting nuclear position as a percentage of the distance along the myotube length (bin width = 10%).

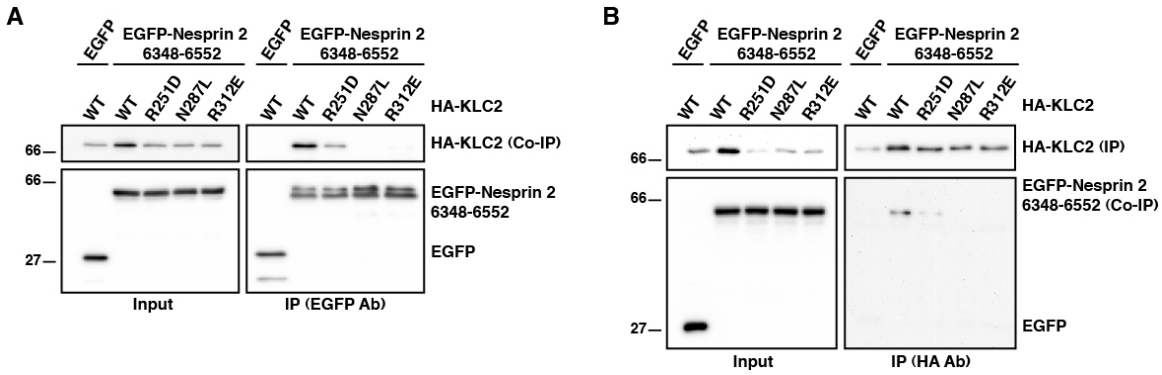

Figure 3.S2. Additional co-immunoprecipitation experiments associated with Figure 3.6.

COS7 cells were transfected with EGFP-nesprin $2^{6348\text{-}6552}$ and HA-KLC2 WT or R251D, N287L and R312E mutants. (A) IP of EGFP-nesprin-2, followed by immunoblot analysis using both anti-EGFP and anti-HA antibodies indicates that point mutations in KLC2 reduce binding to EGFP-nesprin-2. (B) The reciprocal IP of HA-KLC2 WT and mutants reveals the same reduction in binding between EGFP-nesprin-$2^{6348\text{-}6552}$ and KLC2 R251D, N287L and R312E mutants.

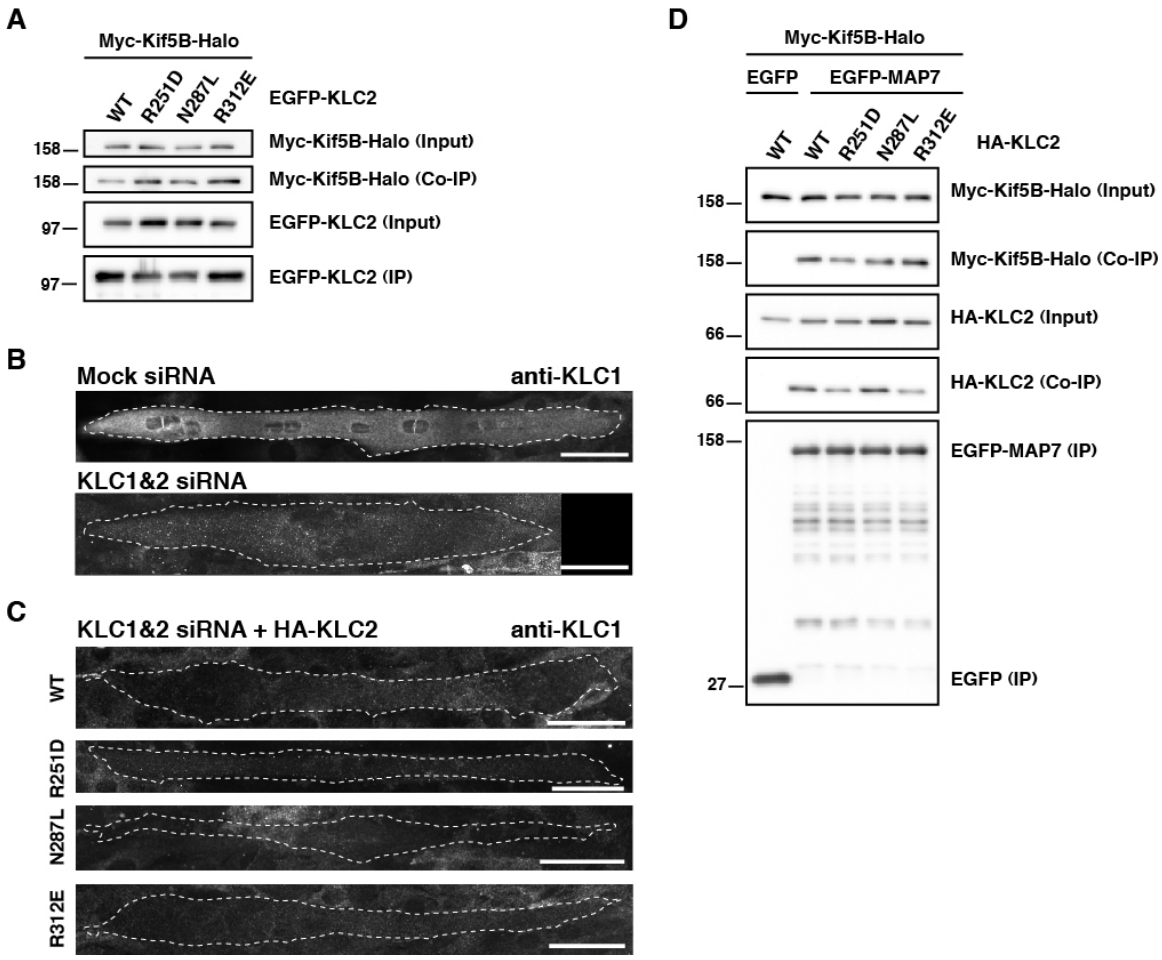

Figure 3.S3. Additional controls associated with Figure 3.7.

