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The Role of Classical Nuclear Import Receptors during Myogenesis

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

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The Role of Classical Nuclear Import Receptors during Myogenesis

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B.S., University of Louisville, 2004

Advisor: Grace K. Pavlath, Ph.D.

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Abstract

The Role of Classical Nuclear Import Receptors during Myogenesis

By Monica Nicole Hall

Skeletal muscle is required for breathing, locomotion and metabolism. In adults, myogenesis, the process of creating muscle is dependent on myogenic stem cells (satellite cells) that have the ability to proliferate, differentiate and fuse into multinucleated cells called myofibers *in vivo* or myotubes *in vitro*. Changes in gene expression during particular stages of myogenesis are due to nuclear factors, such as myogenic transcription factors, entering the nucleus and regulating gene expression. However, little is known regarding the regulation of nuclear import of these nuclear factors in either mono or multinucleated muscle cells. Classical nuclear import is one major pathway used by eukaryotic cells to target proteins to the nucleus and is dependent upon a classical nuclear localization signal (cNLS) within a protein that is recognized by the nuclear import receptor family, karyopherin alpha (KPNA). Six KPNA paralogs exist in mouse: KPNA1, KPNA2, KPNA3, KPNA4, KPNA6 and KPNA7. We established that five KPNA paralogs are expressed by primary mouse myoblasts *in vitro* and that their steady-state levels increase during differentiation. We used RNAi to identify paralog-specific roles for KPNA1 and KPNA2 in muscle cell proliferation, while only KPNA2 had a role in myotube growth. Furthermore, we determined that quiescent (non-proliferating) satellite cells express four KPNA paralogs, *Kpna2*, *Kpna3*, *Kpna4* and *Kpna6*, but not *Kpna1*. Since *Kpna1* is expressed in proliferating satellite cells, we investigated the role of KPNA1 in satellite cell proliferation. Analysis of $Kpna1^{+/-}$ muscles revealed an increase in satellite cell proliferation during muscle maintenance and regeneration compared to $Kpna1^{+/+}$ muscles. We hypothesize that KPNA1 may function in satellite cells to import nuclear proteins required for the negative regulation of proliferation to prevent over-proliferation and potential exhaustion of the satellite cell pool in skeletal muscle. These results identify distinct import pathways that rely on specific KPNAs suggesting that regulation of classical nuclear import has a critical role in controlling gene expression during myogenesis. Uncovering the role of classical nuclear import in regulating the nuclear import of proteins required for proper satellite cell function and myotube growth could provide valuable insight into the critical role of nucleocytoplasmic transport during myogenesis.

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List of Abbreviations

BrdU:	5-bromo-2-deoxyuridine
cNLS:	classical nuclear localization signal
DM:	differentiation media
eMyHC:	embryonic myosin heavy chain
FGF:	fibroblast growth factor
FACS	fluorescence activated cell sorting
GM:	growth media
KPNA:	karyopherin alpha
KPNB1:	karyopherin beta1
NFAT:	Nuclear Factor of Activated T cells
NLS:	nuclear localization signal
NPC:	nuclear pore complex
Nup:	nucleoporin
PI:	propidium iodide
TA:	tibialis anterior
XSA:	cross-sectional area

Chapter 1: Background and Significance

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Chapter 1: Background and Significance

This chapter highlights what is known about nucleocytoplasmic transport in skeletal muscle and considers the unique challenges that muscle stem cells and multinucleated muscle cells encounter in regulating nucleocytoplasmic transport during adult muscle regeneration. First, the structure and function of normal adult skeletal muscle is briefly presented. Second, adult skeletal muscle origins, model systems and morphology are introduced. Third, the challenges of studying satellite cell (muscle stem cell) function are described. Fourth, the role of gene regulation during myogenesis is discussed. Finally, the current knowledge of the emerging importance of nucleocytoplasmic transport in regulating gene expression during myogenesis is reviewed.

1.1 Skeletal Muscle

Skeletal muscle is one of three muscle types that exists in vertebrates along with cardiac and smooth muscle. Skeletal muscle is tethered to skeletal bone and is critical for contractions that enable the voluntary movement of specific bones, as well as facilitating locomotion and breathing. In addition, skeletal muscle mass makes up approximately 50% of total body weight and as such has a significant role in metabolism. A hallmark of adult skeletal muscle is its amazing ability to undergo extensive regeneration upon injury. Therefore, failure in the capacity to regenerate due to disease, injury or aging can affect motility and lifespan. The advancement of therapeutic strategies to treat failing muscle could extend lifespan or at minimum alleviate medical conditions associated with muscle

dystrophy or aging. The development of these therapeutic treatments hinges upon further advancement in our knowledge of gene regulation during adult muscle regeneration.

1.1.1 Skeletal Muscle Structure and Function

Each muscle group is comprised of bundles of cylindrical multinucleated cells called myofibers which are attached to bone by tendons at locations called myotendinous junctions (Figure 1.7.1). Muscles generate force through contractions that are stimulated via nerve impulses that travel through nerves, or motor neurons, to fibers across an interface called the neuromuscular junction. The components of the contractile apparatus are visible at the microscopic level where striations are observed along myofibers due to the presence of many cylindrical myofibrils (Figure 1.7.1). Myofibrils contain many interlocking myofilaments called sarcomeres that are comprised of actin and myosin that slide past each other, thereby shortening the myofiber to generate force. Aside from myofibers and motor neurons, skeletal muscle tissue also contains muscle stem cells or satellite cells which reside between the myofiber cell membrane (sarcolemma) and connective tissue called the basal lamina that encapsulates the myofiber (Figure 1.7.2.A.) (Mauro, 1961). Satellite cells comprise 1-5% of skeletal muscle mass and are essential to myogenesis, the process of creating new muscle (Hawke and Garry, 2001). During myogenesis, satellite cells proliferate and fuse to facilitate the generation of new myonuclei for muscle growth and regeneration (Figure 1.7.2.B.). While this chapter focuses on the role of satellite cells in regeneration, it is generally understood that skeletal muscle also contains non-muscle cell types such as connective tissue and blood vessels, all of which have supporting roles in regeneration and proper tissue function.