(A) COS7 cells were transfected with Myc-Kif5B-Halo and EGFP-KLC2 WT or mutant constructs. IP of EGFP-KLC2, followed by immunoblot analysis using both anti-EGFP and anti-Myc antibodies indicates that point mutations in KLC2 do not prevent binding to Myc-Kif5B-Halo. (B) KLC1 immunofluorescence in myotubes treated with Mock siRNA, KLC 1&2 siRNA and KLC1&2 siRNA + HA-KLC2 WT or R251D, N287L or R312E mutant constructs. Images correspond to the myotubes in Figure 4C & D. Only Mock siRNA-treated myotubes show expression of KLC1 in the cytoplasm and on the nuclear envelope. Images are maximum projections of confocal z-sections. Scale bar = 50 μm. (D) COS7 cells were

transfected with Myc-Kif5B-Halo, HA-KLC2 WT or mutant constructs along with EGFP or EGFP-MAP7. Immunoprecipitation of EGFP-MAP7, followed by immunoblot analysis using anti-GFP, anti-Myc and anti-HA antibodies indicates that point mutations in KLC2 do not disrupt the interaction of MAP7 with Kif5B.

CHAPTER 4: Conclusions and Future Directions

Conclusions

Over 50 years ago, observations of nuclear translocation and rotation were first reported in developing skeletal muscle cells (Capers, 1960; Cooper and Konigsberg, 1961). At the time of these observations, the cellular mechanisms driving these movements were vague at best. However, in the years since these early reports, discovery of the microtubule cytoskeleton and microtubule motors has allowed the elucidation of various modes of microtubule-dependent nuclear movement in many other cell types. Though we learned in 1987 that microtubules are necessary for the movement of nuclei in myotubes (Englander and Rubin, 1987), further investigation of the mechanisms driving nuclear dynamics was lacking. In the present work, we demonstrate that the microtubule motor proteins kinesin-1 and dynein localize to the nuclear envelope in myotubes and drive nuclear translocation and rotation. Furthermore, we show that kinesin-1 binds to the outer nuclear membrane protein nesprin-2 and that nuclear-based kinesin-1 motors are essential for proper nuclear distribution in developing skeletal muscle cells.

Live cell microscopy of cultured C2C12 myotubes reveals complex nuclear movements, including translation with or without rotation. Consistent with previous reports, nuclei rotate along the length of the cell in three-dimensions (rotation axis is perpendicular to the long axis of the myotube), undergo obvious deformation, and are occasionally noted to pass one another as they move through the cell. We verified that nuclear dynamics are dependent on an intact microtubule cytoskeleton, and present evidence that dynamics are unaffected by inhibition of myofibril contraction. We confirmed with immunostaining for tubulin and live imaging of GFP-EB3, that microtubules form a bidirectional dynamic network that predominantly forms a tube surrounding the centrally located nuclei. Moreover, we

show for the first time that kinesin heavy chain and light chain, as well as components of the dynein and dynactin complex localize to the nuclear envelope in myotubes. Depletion of the motors with siRNA not only decreases rates of nuclear translocation and rotation, but also causes aggregation of nuclei, either at the midline of the cells in the absence of Kif5B, or more focally in cell lacking cytoplasmic dynein. These findings led us to propose a model in which Kif5B and dynein drive nuclear movement from the surface of the nuclear envelope and that the variable dynamics of each nucleus are a function of the distribution of opposing motors interacting with the bidirectional microtubule cytoskeleton.

To test our hypothesis that the Kif5B motors present on the nuclear envelope are essential for nuclear distribution in myotubes, we depleted endogenous kinesin heavy chain and used an inducible dimerization system to control recruitment of truncated, active Kif5B motors to the nucleus. Nuclei were only distributed throughout myotubes when HA-Kif5B(1-807)-FRB decorated the nuclear envelope, arguing strongly for the necessity of nuclear-based kinesin motors. Furthermore, we show that Kif5B binds to the nuclear envelope protein nesprin-2 and that this interaction is mediated by the binding of a conserved LEWD motif in nesprin to the TPR domain of kinesin light chain. Expression of a dominant negative nesprin-2α-KASH construct partially displaces Kif5B from the nuclear envelope, and causes nuclei to aggregate. Nuclear aggregation is even more pronounced when KLC expression is depleted with siRNA, and although concurrent expression of wild-type KLC2 rescues nuclear distribution, expression of KLC mutants that prevent localization to the nuclear envelope fails to prevent nuclear aggregation. Together, these data provide substantial evidence for a model in which Kif5B acts from the nuclear surface to move nuclei along the microtubule cytoskeleton in developing muscle cells. In this chapter, I discuss the significance of these findings and provide insight into directions for future research.

The early observations of nuclear movement in muscle cells were performed in primary chick or rat myotubes. Importantly, the nuclear dynamics that we observe in the immortalized C2C12 mouse muscle cell line accurately recapitulate the descriptions provided in the earlier works (Capers, 1960; Cooper and Konigsberg, 1961; Englander and Rubin, 1987). Yaffe & Saxel originally established the C2 cell line almost 40 years ago, and it was subsequently subcloned to yield the rapidly differentiating C2C12 line (Blau et al., 1985; Yaffe and Saxel, 1977). Though these cells do not differentiate into fully mature muscle fibers, they do mimic early muscle cell development in many regards and are amenable to transfection and drug treatments. Notably, many of our findings in the C2C12 cells regarding microtubule dynamics and nuclear positioning have also been recently reported in primary mouse myotubes and in Drosophila embryos (Cadot et al., 2012; Folker et al., 2014; Metzger et al., 2012), providing validation of the cells as a model for the study of nuclear dynamics. Ideally, these observations of nuclear movement will one day be confirmed in developing mammalian muscle cells *in vivo*. Current technologies allow live imaging of adult mouse muscle fibers (Oddoux et al., 2013), but as imaging technologies advance, we may gain better access to dynamic processes occurring during embryonic and postnatal muscle development. Despite this current limitation, the central aggregation of myonuclei found in the muscle-specific conditional Kif5B knock-out mouse (Wang et al., 2013a), strongly argues for the necessity of kinesin-1 in driving myonuclear positioning *in vivo*.

Aberrant positioning of nuclei in muscle cells is correlated with muscle dysfunction. In the ensconsin-null flies, larval crawling is significantly slower (Metzger et al., 2012), and the muscle-specific Kif5B-null mice exhibit severe limb dystrophy and do not survive as they are unable to move and can not maintain respiration (Wang et al., 2013a). Nuclei are also mispositioned in patients with muscle diseases. For instance, the centronuclear myopathies, as their name suggests, are characterized, in part, by numerous centrally-localized nuclei. It is believed that the congenital X-linked myotubular myopathy is the result of arrested

development of muscle cells, and it is not clear whether fibers ever develop to the point where peripheral nuclei are observed. It is unclear whether growth arrest also underlies the autosomal forms of centronuclear myopathy, but prominent central localization of nuclei remains a hallmark of the diseases (Pierson et al., 2005). Additionally, aggregates of nuclei at the cell periphery have been noted in patients with Emery-Dreifuss muscular dystrophy (Mattioli et al., 2011).

While the absence of synaptic nuclei has clear repercussions for synaptic maintenance and transmission (Jevsek et al., 2006; Mejat et al., 2009; Merlie and Sanes, 1985; Zhang et al., 2007b), the resulting effects of aggregation of extrasynaptic nuclei at the myotube periphery or abnormally elevated levels of centrally localized nuclei are less obvious. According to the myonuclear domain theory (Hall and Ralston, 1989; Pavlath et al., 1989; Ralston and Hall, 1989), aggregation of nuclei might alter the dispersal of mRNA and proteins such that certain areas of the fiber are deficient. Nuclei located in the center of the fiber may experience different forces during muscle contraction than those properly anchored at the periphery, leading to increased nuclear fragility. This may have consequences for genome stability and/or gene expression that could have downstream effects on muscle fiber structure and function. While the aberrant position of nuclei may simply be a symptom of some of these diseases, a growing number of mutations have been found in nuclear envelope proteins involved in nuclear movement and positioning (Mejat and Misteli, 2010), suggesting that in these instances, mispositioned nuclei may be more directly associated with the disease phenotype.

As we learn more about the mechanisms of nuclear movement during muscle cell development and anchoring of nuclei in mature fibers, the consequences of improperly positioned nuclei on muscle function will also become more apparent. Hopefully, this will allow us to improve therapies for muscle diseases by preventing or alleviating the negative consequences of mispositioned nuclei. To this end, the findings described in this

dissertation substantially advance our understanding of nuclear dynamics and positioning within developing skeletal muscle cells.

Furthermore, our work indicates that the mechanisms driving nuclear dynamics in myotubes are very similar to those used by other cells. Our discovery of the conserved kinesin light chain-binding motif in nesprin-1 and nesprin-2, has broad implications for nuclear positioning in other tissues, as these proteins are widely expressed throughout the body. The more we learn about how kinesin-1 and dynein bind to the nuclear envelope in muscle, the more hypotheses we can generate for nuclear movement and positioning in other cell types, such as neurons and epithelial cells where nuclear positioning is also essential for normal tissue development and cell function.

Remaining Questions and Future Directions

Though this work has significantly enhanced our understanding of how nuclei move through developing myotubes, there is still a great deal to understand about nuclear positioning in muscle. Going forward, some of the pertinent areas that should be addressed include:

The role of MAP7/Ensconsin in nuclear dynamics

In the model proposed by Metzger et al. (2012), Kif5B and MAP7 interact to cross-link and slide anti-parallel microtubules, thereby pushing the attached nuclei apart. Evidence for this model is based on yeast 2-hybrid and co-immunoprecipitation data showing an interaction between the two proteins as well as data that a chimera construct including the motor domain of Kif5B and the microtubule-binding domain of MAP7 (K560-EMTB) rescue the nuclear positioning phenotype caused by MAP7 depletion (Metzger et al., 2012). While other studies have found that ensconsin recruits the motor to microtubules and activates kinesin-1 activity, these studies have not been able to find a stable interaction between

ensconsin and kinesin-1, suggesting that the two proteins may only interact transiently (Barlan et al., 2013; Sung et al., 2008). While this argues against the MAP7-Kif5B sliding mechanism, it is important to note that kinesin-1 has a second microtubule-binding domain in the tail region, and it has been reported that kinesin-1 itself can cause sliding of microtubules (Barlan et al., 2013; Jolly et al., 2010; Straube et al., 2006; Urrutia et al., 1991; Vale et al., 1985a). Therefore, MAP7 may serve to alleviate kinesin-1 auto-inhibition, allowing kinesin-1 to slide microtubules (Barlan et al., 2013). Going forward, it will be important to test whether Kif5B does slide microtubules in myotubes, and whether this is dependent on the microtubule-binding domain of MAP7, or just activation of the motor by the kinesin-binding domain of MAP7. If Kif5B does cross-link and/or slide microtubules in myotubes, does this influence nuclear movement? While our data indicate that Kif5B is necessary on the nuclear envelope for proper nuclear distribution, it does not rule out an additional role for kinesin-dependent microtubule sliding in myotube development. Possible ways to test whether microtubule-microtubule sliding influences nuclear dynamics might include preventing sliding by inducing cross-linking of microtubules, or removing the microtubule-binding domain in the tail of Kif5B. Alternatively, sliding activity could be enhanced through ectopic expression of the mitotic Eg5 kinesin.

While I have shown that the localization of Kif5B to the nuclear envelope is necessary for proper nuclear distribution, it is currently unclear whether binding of KLC to nesprins on the envelope is sufficient for motor activation. Though binding of the tryptophan-acidic motifs in calsyntenin-1 to the TPR domain of KLC activates kinesin-1 (Kawano et al., 2012), it is unknown whether all WD-containing KLC-binding proteins have the same effect. Interestingly, nuclei decorated with the constitutively active HA-Kif5B(1-807)-FRB construct can often be found at the extreme ends of the myotubes (Figure 3.5). This exaggerated positioning compared to nuclei in untreated myotubes suggests that endogenous Kif5B may not always be active while bound to the nucleus. This raises the possibility that MAP7 may

activate Kif5B on the nucleus; in the absence of MAP7, nuclear-bound Kif5B would be autoinhibited and unable to drive nuclear dynamics, explaining the need for both Kif5B and MAP7 in developing myotubes. Furthermore, because MAP7 decorates microtubules, activation of motors by MAP7 could occur readily when microtubules are proximal to the nuclear envelope, but in their absence the motors are inhibited, reducing futile ATP consumption. Differential activation of motors on one side of the nucleus might also account for nuclear rotation. The question of kinesin-1 activation on the nuclear envelope could be addressed by eliminating the need for motor activation through the use of hinge-less, unfolded kinesin constructs (Barlan et al., 2013) that retain their targeting to the nuclear envelope, and assessing the effects on nuclear dynamics and distribution. Additionally, the use of fluorescence resonance energy transfer (FRET) sensors placed on the kinesin motor domain and tail domain may help elucidate the distribution of active and inactive motors on the nuclear envelope (Cai et al., 2007).

Are nesprins the only Kif5B cargo adaptors on the nucleus?