1.1.2 Adult Skeletal Muscle Origin, Model Systems and Morphology

Muscle regeneration in adult mammal resembles to some extent the development of skeletal muscle during embryogenesis where mononucleated muscle progenitors fuse with one another to form functionally mature multinucleated myofibers. Skeletal muscle formation of the trunk and limbs occurs during embryonic development through the specification of multipotent mesodermal cells originating from the somites, segmented structures that form from the paraxial mesoderm (Kang and Krauss, 2010). Specification of these cells into a myogenic lineage occurs with the upregulation of myogenic regulatory factors as a result of positive and negative cues during development (Asakura et al., 2001). During initial muscle formation, proliferative muscle precursor cells, termed myoblasts, withdraw from the cell cycle and begin to express additional myogenic regulatory proteins to become terminally differentiated myocytes. Myofiber formation in the trunk and limb occurs through a highly regulated process that involves myogenic cells migrating out of the somites as well as migrating into the limb buds to form different muscle groups (Kang and Krauss, 2010). Myocytes then fuse with each other to generate the first multinucleate myofibers that mature into contracting muscle fibers. During development, a distinct population of myogenic cells, called satellite cells, associate with growing myofibers and remain in a quiescent state or non-proliferative state to provide myonuclei for postnatal growth and adult regeneration (Mauro, 1961). Satellite cells, as their name suggests, are found at the periphery of each myofiber and are collectively referred to as the muscle satellite cell pool (Fig. 1.1.2.A.). The satellite cell pool is essential to myogenesis and must be maintained within muscle tissue throughout the adult

lifespan to generate myonuclei for future cycles of regeneration since myofibers are post-mitotic in mammals.

Our understanding of adult muscle regeneration has occurred through advancements in molecular biology techniques and the use of rodent model systems. Rodent models of muscle injury *in vivo* involve injecting chemicals, physical injury, forced exercise or muscle cell transplants into skeletal muscle to induce regeneration in a controlled, predictable manner. In addition, several rodent muscle dystrophy models exist with varying degrees of regenerative capacity as alternatives for studying regeneration. In general, rodent injury models allow for a scheduled analysis of the different stages of skeletal muscle regeneration. These *in vivo* studies of skeletal muscle regeneration include, but are not limited to, isolation of satellite cells or bulk muscle to analyze its molecular content or enzymatic function as well as analyzing muscle morphology by microscopy.

Distinct skeletal muscle morphologies exist for healthy, diseased or injured muscle as detected through the use of well-established histological techniques. The morphology of uninjured muscle is characterized, in histological cross section, as intact myofibers with myofiber nuclei residing at the myofiber cell periphery or plasma membrane. Satellite cells are distinguished from myonuclei by their location between the basal lamina and the myofiber sarcolemma (Mauro, 1961). The turnover of myonuclei in uninjured muscle is estimated to be around two percent of total myonuclei per week in a rodent model (Schmalbruch and Lewis, 2000). Overall, adult uninjured skeletal muscle is generally characterized as a stable tissue that does not undergo significant changes in morphology. The major morphological characteristics of injured or diseased skeletal

muscle are represented by disruptions in the myofiber membrane, the appearance of myonuclei within the center of the myofiber, smaller caliber fibers (decreased cross-sectional area) and an increase in the number of both muscle and non-muscle mononucleated cells. The increase in non-muscle cells is in part due to an infiltration of immune cells which are thought to have a significant role in regulating muscle repair (Husmann et al., 1996; Tidball and Villalta, 2010). Additional cell types are observed, some of which are involved in the replacement of connective tissue, blood vessels and neurons, since these cells are also required to restore proper muscle structure and function.

Morphological observations of damaged muscle reveal two phases of muscle regeneration which are termed the degenerative phase and the regenerative phase (Charge and Rudnicki, 2004). Muscle degeneration is characterized by disruption or damage to the myofiber sarcolemma as well as myofiber necrosis. Damaged myofibers release factors that induce muscle cells and inflammatory cells to engage in muscle repair (Charge and Rudnicki, 2004). Subsequently, the invasion of non-resident cells is observed, including immune cells that have roles in inflammation and infection as well as phagocytotic macrophages that clear debris (Arnold et al., 2007; Tidball and Villalta, 2010). The muscle regeneration phase is characterized by the cellular proliferation of myogenic cells that provide a new source of myonuclei, the de novo formation of myofibers and the fusion of myocytes into damaged myofibers (Charge and Rudnicki, 2004). Lastly, new myofibers increase in size and myonuclei move to the myofiber membrane periphery. Ultimately, upon completion of regeneration, the injured muscle tissue becomes morphologically, and functionally, indistinguishable from normal muscle.