I have shown that kinesin light chain binds to the LEWD motif in nesprin-2 and that KLC mutations that disrupt this interaction prevent the localization of Kif5B to the nuclear envelope in myotubes. Additionally, I showed that expression of the dominant negative nesprin-2α-KASH construct in myotubes reduces the level of KLC and KHC on the nuclear envelope, decreases nuclear rotation and increases nuclear aggregation. Together these data argue that nesprins act as kinesin-1 cargo adaptors for the nucleus. While the incomplete removal of Kif5B from the nuclear envelope with the dominant negative KASH construct (Figure 2.7), could simply indicate that we did not fully displace all of the nesprins from the envelope (a possibility we were unable to assess due to our lack of reliable nesprin antibodies), it could also indicate that there are additional KLC binding proteins on the nuclear envelope. Importantly, this protein or proteins must also bind KLC in the WD-motif

binding groove since kinesin-1 was completely absent from the envelope when KLC was mutated.

A possible protein that could serve as an additional cargo adaptor for the nucleus is RanBP2. This protein, which is also known as Nup358, serves as a kinesin-1 cargo adaptor for mitochondria (Cho et al., 2007). However, it is also a part of the nuclear pore complex and has already been shown to recruit kinesin to the nuclear surface specifically during G2 of the cell cycle (Splinter et al., 2010). While RanBP2 interacts with the stalk and tail domains of Kif5B and Kif5C (Cai et al., 2007), the complex also contains KLC (Cai et al., 2001). Interestingly, an examination of the sequence of the mapped kinesin binding domain of RanBP2 reveals a LEWD motif, suggesting that KLC may also mediate the binding of kinesin-1 to RanBP2. Furthermore, RanBP2/Nup358 was recently found to be upregulated during myogenesis, such that more RanBP2 is found on the nuclear envelope in myotubes compared to myoblasts (Asally et al., 2011). Given these observations, it will be important to determine whether Kif5B is not only binding to nesprins on the nuclear envelope in developing muscle cells, but whether it is also binding RanBP2. Currently, we have experiments underway to more completely deplete nesprin-1 and nesprin-2 from the nuclear envelope using siRNA in conjunction with the dominant negative KASH construct. Should we find that substantial Kif5B levels remain on the nuclear envelope following this treatment, the obvious next step is to deplete RanBP2 in order to determine whether it also serves as a kinesin-1 cargo adaptor for the nucleus in developing muscle cells.

How does dynein localize to the nuclear envelope?

The current data indicates that dynein and dynactin also localize to the nuclear envelope in myotubes (Cadot et al., 2012; Folker et al., 2014; Wilson and Holzbaur, 2012). However, we know very little about the adaptor proteins that recruit dynein to the nuclear surface in muscle cells. In the brain, there is evidence that dynein/dynactin binds to nesprin-

1 and nesprin-2 and mediates interkinetic nuclear migration as well as nuclear movements during neuronal migration (Yu et al., 2011; Zhang et al., 2009b). Though our GST-binding assays showed robust binding of Kif5B to residues 6348-6552 of nesprin-2 (Figure 2.7), dynein did not bind to this fragment. Further binding assays should be performed to verify and map the association of dynein/dynactin with nesprins in muscle cells and other tissues.

RanBP2 may also indirectly recruit dynein to the nuclear envelope in myotubes. In addition to binding kinesin-1, RanBP2 also binds the motor adaptor protein BICD2 (Splinter et al., 2010). The N-terminus of BICD2 binds to dynein and dynactin and recruits the motor complex to organelles, including the nucleus (Hoogenraad et al., 2001; Hu et al., 2013; Kapitein et al., 2010b; Splinter et al., 2010). As noted above, the expression of RanBP2 increases during muscle cell differentiation (Asally et al., 2011), and thus may aide in recruiting the dynein complex to the nuclear envelope in myotubes. The nuclear pore complex protein Nup133 might also anchor dynein to the nuclear envelope in myotubes, as it has been previously shown to anchor dynein/dynactin through a complex involving CENP-F and NudE/NudEL (Bolhy et al., 2011). Finally, dynein light intermediate chain 1 binds to the centrosomal protein pericentrin (Tynan et al., 2000), which redistributes to the nuclear envelope during muscle cell differentiation (Bugnard et al., 2005; Srsen et al., 2009). Therefore, pericentrin may recruit dynein to the nuclear envelope in myotubes.

Are microtubule minus ends released from the nuclear envelope?

In the microtubule sliding model of myonuclear movement proposed by Metzger et al. (2012), as well as the dynein-mediated pulling mechanism proposed in their subsequent paper (Cadot et al., 2012), it is essential that at least some microtubules remain attached at their minus ends to the nuclear envelope. While microtubule nucleation from the nuclear envelope following nocodazole wash-out is readily observed (Tassin et al., 1985) and imaging of EB3-GFP comets shows constant nucleation of microtubules at a rate of 1-10 per

100 seconds in unperturbed myotubes (Zaal et al., 2011), to the best of my knowledge, there has been no examination of whether the microtubule minus-ends remain attached at the nuclear surface. Given that microtubule minus ends are released from the centrosome in migrating cells (Abal et al., 2002) and the polarized microtubule array in epithelial cells is thought to be derived from microtubules released from the centrosome (Keating et al., 1997; Mogensen, 1999), it is very likely that microtubules are also released from the nuclear surface in myotubes. While the high density of microtubules in unperturbed myotubes hampers tracing the origin of individual microtubules, it may be possible to begin to address this question by manipulating the levels of the microtubule severing protein fidgetin, which localizes to the centrosome and controls the number and length of astral microtubules in mitotic cells (Mukherjee et al., 2012).

Additionally, though it is likely that at any given time, a population of microtubule minus-ends are still attached to the nuclear envelope, it is probable that these microtubules are severed at sites distant from the centrosome by enzymes such as katanin or spastin (Evans et al., 2005; McNally et al., 1996). While short microtubules may extend from one nucleus to another in a myotube, it is difficult to imagine how nuclei tens of microns away from one another could be connected by nuclear-bound microtubule arrays. Furthermore, it remains unclear how sliding of nuclear-bound microtubules would account for the nuclear rotation, deformation, and passing events that we observe in the myotubes.

The contribution of cytoplasmic flow to nuclear dynamics in myotubes

During the course of imaging nuclear dynamics, we often observed directed steams of cytoplasm around the nuclei (Video 1). Movement of small phase-dense organelles appeared to be swept along in these streams, rapidly moving along the long-axis of the myotubes. Interestingly, the predominant direction of nuclear rotation often coincided with the direction of the streaming, suggesting that fluid flow in the myotube may also influence

nuclear dynamics. Additionally, depolymerization of the microtubules with nocodazole decreased the rate and changed the visual appearance of the cytoplasmic flow in myotubes.