Alternatives to studying muscle regeneration *in vivo* include two well established *in vitro* myogenesis models. One model involves culturing primary or immortalized mouse myoblasts, the progeny of satellite cells, and inducing them to differentiate into multinucleated myotubes by changes in culture media (Rando and Blau, 1994). Cell culture media lacking serum induces an orderly pattern of terminal differentiation, myoblast fusion and myotube formation analogous to myogenesis *in vivo*. This model system has allowed for major advances in our understanding of many molecular pathways involved with regulating myogenesis. In addition, this model perpetually lends itself to the ever-growing arsenal of molecular biology techniques. A second model utilizes activating satellite cells in association with their myofibers and involves the gentle isolation and culturing of fresh myofibers (Bischoff, 1975; Rosenblatt et al., 1995). Studying satellite cells in this manner provides the benefit of culturing while simulating to a degree the muscle stem cell or satellite cell niche *in vivo* since the niche has been shown to contribute to satellite function (Kuang et al., 2008). However, the study of satellite cells *in vitro* is limited to short term culturing since the isolation and culture process results in satellite cell activation and progression of the cell into the myogenic program, however, *in vivo* systems enable the study of processes not easily studied *in vivo*. Overall, rodent model systems of adult skeletal muscle regeneration prove invaluable to unraveling the complexities of gene regulation during myogenesis.

1.1.3 Challenges of Studying Satellite Cell Function

Satellite cells or muscle stem cells are essential to muscle regeneration since they are the primary source of myonuclei during regeneration (Mauro, 1961). During postnatal growth or adult muscle regeneration, satellite cells activate or exit quiescence and have the ability to proliferate or undergo self-renewal. The progeny of satellite cells, myoblasts, are capable of proliferating or differentiating into myocytes which fuse with each other to form nascent myofibers or with existing myofibers (Figure 1.7.2.B.). Satellite cells are defined anatomically and functionally through their location outside of the fiber sarcolemma and beneath the basal lamina in addition to their capacity to generate muscle by participating in myogenesis (Mauro, 1961). Morphological features of satellite cells are consistent with their quiescent state and include a smaller nucleus size, an increased nuclear to cytoplasmic ratio, and reduced organelle content compared to proliferating myoblasts (Charge and Rudnicki, 2004). The satellite cell pool or total number of satellite cells present to participate in myogenesis becomes smaller with age. Therefore, a decrease in regenerative capacity is also observed with age (Gibson and Schultz 1983; Charge and Rudnicki 2004). The reason for the decrease in satellite cell pool size is unclear, but several hypotheses exist including satellite cell replicative senescence and/or failure of the satellite cell niche to provide proper signals (Conboy et al., 2005; Decary et al., 1997; Gopinath and Rando, 2008; Kuang et al., 2008). Since the satellite cell is the primary unit in generating skeletal muscle, the mechanisms for satellite cell quiescence and activation during aging and disease are a major focus of study.

The study of satellite cells and their role in muscle regeneration serves as a paradigm for the study of stem cell lineage commitment and cell differentiation. Our

understanding of satellite cell function as well as muscle regeneration have also benefited from the use of rodent models. Controlled cell ablation and lineage tracing experiments have highlighted the complexities of satellite cell lineage commitment and differentiation as well as the role of the satellite cell niche, or environment, in controlling muscle development and regeneration. Characterization of the satellite cell pool can be performed in numerous ways including isolation of cells through fluorescence-activated cell sorting (FACS) using a panel of different cell-surface markers (Kuang et al., 2008). Isolated satellite cells can be analyzed for their molecular content or their ability to participate in myogenesis in culture. Other studies investigating the myogenic potential of satellite cells involve irradiating mice to destroy the endogenous satellite cell population to prevent their contribution to growth, followed by transplantation of a genetically different population of satellite cells to assess their ability to contribute to muscle regeneration in the irradiated animal (Gross, Bou-Gharios et al. 1999; Gross and Morgan 1999). These studies are also useful in studying satellite cell self-renewal since satellite cells, like other stem cell populations, undergo self-renewal to replenish the pool for subsequent rounds of growth or regeneration (Collins et al., 2005; Collins and Partridge, 2005; Sacco et al., 2008). Cultured satellite cells also have the potential to differentiate into non-skeletal muscle lineages such as adipogenic and osteogenic lineages upon induction, although, the potential appears to be restricted to mesenchymal lineages (Kajimura et al., 2009; Shefer et al., 2004). In addition, the *in vivo* contribution of satellite cells to bone and fat appears to be marginal except in bone disease or in muscular dystrophy such as Duchenne muscular dystrophy where an increase in adipocytes is observed (Schmalbruch, 1984).

Characterization of the satellite cell pool through FACS has revealed a heterogeneous population with different subpopulations having varied capacities for proliferation and muscle regeneration (Kuang et al., 2007). While Pax7 marks the satellite cell pool, there is a lack of markers for the specific and often overlapping stages of satellite cell lineage progression and/or self-renewal. The lack of satellite cell markers for specific stages of satellite cell function presents a great challenge in studying satellite cell biology. The identification of cell surface antigens and/or proteins that mark specific stages of satellite cell lineage progression or renewing satellite cells will greatly enhance our understanding of satellite cell biology and function. To further complicate satellite cell functional studies, other non-muscle resident and muscle resident stem cells have been identified as contributing to the satellite cell pool and participating in muscle regeneration, albeit at a marginal level. Several studies indicate that mesenchymal stem cells, vascular endothelial cells, pericytes, and interstitial cells have some capacity to participate directly or indirectly in replenishing the satellite pool or muscle regeneration (Andersen et al., 2009; Brachvogel et al., 2005; Corbel et al., 2003; Ferrari et al., 1998). Further investigation of muscle and non-muscle cell subpopulations within skeletal muscle as well as their origins is tantamount to understanding the role of satellite cells in the maintenance and muscle regeneration. Lastly, the therapeutic potential of satellite cell transplantation to treat muscle dystrophies is contingent on full characterization of these subpopulations to determine which subpopulation(s) have the best regenerative capacity upon transplantation into diseased muscle.

1.1.4 Gene Regulation during Myogenesis

Control of gene expression during myogenesis involves the spatial and temporal control of nuclear factors or transcription factors that interact with the transcriptional machinery. At particular stages of myogenesis, extrinsic or intrinsic cell signals induce complex transcriptional regulatory cascades that depend upon specific molecular regulatory factors. While regulatory networks in skeletal muscle development resemble that of networks in adult muscle regeneration, recent studies have uncovered distinct genetic requirements for some transcription factors during development, post-natal growth and adult muscle regeneration (Carvajal and Rigby, 2010). For example, during development, the transcription factor Pax7 has a critical role in muscle cell lineage determination, but is not required in adult skeletal muscle (Lepper et al., 2009). The distinct genetic requirement for transcription factors at one stage of myogenesis, but not another, highlights the importance of proper transcription factor expression as well as the proper localization of a transcription factor with the cell's transcriptional machinery.

Myogenesis requires both muscle-specific and non-muscle regulatory factors. Myogenic regulatory factors termed MRFs are the best characterized factors that are essential to different stages of myogenesis. These include MyoD, myogenin and MRF4 from the basic helix-loop-helix family. These transcription factors activate myogenic regulatory cascades in response to extrinsic factors such as growth factors and other signaling molecules (Kuang et al., 2008). Distinct subpopulations of satellite cells require either MyoD or Myf5 for normal skeletal muscle development (Gensch et al., 2008; Haldar et al., 2008; Rudnicki et al., 1993). Meanwhile, MyoD is expressed by proliferating myoblasts, while cells committing to myogenic differentiation express

Myogenin and MRF4 (Charge and Rudnicki, 2004; Meadows et al., 2008). Non-muscle specific transcription factors have also been shown to have critical roles in myogenesis and adult muscle regeneration, such as nuclear factor of activated T cells, NFATs (Abbott et al., 1998; Horsley et al., 2001), Notch (Conboy and Rando, 2002) and NF-κB (Guttridge et al., 2000). In summary, myogenesis requires the coordinate activation and repression of a multitude of genes during the myogenic continuum of quiescent satellite cells to mature multinucleated myofibers. Further advances in our understanding of how gene regulation is controlled in satellite cells and during myogenesis will better enable us to treat aging and diseased muscle.

1.2 Nucleocytoplasmic Transport during Skeletal Myogenesis

Myogenesis requires the activation and repression of a multitude of genes during myogenesis. How satellite cells differentially regulate nucleocytoplasmic transport of cargo proteins critical for regulating gene expression during quiescence, activation and differentiation is unknown. In addition, how a myofiber with hundreds of nuclei coordinates and regulates nucleocytoplasmic transport is not clear. This section describes what is known regarding the role of nucleocytoplasmic transport in regulating gene expression during skeletal myogenesis.

1.2.1 The Nuclear Envelope

The nuclear envelope of eukaryotic cells provides separation of the genetic material and transcriptional machinery within the nucleus from the translational machinery in the cytoplasm enhancing regulation of gene expression. The nuclear

envelope is comprised of two lipid membrane bilayers, the outer nuclear membrane which is contiguous with the endoplasmic reticulum and the inner nuclear membrane which faces the nucleoplasm (Hetzer and Wente, 2009). The inner nuclear membrane contains integral transmembrane proteins that interact with lamins within a nuclear lamina meshwork of intermediate-type V filaments that lines the inner nuclear membrane. The nuclear lamina contributes to nuclear envelope stability and provides a platform for proteins involved in chromatin anchoring, DNA replication and gene transcription (Kind and van Steensel, 2010).

Several studies have revealed a critical role for inner nuclear envelope proteins in regulating the expression of muscle-specific genes during muscle differentiation (Datta et al., 2009; Huber et al., 2009; Liu et al., 2009; Ostlund et al., 2009). For example, loss of function or mutation of lamins or lamin-associated inner nuclear membrane proteins can result in tissue-specific diseases which are referred to as nuclear envelopathies (Holaska, 2008; Mattout et al., 2006). These diseases encompass a wide range of clinical phenotypes with different envelopathies affecting different tissues including muscle. Mutations in the nuclear envelope transmembrane protein emerin are associated with Emery-Dreifuss muscular dystrophy(Manilal et al., 1996), while mutations in lamin A/C lead to two muscular dystrophies, Emery-Dreifuss muscular dystrophy and Limb-Girdle muscular dystrophy 1B (Muchir et al., 2000). Emerin sequesters β-catenin and Lim domain protein, LMO7, at the inner nuclear periphery to regulate their participation in gene transcription; therefore disease-causing mutations in emerin may disrupt access of these proteins to the transcriptional machinery (Holaska et al., 2006; Markiewicz et al., 2006). In addition, nuclear envelope transmembrane proteins, termed NETs, have been