Cytoplasmic flow influences nuclear dynamics and positioning in a number of other cell systems. For example, in the filamentous fungi, *Ashbya gossypii,* nuclei are not only subject to dynein-dependent microtubule-based pulling forces, but are also moved through hyphae by bulk cytoplasmic fluid flow driven by osmotic pressure gradients (Lang et al., 2010). These fluid flows not only drive colony growth, but physical mixing of the nuclei is believed to preserve genetic diversity within the colony (Roper et al., 2013). In plants, including *Chara* and *Arabidopsis*, cytoplasmic flow is driven by actin polymerization as well the movement of myosin XI motors along bundles of actin filaments. Active transport of organelles by the motors produces hydrodynamic flow that can efficiently transport cytosolic molecules and unbound organelles (Blau et al., 1985; Esseling-Ozdoba et al., 2008).

Motor-driven fluid flow is also important during oogenesis in Drosophila for mixing the contents of maturing oocytes. However, unlike plants, this flow is driven kinesin-1 motor activity along the microtubule cytoskeleton (Palacios and St Johnston, 2002; Serbus et al., 2005). Loss of kinesin-1 activity slows or blocks streaming (Ganguly et al., 2012; Serbus et al., 2005), and results in mispositioning of the nucleus in the oocyte (Williams et al., 2013). It is also interesting to note that ensconsin/MAP7 mutants also block cytoplasmic streaming leading to similar defects in nuclear position (Sung et al., 2008).

Based on these findings in the fly, it is tempting to speculate that the cytoplasmic flow we observe in myotubes might also be driven by the activity of Kif5B, however, we have not yet been able to suitably evaluate this possibility. More careful analysis of streaming in myotubes, including the use of tracer particles and current image analysis techniques for fluid dynamics should be performed, and the role of Kif5B and MAP7 in streaming should be evaluated. While we have shown that Kif5B motors are essential on the nuclear envelope for nuclear distribution, we have not ruled out an additional role for Kif5B in driving fluid flow. It

is also interesting to consider whether the nuclear passing events that occur in myotubes may be driven at least in part by cytoplasmic flow, and whether passing serves to spread newly incorporated nuclei amongst the earlier incorporated myonuclei, thereby ensuring that gene expression patterns do not vary dramatically throughout the myotube as it develops.

How do nuclei stop translocating and become anchored?

As the myotube matures, the nuclei move from the interior of the cell to the periphery, and become anchored. A number of proteins, including nesprins and the intermediate filament protein desmin, have been proposed to help anchor nuclei (Chapman et al., 2014; Grady et al., 2005; Ralston et al., 2006; Shah et al., 2004; Zhang et al., 2010; Zhang et al., 2007b), but we do not yet understand how the nuclei stop moving and achieve their final position within the myofiber.

Examining nuclear clustering at the developing neuromuscular junction is the most straightforward place to begin these investigations because work in primary chick myotubes has revealed that when translocating nuclei encounter a cluster of acetylcholine receptors on the cell membrane, nuclei stop moving (Englander and Rubin, 1987). If the AChR cluster disperses, the nuclei resume movement, suggesting that cytoskeletal rearrangements known to be induced by AChR clustering (Brosamle and Kuffler, 1996; Connolly and Graham, 1985; Lubit, 1984; Schmidt et al., 2012; Shi et al., 2012; Wu et al., 2010), restrict nuclear mobility and trap nuclei at the developing NMJ. In support of this hypothesis, proteolytic dissociation of mature muscle fibers, but not denervation *in vivo*, results in dispersal of the synaptic nuclei, likely through degredation of the extracellular matrix and associated disorganization of the underlying postsynaptic cytoskeleton (Brosamle and Kuffler, 1996). Despite these findings, it is not known which cytoskeletal elements are necessary for restricting the moving nuclei, and whether the nuclei stop because the cytoskeletal network is so dense it blocks

the path, or because there are specific interactions between proteins on the nuclear envelope and the cytoskeleton under the developing synapse, or both.

Initial investigation of these questions may be addressed in cultured cells following induction of AChR clustering by defined application of the neural form of the proteoglycan agrin to the myotube membrane (Bromann et al., 2004; Godfrey et al., 1984; Schmidt et al., 2012; Tourovskaia et al., 2008). However, these experiments should ideally be performed *in vivo,* or at least in a co-culture system with motor neurons, allowing for the formation of a synapse (Morimoto et al., 2013). Examination of the actin, microtubule and desmin cytoskeleton at the developing synapse over time, combined with perturbation of these networks with various drugs such as nocodozole, phalloidin, or latrunculin, should be performed to assess the contribution of these networks on local nuclear dynamics. Additionally, a more complete examination of the nesprin isoforms present on the nuclei at developing and mature synapses may yield further insight into the mechanisms of nuclear anchoring (Apel et al., 2000; Grady et al., 2005; Randles et al., 2010).

Nuclear movement and positioning during muscle regeneration

Following significant damage to the muscle, fibers are either repaired, or new myotubes are generated. During fiber repair, the damaged segment is first resealed by the sarcolemma, debris is removed by leukocytes, and satellite cells are activated, resulting in both proliferation and subsequent fusion of differentiated myoblasts to the damaged fiber (Papadimitriou et al., 1990; Yin et al., 2013). In either newly generated myotubes or repaired fibers, incorporated nuclei undergo similar movements as those nuclei in the developing muscle, in that the nuclei first move to the center of the myotube or fiber and are then later moved out to the cell periphery (Dubowitz and Sewry, 2007; Lash et al., 1957; Li et al., 2011; Schmalbruch, 1976).

While it is likely that the mechanisms driving nuclear movements in developing myotubes are also responsible for nuclear positioning in regenerating muscle, this remains to be tested. It will be important to determine how the microtubule cytoskeleton is reorganized in the myofiber following muscle damage and whether Kif5B and dynein are recruited to the surface of newly incorporated nuclei. It has been noted that the central nuclei in regenerating mouse fibers are strongly nesprin-1 positive (Apel et al., 2000), similar to the nuclei in immature myotubes, suggesting that motors likely decorate these nuclei as well.

Studying fiber regeneration in healthy individuals may help us to understand why nuclei are first positioned in the center of the fiber and whether this localization is necessary for the subsequent repair of muscle architecture in the damaged region. It may also provide additional insight into the consequences of improperly positioned nuclei on muscle function, and help us to better understand the cost of continual rounds of fiber repair in muscle disease pathology.

CHAPTER 5: Methods and Materials

Reagents

The following constructs were generously provided: pRFP-Lmna and pGFP-emerin (Dr. Howard Worman, Columbia University); mCherry-GPP130 (Dr. Adam Linstedt, Carnegie Mellon University); DsRed1-Centrin-2 (Dr. Joseph Gleeson, University of California San Diego); GFP-KIF5C tail (Dr. Mitsutoshi Setou, Hamamatsu University School of Medicine), EGFP-Nesprin2α-KASH (human, residues 483-542) (Dr. Catherine Shanahan, King's College London); YFP-α-actinin (Dr. Joeseph Sanger, SUNY Upstate Medical University); GFP-EB3 (Dr. Anna Akhmanova, University Utrecht); HA-Kif5B(1-807)-FRB (human) and PEX3-mRFP-FKBP12 (human PEX3; Casper Hoogenraad, Utrecht University); EGFP-nesprin-2 constructs (human, residues 6146-6799 and 6348-6552)(Angelika Noegel, University of Cologne); HA- and EGFP-tagged mouse KLC2 WT and R251D, N287L, R312E mutant constructs (Mark Dodding, King's College London); pRK5-Myc-KifB and –Kif5C (mouse)(Josef Kittler, University College London); EGFP-MAP7 (mouse)(Edgar Gomes, Pierre and Marie Curie University Paris).

The following constructs were generated in the Holzbaur Laboratory by Mariko Tokito. FKBP-EGFP-KASH was generated by polymerase chain reaction using the 2xFKBP12 fragment from PEX3-mRFP-FKBP and subcloned into the EGFP-nesprin-2α-KASH vector. Myc-Kif5B-Halo was generated by first inserting the Halo-tag from the pHTC HaloTag CMV-neo vector (Promega) at the C-terminus of pRK5-Myc-Kif5C and then Kif5C was replaced with Kif5B from pRK5-Myc-Kif5B. EGFP-nesprin-$2^{6146-6799}$ and EGFP-nesprin-$2^{6348-6552}$ WD/AA mutants were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit with the mutagenic primer (5'-CCCCCTGGAG**GC**GG**C**CCACACAGGC3-3'). The mutant fragment was sequenced in

its entirety and sub-cloned into EGFP-nesprin-2$^{6146-6799}$ and EGFP-nesprin-2$^{6348-6552}$. Final WD/AA mutagenic constructs were sequenced for accuracy.

All siRNA oligos were from GE Healthcare Dharmacon.

siRNA oligos used in the experiments in Chapter 2 were directed against mouse KIF5B (GenBank NM_008448, EG-ID 16573, 5'-CAACAGACAUGUCGCAGUU-3', targets ORF), scrambled control: (5'-GAACGAAUGUCGCUUACCAUU-3'), and mouse dynein heavy chain (GenBank NM_030238, 5'-GAAAUCAACUUGCCCGAUAUU-3' targets ORF), scrambled control: (5'GACCGAUAAUCAAACCUGUUU-3').

siRNA oligos used in the experiments in Chapter 3 were directed against mouse Kif5B (Gene ID 16573, 5'-GGAGGAGGCUCAUUUGUUC-3', targets ORF in the tail domain of the mouse transcript that is absent from the truncated, human Kif5B(1-807) dimerization construct); mouse KLC1 (NM_008450, EG-ID16593, 5'-GAGUAUGGCGGCUGGUAUA-3', targets ORF); mouse KLC2 (NM_008451, EG-ID 16594, 5'-CUGUAGAAAUAAAGACGAU-3', targets 3'UTR).

Primary antibodies include anti-alpha-tubulin (YL1/2, AdD Serotec), anti-GFP (GFP-1020, Aves Labs, Inc.), anti-GFP (JL8, Clontech), anti-DHC (R-325, Santa Cruz Biotechnology, Inc.), anti- KIF5B (ab15705, Abcam) anti-α-actinin (Clone EA-53, Sigma-Aldrich), anti-KLC 1/2 (63-90, from Dr. Scott Brady, University of Illinois at Chicago), anti-KLC1 (ab174273, Abcam), anti-KHC (MAB1614, Clone H2, Millipore), anti-KHC (SUK4, Abcam), anti-DIC (MAB1618, Millipore), anti-p150^{Glued} (BD Biosciences), anti-GAPDH (mAb 9484, Abcam), anti-HA (Covance), and anti-Myc (Abcam Ab 9106). Alexa fluorophore-conjugated secondary antibodies and Hoechst 33342 (H21492) were from Molecular Probes (Invitrogen). HRP-conjugated secondary antibodies and Cy2-conjugated goat anti-chicken

antibodies were from Jackson ImmunoResearch Laboratories, Inc. A/C Heterodimerizer ligand (0.5 mM) was obtained from Clontech.

C2C12 Cell Culture, transfections and drug treatment

Mouse C2C12 myoblasts (American Type Culture Collection, Manassas, VA, CRL-1772) were maintained at 37°C/5% CO_2 in growth medium (Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamax and 10% fetal bovine serum). To induce myogenic differentiation, myoblasts were grown to ~70% confluence and switched to differentiation medium (DMEM with 2 mM glutamax and 10% horse serum) for 3-7 days. Media was replaced every 24 hours. For live cell imaging, cells were grown on either 35 or 50 mm glass bottom dishes (FluorDish, World Precision Instruments) coated with 5ug/cm^2 rat tail collagen, type 1 (BD Biosciences). For immunofluoresence analyses, cells were grown on collagen-coated 1-cm^2 squares of ACLAR embedding film (Ted Pella, Inc.).

DNA transfections were performed with Lipofectamine 2000 (Invitrogen). For RNAi knockdown, cells were transfected with siRNA duplexes (Thermo Scientific Dharmacon) at a final concentration of 50nM using Lipofectamine RNAiMax (Invitrogen). Unless otherwise noted, cells were transfected four days after induction of differentiation and imaged 72 hours post-transfection. siRNA knockdown was assessed by lysing cells in HEM lysis buffer with 1% Triton X-100 and protease inhibitors (Roche); total protein was measured using the BCA protein assay kit (Pierce). Equal total protein was separated by SDS-PAGE, transferred to Immobilon–PVDF membrane (Millipore, Billerica, MA), and subjected to immunoblot analysis. Chemiluminescence was enhanced by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to film or detected with the G:Box and GeneSys

digital imaging system (Syngene). Densitometry was performed with Image-J (Fiji) (National Institutes of Health, Rockville, MD).