identified, a subset of which are hypothesized to have skeletal muscle-specific roles since they are highly expressed in skeletal muscle tissue compared with other mouse tissues (Chen et al., 2006; Schirmer et al., 2003). Specific roles for NETs appear to exist in signaling pathways during muscle differentiation (Datta et al., 2009; Huber et al., 2009; Liu et al., 2009). For example, during differentiation of C2C12 cells, a mouse muscle cell line (Yaffe and Saxel, 1977), depletion of NET25 led to elevated mitogen-activated kinase (MAPK) signaling which delayed myogenesis (Huber et al., 2009). In contrast, depletion of NET39 accelerated myogenesis through diminished mammalian target of rapamycin (mTOR) signaling and increased Insulin-like growth factor 2 (IGF-2) production (Liu et al., 2009). Together, these studies expose a crucial role for nuclear envelope proteins in regulating gene expression during muscle differentiation. Mutations in nuclear envelope transmembrane proteins may alter signaling at the nuclear envelope and may contribute to the altered gene expression observed in laminopathies affecting skeletal muscle. Further studies will likely uncover functional roles for other nuclear envelope proteins in regulating skeletal muscle gene expression.

1.2.2 Nuclear Pore Complexes

The nuclear envelope is perforated by nuclear pore complexes (NPC) which fuse the outer nuclear membrane and inner nuclear membrane together to create channels for nucleocytoplasmic transport (Figure 1.7.3) (Lim and Fahrenkrog, 2006). Nuclear pore complexes are multiprotein suprastructures (~50 MDa) which provide channels for the nucleocytoplasmic exchange of ions and macromolecules (Alber et al., 2007). While smaller ions and molecules can diffuse through the NPC, molecules larger than ~40 kDa

require a targeting signal and a soluble transport receptor to mediate transport through the NPC (Freitas and Cunha, 2009; Rabut et al., 2004; Weis, 2003). The NPC is comprised of ~30 types of nucleoporins or Nups, many of which are present in multiple copies, consistent with the 8-fold symmetry of the NPC (Frenkiel-Krispin et al., 2010). Based on the current model of the NPC, Nups are categorized as scaffold, transmembrane or peripheral Nups (Figure 1.7.3) (Fernandez-Martinez and Rout, 2009). Scaffold Nups, also termed core Nups, provide structure to the NPC core by forming a cage-like scaffold, while transmembrane Nups located at the nuclear envelope-NPC interface, function in NPC biogenesis and nuclear envelope anchoring (Strambio-De-Castillia et al., 2010). Peripheral Nups within the NPC function in cargo transport, chromatin anchoring and gene transcription. Peripheral Nups lining the pore channel contain phenylalanine-glycine (FG) repeats that extend into the channel to function in NPC permeability and mediate facilitated transport of macromolecules (Strambio-De-Castillia et al., 2010). The physical mechanics of NPC permeability and transport are still unclear with several models proposing varying arrangements of FG-Nups during transport; however, the functional role of individual FG-Nups in mediating transport and regulating different transport pathways has been well established (Walde and Kehlenbach, 2010).

NPCs within the nuclear envelope display variability in density and distribution between cell types and even within a single cell (Hetzer and Wente, 2009). For example, NPC density differs between Xenopus oocytes (>50 NPCs/mm^2) and C2C12 muscle cells (5 NPCs/mm^2) by 10-fold (D'Angelo et al., 2009; Hetzer and Wente, 2009), meanwhile a 50% increase in NPC density was observed as mouse embryonic stem cells differentiated into cardiomyocytes (Perez-Terzic et al., 2007). In addition, differences in NPC density

across the nuclear envelope have been observed in *Saccharomyces cerevisiae* which suggests nuclear transport may be spatially regulated across the nuclear envelope (Winey et al., 1997). NPC nucleoporin composition also varies between cell types where multiple Nups involved in nucleocytoplasmic transport display differential expression between tissues (Hetzer and Wente, 2009; Smitherman et al., 2000; Tran and Wente, 2006). In skeletal muscle, the transcripts for numerous Nups are upregulated during satellite cell activation suggesting that an increase in NPC biogenesis occurs in proliferating satellite cells (Fukada et al., 2007; Pallafacchina et al., 2010). Specific roles for nucleoporins appear to exist in signaling pathways during muscle differentiation. For example, a study using C2C12s uncovered a role for nucleoporin Nup358 in proper NPC architecture and myotube formation (Asally et al., 2011). Meanwhile, *Tetrahymena thermophila*, a binucleated ciliated protozoa expresses a subset of Nups that differentially localize to either the macronucleus or the micronucleus to regulate transport of cargoes involved in nucleus-specific functions (Malone et al., 2008). Variations in NPC density, distribution and nucleoporin composition between cell types or nuclei sharing a common cytoplasm, suggest that NPCs and nucleoporins are regulated to accommodate ever changing demands on nuclear transport in both mono and multinucleated cells.

In proliferating eukaryotic cells, new NPCs are formed during mitosis and interphase which allows for regular replacement of Nups (Doucet and Hetzer, 2010). Therefore, de novo assembly of NPCs would not be predicted to occur in post-mitotic cells. Experiments examining the synthesis of scaffold and peripheral Nups in post-mitotic C2C12 myotubes, revealed that some peripheral Nups, such as NUP153 and NUP50, are continuously synthesized, whereas scaffold Nups, such as NUP107 and

NUP160, are transcriptionally down-regulated in myotubes and are consequently not replaced (D'Angelo et al., 2009). Accumulation or loss of damaged scaffold Nups in non-dividing cells, such as muscle, could result in dysfunctional NPCs and loss of integrity of the nucleocytoplasmic barrier. Indeed, oxidized scaffold Nups in neuronal nuclei from aged rats are associated with leakage of cytoplasmic proteins into the nucleus (D'Angelo et al., 2009). These studies have significant implications for the functional integrity of the NPC in quiescent satellite cells and post-mitotic multinucleated muscle cells. Further studies are required to determine if Nups in NPCs become damaged in skeletal muscle and whether the nucleocytoplasmic barrier is altered and contributes to muscle dysfunction during disease and aging.

1.2.3 Nuclear Import Pathways

Nuclear transport is a process whereby proteins or other macromolecules traverse the NPC either by directly interacting with peripheral FG-Nups within the NPC channel or by binding to import or export receptors that mediate transport through the NPC (Walde and Kehlenbach, 2010). The majority of nuclear transport receptors are karyopherin family members termed importins, transportins or exportins which mediate transport across the NPC in an energy-dependent manner (Tran and Wente, 2006; Weis, 2003). Transport receptors tend to be divided into importins, which bind a nuclear localization signal (NLS) within a cargo protein to target it into the nucleus and exportins, which bind a nuclear export sequence (NES) within a cargo protein to target it for export to the cytoplasm (Cook and Conti, 2007; Kalderon et al., 1984a; Kalderon et al., 1984b; Kutay and Guttinger, 2005; Lange et al., 2007). Karyopherin import receptors

fall within two families, the karyopherin alpha family and the karyopherin beta family (Wagstaff and Jans, 2009). Increasing evidence suggests that transport receptors have critical roles in controlling the nuclear import and export of key proteins involved in genetic reprogramming and cell adaptation in a large number of cell types and tissues (Kohler et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007). Below we describe the mechanisms of karyopherin-dependent nuclear transport and detail what is currently known about these transport receptors and pathways in skeletal muscle.

A. Classical Nuclear Import: Karyopherin Alpha Family

Classical nuclear import, which is the best characterized of the nuclear transport pathways, is an active process that depends on karyopherin alpha and beta family members as well as a classical nuclear localization signal sequence (cNLS) defined by a string of basic residues contained within a protein (Hodel et al., 2001; Kalderon et al., 1984b; Robbins et al., 1991). In *Mus musculus*, 35-55% of nuclear proteins may depend on classical nuclear import for nuclear targeting as determined using a bioinformatics approach (Marfori et al., 2010). Karyopherin alphas recognize two types of cNLS, a monopartite signal comprised of a single string of basic amino acid residues or a bipartite signal containing two strings of basic variable residues flanking a 10-12 amino acid linker region (Kalderon et al., 1984b; Robbins et al., 1991). The prototypical sequence for the monopartite is the SV40 large-T antigen sequence, PKKKRKV, and for the bipartite, the nucleoplasmin sequence KRPAATKKAGQAKKKK (Hodel et al., 2001; Kalderon et al., 1984b; Robbins et al., 1991). Classical nuclear protein import is

mediated by a heterotrimeric complex of karyopherin alpha (KPNA), which recognizes and binds the cNLS signal within a cargo protein, and karyopherin beta1 (KPNB1), which binds KPNA and interacts with FG-Nups within the NPC to mediate nuclear import (Figure 1.7.4) (Matsuura et al., 2003; Matsuura and Stewart, 2005). Once in the nucleus, a small GTPase, Ran-GTP, binds KPNB1, triggering disassembly of the trimeric complex and subsequent cargo release (Cook et al., 2007). Disassembly of the import complex is also facilitated by CAS, the export receptor for KPNA and another member of the karyopherin family (Kutay et al., 1997), and the nucleoporin, NPAP60L (Ogawa et al., 2010). Upon cargo release, KPNA is recycled back to the cytoplasm by CAS, while KPNB1 is returned to the cytoplasm in complex with Ran-GTP (Hood and Silver, 1998; Kutay et al., 1997). Thus, classical nuclear import cycles and directionality depend upon the GTPase, Ran, which facilitates assembly and disassembly of transport complexes (Cook and Conti, 2007). Ran-GTP, but not Ran-GDP, triggers cargo release in the nucleus upon binding to KPNB1, therefore, the directionality of import is driven by the presence of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm (Lonhienne et al., 2009). Ran-GTP levels are maintained in the nucleus through the nuclear import of Ran-GDP (Ribbeck et al., 1998) and conversion to Ran-GTP by the nuclear localized Ran guanine nucleotide exchange factor (RanGEF) (Cook et al., 2007; Smith et al., 1998). Meanwhile, in the cytoplasm Ran-GTP is hydrolyzed to Ran-GDP by the Ran GTPase-activating protein (RanGAP).

KPNB1 is the sole member of the karyopherin beta family to participate in classical nuclear import with KPNA (Liu and Liu, 2007). KPNB1 and other karyopherin beta family members participate in non-classical nuclear import which involves either

direct NLS binding or the use of non-karyopherin alpha adaptor proteins. Modeling studies for cNLS import reveal that the addition of the KPNA adaptor to the transport cycle, as opposed to direct protein import by KPNB1 alone, reduces import efficiency (Riddick and Macara, 2007); however the loss of import efficiency is offset by the addition of multiple KPNA adaptor paralogs that allows for additional points of control over the nuclear localization of cNLS proteins.