For motor recruitment assays in Chapter 3, cells were transfected with DNA ~84 hours prior to cell fixation or live imaging and treated with siRNA ~12 hours following DNA delivery (72 hour siRNA treatment). Control conditions were similarly treated with Lipofectamine 2000 and RNAiMax. Unless otherwise noted, cells were treated with 500 nM A/C Heterodimerizer ligand (Clontech) continuously starting 1 hr after DNA transfection. Assay results were similar when the order of DNA and siRNA addition was reversed, however, expression levels of the transfected constructs were less robust. For kinesin light chain assays, cells were concurrently transfected with DNA and siRNA (KLC1 & KLC2 at 1:1 ratio) 72 hours prior to cell fixation or live imaging. Labeling of cells transfected with Myc-Kif5B-Halo was performed with HaloTag TMR Ligand (Promega G8252) according to the manufacturer's instructions.

Myotubes were treated with nocodazole at 10 or 20 μg/ml (Sigma-Aldrich) or DMSO control for 30 min at 37°C. Myotubes were treated with 50 μM N-benzyl-ptoluene sulphonamide (BTS; Tocris Bioscience) in DMSO to inhibit myofibril contraction.

Immunofluorescence

Myotubes grown on ACLAR film were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Alternatively, myotubes were fixed with ice-cold methanol with 1 mM EDTA or 1:1 acetone-methanol as noted. Fixed cells were incubated with primary and secondary antibodies as noted then stained with Hoechst 33342 (0.5 μg/ml) and mounted on 40mm glass coverslips with ProLong Gold antifade reagent (Invitrogen).

GST-pulldown assays

GST-Nesprin2 constructs from Angelika Noegel and Iakowos Karakesisoglou (Schneider et al. 2011) were expressed in BL21 DE3 *E. coli* and purified by binding to Glutathione-Sepharose^{TM} 4B (Amersham). C2C12 myotube lysates were prepared using HEM lysis buffer (50 mM HEPES, 25 mM NaCl, 1 mM EDTA, 1 mM MgCl2, pH 7.0 plus 0.1% Triton X-100 and protease inhibitors (Roche)). Lysates were incubated overnight at 4°C with equal amounts of purified GST fusion proteins coupled to GST-Sepharose beads. Interacting proteins were eluted with 40 mM Glutathione, pH 7.5, and analyzed by western blot.

Co-Immunoprecipitation

COS7 cells (American Type Culture Collection, CRL-1651) were transfected using Fugene6 (Roche) according to the manufacturer's instructions and harvested 15-16 hours after transfection. Two 10-cm plates of cells per condition were lysed with 300 μl ice-cold HEM (50 mM HEPES, 25 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, pH 7.0) buffer with 0.1% Triton X-100 and protease inhibitor cocktail (Roche). Cells were sheared by 5 passages through a 20-gauge needle and incubated on ice for 30 minutes, followed by centrifugation at 13,000 g for 15 minutes at 4°C. The resulting supernatant was incubated for 60 minutes with Protein- G Dynabeads that were pre-incubated with either anti-HA (Covance) or anti-EGFP (JL8, Clontech) antibodies. Beads were washed and boiled in 50 μl SDS-loading buffer. Samples were analyzed by western blot. EGFP was detected on immunoblots using anti-EGFP raised in chicken (Aves Lab, Inc.) to avoid detection of the mouse anti-EGFP IgG bands.

Microscopy

All live cell imaging was performed in an environmental chamber set to 37°C; cells were imaged in phenol red-free DMEM with 25 mM HEPES (Gibco), 10% horse serum, and 2mM Glutamax, overlaid with mineral oil (Sigma-Aldrich). Where noted, DNA was labeled with Hoechst 3342 (0.5 μg/ml) for 20 minutes prior to imaging.

For nuclear translocation analyses in Chapter 2, imaging was performed on a Leica DMI6000B inverted epifluorescence microscope using an Apochromatic 63x 1.4 NA oil-immersion objective with 1.6x magnifier (Leica Microsystems). Digital images were acquired with a Hamamatsu ORCA-R^2 charge-coupled device camera using LAS-AF software (Leica Microsystems). Phase images were taken every 30 sec for 60 or 180 min.

All fluorescence imaging was performed on a Perkin Elmer UltraView Vox Spinning Disk Confocal with a Nikon Ti Microscope equipped with PFS, a motorized stage, and 40x/1.30 NA, 60x/1.49 NA, and 100x/1.49 NA oil-immersion apochromatic objectives (Nikon). Digital images were acquired with a Hamamatsu EMCCD C9100-50 camera and Volocity 3D Image Analysis Software (Improvision/Perkin Elmer).

For nuclear rotation analyses in Chapter 2, Z-series encompassing the depth of each myotube (~15-30 μm) were obtained with a 0.5 μm step size. Images were taken at a rate of 1 z-series per minute for 15 minutes. For dual-color movies, z-series of each fluorophore were taken consecutively to minimize exposure times.

GFP-EB3 analyses in Chapter 2 were performed using an Apochromatic 100x 1.49 NA oil-immersion objective (Nikon). Images were taken in a single z-plane at a rate of 1 frame every 3 seconds.

In Chapter 3, long-term imaging of PEX3-mRFP-FKBP in whole myotubes was performed at 40x, z-series encompassing the entire depth of each myotube (~20-30 μm, 1

μm z-step), were obtained every 15 minutes with from adjacent areas with 10% overlap using the motorized stage. Images were stitched together digitally in Volocity to obtain composite images. Fast imaging of PEX3-mRFP-FKBP was performed 60x in a single z-plane at a rate of 20 frames/minute. Following labeling of the Halo-tag with TMR ligand, single z-plane images of EGFP-KLC2 and Myc-Kif5B-Halo-TMR were obtained in live cells at 60x.

Images of fixed myotubes were acquired at 40x, 60x, or 100x. Z-series encompassing the entire depth of each myotube (~15-30 μm) were taken at a step-size of 0.5 μm. For analysis of nuclear distribution in Chapter 2, z-series (1 μm z-step) of adjacent areas were obtained at 40x with 20% overlap using the motorized stage. Images were stitched together digitally in Volocity to obtain composite images of ~1000 x 1000 μm. Similar imaging parameters were used for the nuclear distribution analyses in Chapter 3, however, for induced motor recruitment assays in Figure 3.2, images were acquired at 60x for better visualization of the presence or absence of HA signal on the nuclear envelope.

Image analysis

To assess nuclear translocation, the centroid of each nucleus was manually tracked using LAS-AF software (Leica Microsystems); mean velocity reported reflects both translocating and non-moving nuclei. To quantify nuclear rotation, bright Hoechst-dye-positive chromocenters in individual nuclei were automatically tracked in XYZ over time using Volocity 3D Image Analysis Software (Improvision/Perkin Elmer). Principal component analysis was used to find the angular velocity and translational speed from the tracked chromocenter positions. Analysis routines were implemented in Matlab. For analyses of nuclear rotation in treated myotubes, angular velocity was first quantified in a subset of nuclei exhibiting a wide range of rotation. These nuclei then served as standards for comparison

as rotation of the remaining nuclei was visually assessed and binned into 30° categories. The direction of GFP-EB3 comets was quantified from the resulting overlay images of three sequential pseudo-colored frames (Image-J). Comet direction was evaluated with regard to the long-axis of the myotubes.

Nuclear distribution was assessed in stitched images of fixed myotubes using Volocity 3D Image Analysis software. The position of the ends of the myotube and the centroid of each nucleus in the myotube was defined XY; only unbranched myotubes (<800 μm in length, Chapter 2; <600 μm in length, Chapter 3) were included in the analysis. Nuclear position was linearized along the myotube length and distance between adjacent nuclei was calculated. Nuclear position as a percent of the distance along the myotube length was also determined.

Movement of peroxisomes was tracked using the manual tracking function in Fiji. Line scan analysis was also performed using Fiji; a 100-pixel line was drawn from the cytoplasm into a nucleus at a right angle, such that the center of the line coincided with the edge of the nucleus, as demarcated by the Hoechst dye signal. The fluorescence intensity along this line was obtained in all pertinent channels for each nucleus. Data was normalized to the first 10 points of each line to account for variation in the cytoplasmic signal.

All graphs and statistical analyses were performed using GraphPad Prism V5.0 (GraphPad Software, Inc.). Figures were assembled using Fiji and the Adobe Photoshop and Illustrator packages (Adobe, CA, USA).

Sequence Alignments

Sequence alignments were performed in Geneious Basic 4.8.3 (Biomatters, Ltd.).

APPENDIX: Supplemental Video Legends

Video 1. Nuclei translocate and rotate in developing myotubes. Time-lapse phase microscopy of nuclear migration in a D7 C2C12 myotube. Frames were taken once every 30 seconds for 3 hours, movie plays at 20 frames per second. Scale = 10 μm. Still images from this movie are shown in Figure 1F.

Video 2. Nuclei rotate in three dimensions in myotubes. Representative examples of rotating nuclei stained with Hoechst 33342 to label DNA (blue). Images were acquired by time-lapse microscopy using a spinning-disk confocal microscope. Maximum projections of z-stacks are shown. Frames were taken once a minute for 15 minutes, movies play at 5 frames per second. Scale = 2 μm. Still images from the left two movies are shown in Figure 2A.

Video 3. Nuclei rotate as intact structures in myotubes. Representative examples of rotating nuclei in myotubes transfected with RFP-laminA (red), GFP-Emerin (green), DsRed-Centrin-2 (red) or mCherry-GPP130 (red). DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. Maximum projections of z-stacks are shown. Frames were taken once a minute for 15 minutes, movies play at 5 frames per second. Scale = 2 μm. Still images from these movies are shown in Figure 2B.

Video 4. Microtubules grow bidirectionally in the center of myotubes and are polarized at myotube ends. Myotubes were transfected with GFP-EB3 (green). DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. Frames were taken once every 3 seconds for 2 minutes, movies play at 5 frames per second. Left: center of myotube, scale = 10 μm. Right: end of myotube, scale = 5 μm. Still images from these movies are shown in Figure 3C(1,2).

Video 5. Microtubules are dynamic between nuclei and across the nuclear surface. Myotubes were transfected with GFP-EB3 (green). DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. Frames were taken once every 3 seconds for 2 minutes, movies play at 5 frames per second. Left scale = 10 μm, right scale = 2 μm. Still images from these movies are shown in Figure 3C(3,4).

Video 6. Microtubules are necessary for nuclear rotation in myotubes. Myotubes were treated with DMSO or with nocodazole (10μg/ml) at 37°C for at least 30 minutes prior to imaging. DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning-disk confocal microscope. Maximum projections of z-stacks are shown. Frames were taken once a minute for 15 minutes, movies play at 5 frames per second. Scale = 10 μm. Still images from these movies are shown in Figure 4C.

Video 7: Nuclei still translocate and rotate when microtubule dynamics are reduced. Myotubes were transfected with GFP-EB3 (green). DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. After initially imaging EB3 dynamics in an untreated myotube (left), a low-dose (100ng/ml) of nocodazole was added to the plate to inhibit microtubule growth (center & right). In the left two panels, frames were taken once every 3 seconds for 45 seconds. In the right panel, z-series were taken once a minute for 15 minutes, maximum projections of the z-stacks are shown. All movies play at 5 frames per second. Scale = 10 μm.

Video 8. Nuclear rotation is abolished in KIF5B-deficient myotubes. Myotubes were treated with siRNA against KIF5B or a scrambled control (SCR) for 72 hours. DNA was labeled with Hoechst 33324 (blue). Images were acquired by time-lapse microscopy using a spinning-disk confocal microscope. Maximum projections of z-stacks are shown. Frames were taken once a minute for 15 minutes, movies play at 5 frames per second. Scale = 10 μm. Still images from these movies are shown in Figure 6C.

Video 9. Microtubules grow off-axis around the nuclei aggregated in the center of myotubes treated with KIF5B siRNA but are still polarized at myotube ends. Myotubes were transfected with siRNA against KIF5B and with GFP-EB3 (green). DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. Frames were taken once every 3 seconds for 2 minutes, movies play at 5 frames per second. Left: center of myotube. Right: end of myotube. Scale = 10 μm.

Video 10. Myofibrils contract in D7 myotubes. Myotubes were transfected with YFP-α-actinin (green) to label z-disks of myofibrils. DNA was labeled with Hoechst 33342 (blue). Images were acquired by time-lapse microscopy using a spinning disk confocal microscope. Frames were taken once a second for 1 minute, movie plays at 5 frames per second. Scale = 10 μm.

Video 11. Nuclei still translocate and rotate when myofibril contraction is inhibited. A myotube was imaged by time-lapse phase microscopy in the presence of BTS (50 μM) to inhibit the activity of skeletal muscle myosin. Frames were taken once every 30 seconds for 1 hour, movie plays at 20 frames per second. Scale = 10 μm.

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