Saccharomyces cerevisiae contains a single, essential karyopherin alpha import receptor. Six KPNA paralogs are found in mouse: KPNA1, KPNA2, KPNA3, KPNA4, KPNA6 and KPNA7 (Hu et al., 2010; Tsuji et al., 1997). Seven KPNA paralogs exist in human with which the mouse homologues share 90% amino acid identity (Kelley et al., 2010; Kohler et al., 1997; Kohler et al., 1999; Tsuji et al., 1997). Confusion regarding karyopherin alpha protein nomenclature between species exists in the literature. While karyopherin alpha gene names (*KPNA* and *Kpna*) between human and mouse are consistent, protein nomenclature using the terms importin alpha or importins does not match between these two species (Figure 1.7.5). In this dissertation we refer to KPNA paralogs using the KPNA/kpna nomenclature, instead of importin alpha, to minimize confusion when discussing KPNA paralog function. KPNA paralogs in mouse and human are categorized into three subtypes based on percentage of amino acid identity. Mouse subtypes are Subtype S: KPNA1 and KPNA6; Subtype P: KPNA2; and Subtype Q: KPNA3 and KPNA4. Subtype members share 80% to 90% amino acid identity, whereas different subtypes share 40% to 50% amino acid identity (Tsuji et al., 1997). All KPNA paralogs function as nuclear import receptors, but paralogs may differ in their cNLS binding affinities and/or specificities for cNLS proteins (Hodel et al., 2001; Kohler

et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Timney et al., 2006; Yasuhara et al., 2007). For example, human regulator of chromosome condensation 1, RCC1, which is the RanGEF, depends solely on KPNA4 to access to the nucleus, while other cNLS proteins, such as RNA Helicase A, may utilize multiple KPNA paralogs to access the nucleus, but may have preference for one paralog over another (Aratani et al., 2006; Quensel et al., 2004). *In silico* experiments suggest that the rate of nuclear import of a cNLS cargo is limited by the levels of karyopherin alpha and Ran (Riddick and Macara, 2005).

The steady-state levels of different KPNA paralogs can vary both among tissue types and within a single tissue during differentiation suggesting distinct roles for individual KPNA paralogs in importing key factors required for cell function and differentiation (Goldfarb et al., 2004; Mason and Goldfarb, 2009; Okada et al., 2008; Poon and Jans, 2005). For example, during mouse spermatogenesis KPNA paralogs are expressed with unique cellular and temporal expression profiles at discrete stages of development (Hogarth et al., 2006). In contrast, during neural differentiation of mouse embryonic stem cells *in vitro*, the steady-state levels of one KPNA paralog increase, while that of another paralog decrease, thereby allowing for differential nuclear import of transcription factors involved in maintaining either the undifferentiated or differentiated state (Yasuhara et al., 2007). This KPNA paralog switching was proposed by the authors of this study as a general mechanism that enables cells to coordinate differentiation by controlling the subcellular localization of transcription factors. In support of the subtype switching model, KPNA steady-state profiles in multiple differentiating human and mouse cell types are characterized by increases in expression of one KPNA paralog with

concomitant decreases in another paralog (Kamei et al., 1999; Kohler et al., 1997; Kohler et al., 2002; Okada et al., 2008). These studies suggest that the role of KPNAs in cell differentiation may differ between cell types that express different cNLS-containing cargo proteins.

Additional evidence for the non-redundant roles of individual KPNA paralogs in cellular physiology stems from loss of function experiments in model organisms. *Kpna1* null *Drosophila melanogaster* developed normally but displayed defects in gametogenesis resulting in sterility in both males and females (Ratan et al., 2008). Similarly, male and female sterility occurred in *Kpna2* null flies (Mason et al., 2002). The sterility in females could be rescued only by *Kpna2* transgenes, whereas the sterility in males could be rescued by *Kpna1, Kpna2* or *Kpna3* transgenes suggesting distinct requirements for KPNA2 in male and female gametogenesis (Mason et al., 2002). In contrast, *Kpna3* null flies displayed defects throughout development, whereas later stages of development could only be rescued with *Kpna3* but not *Kpna1* or *Kpna2* transgenes (Mason et al., 2003). RNAi experiments in *Caenorhabditis elegans* demonstrated that KPNA3 but not KPNA2 is required for oocyte development (Geles and Adam, 2001). Together, these results in genetic model organisms support the notion that KPNA paralogs have evolved distinct functions in different cell types during development.

Loss of function experiments *in vitro* also provide support for distinct roles of KPNA paralogs in controlling cell proliferation and differentiation. During neural differentiation of mouse embryonic stem cells, depletion of KPNA1 by RNAi-mediated knockdown resulted in accelerated neural differentiation, while loss of KPNA5 delayed differentiation (Yasuhara et al., 2007). Classical nuclear import has been implicated in

transporting cargoes across large distances or from specific sites in the cell to the nucleus. This phenomenon has been most extensively studied in neurons (Lai et al., 2008; Mikenberg et al., 2007; Thompson et al., 2004), but emerging evidence supports the presence of such spatial signaling in skeletal muscle. In rodent hippocampal neurons, classical nuclear import mediates the transport of cNLS cargoes from the synapse to the nucleus upon receptor activation (Thompson et al., 2004). In skeletal muscle cells, KPNA mediates the nuclear import of myopodin, an actin bundling protein (Faul et al., 2007), which has been shown to shuttle between the sarcomeric Z-disc and the nucleus in a differentiation and stress-dependent manner (Weins et al., 2001). However, the role of myopodin nucleocytoplasmic shuttling during cell differentiation and stress is unclear. In contrast, a Z-disc associated protein with known nuclear function is muscle limb protein (MLP), which acts as a mechanosensor in rat cardiomyocytes (Boateng et al., 2009). Loss of cNLS-dependent import of MLP results in disarranged sarcomeres. Another cargo that undergoes similar shuttling is serum response factor (SRF), a transcription factor required for skeletal muscle growth that shuttles between the sarcomere and the nucleus (Li et al., 2005). The nuclear import of SRF occurs via KPNA1/KPNB1 of the classical nuclear import pathway (McConville et al., 2010). These findings suggest that nucleocytoplasmic import has a significant role in transmitting signals from sarcomeres to the nucleus in response to stimuli that induce muscle cell remodeling to adapt to cellular stress. Further studies should shed light on the role of classical nuclear import in overcoming the unique spatial challenges of signaling in a multinucleated muscle cell.

B. Karyopherin Beta Family Members Mediate Import and Export

Karyopherin beta family members comprise the majority of nuclear transport receptors which includes karyopherin beta import receptors (importins or transportins) involved in protein import and exportins involved in protein export (Cook and Conti, 2007; Cook and Conti, 2010). Similar to KPNA-dependent nuclear import, the karyopherin beta nuclear import pathway is dependent upon energy and the Ran gradient for directionality (Lonhienne et al., 2009). Karyopherin beta import receptors bind directly to non-classical nuclear localization signals (NLSs) in cargo proteins to mediate import (Fig. 2) or may use a non-karyopherin alpha adaptor protein for NLS cargo recognition (Cook et al., 2007; Kutay and Guttinger, 2005; Lange et al., 2007). Currently, only karyopherin beta2-dependent cargoes have defined NLSs termed PY-NLS, while cargoes depending on other karyopherin beta family members do not have recognizable amino acid sequences that comprise NLS motifs (Marfori et al., 2010).

In total, 14 karyopherin beta import receptors exist in *S. cerevisiae*, while humans have over 19 karyopherin betas (Chook and Suel, 2010). Karyopherin beta family members display protein homology ranging from 15-20% and function in the import of distinct sets of proteins, RNAs and Nups (Chook and Suel, 2010; Marfori et al., 2010). Karyopherin beta1, the best characterized member of the karyopherin family, is one of four essential karyopherin beta family members in *S. cerevisiae* (Chook and Suel, 2010). In contrast to classical nuclear import, karyopherin beta1-dependent nuclear import does not occur through a conserved receptor-cargo binding conformation which provides karyopherin beta1 with the flexibility to bind a wide variety of cargoes containing unique classes of NLS signals (Fiserova et al., 2009; Marfori et al., 2010).

Several studies provide evidence that karyopherin beta family members have critical roles in regulating the cellular localization of different NLS proteins involved in myogenesis and neuromuscular junction physiology (Giagtzoglou et al., 2009; Higashi-Kovtun et al., 2010; Mosca and Schwarz, 2010; van der Giessen and Gallouzi, 2007). In C2C12 myoblasts, the nuclear import of the RNA-binding protein HuR depends upon the karyopherin beta family member transportin-2 (van der Giessen and Gallouzi, 2007). HuR is a RNA-binding protein involved in regulating the stability of mRNA transcripts encoding MyoD and Myogenin which are myogenic transcription factors required for differentiation (Figueroa et al., 2003; van der Giessen and Gallouzi, 2007). During differentiation, cleavage of HuR prevents its nuclear import by transportin-2 which results in the stabilization of MyoD and Myogenin mRNAs and enhancement of myogenesis (Mazroui et al., 2008). Several studies using a Drosophila model system have uncovered roles for karyopherin beta family members in regulating the import of proteins involved in postsynaptic membrane development and neurotransmitter release at the neuromuscular junction (Giagtzoglou et al., 2009; Higashi-Kovtun et al., 2010; Mosca and Schwarz, 2010). Wingless signaling at the neuromuscular junction causes cleavage and release of the C terminus of Frizzled2 (Fz2-C), which is then imported into the nucleus by KPNA2/KPNB1, or by the karyopherin beta family member karyopherin-beta11 (Mosca and Schwarz, 2010). In Drosophila mutants lacking either KPNA2 or karyopherin-beta11, a reduction in the nuclear import of Fz2-C and defects in the post-synaptic membrane were observed suggesting that multiple transport pathways are required for membrane development at the neuromuscular junction. Another karyopherin beta involved in neuromuscular junction physiology is Drosophila karyopherin beta13

which controls neurotransmitter release and intracellular $Ca2^{+}$ levels at the neuromuscular junction (Giagtzoglou et al., 2009). These data suggest that karyopherin beta family members regulate myogenesis, neuromuscular development and neurotransmitter release by importing a variety of proteins, including RNA-binding proteins and cell surface receptor components.

Karyopherin beta family members also play roles in facilitated nuclear export of proteins to the cytoplasm (Cook and Conti, 2010; Wente and Rout, 2010). The best understood export pathway is the recognition of a classical nuclear export signal (NES) by the karyopherin beta, CRM1 (Figure 1.7.4). The classical NES signal consists of a short string of hydrophobic leucine-rich residues, which are difficult to identify because they share sequence similarity with the hydrophobic cores of most proteins (Cook and Conti, 2010). Prototypical sequences for an NES are the cyclin D NESs, RFLSLEPL and TPTDVRDVDI as well as the mitogen-activated protein kinase kinase (MAPKK) NES, LQKKLEELEL (Kutay and Guttinger, 2005; Poon and Jans, 2005). Export receptors or exportins recognize and bind export cargoes while bound to the GTPase, Ran-GTP, in an obligate trimeric complex (Cook and Conti, 2007; Kutay and Guttinger, 2005). As with facilitated nuclear import, directionality of export is driven by the compartmentalization of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm.

At least six exportin genes are found in mouse and human (Okada et al., 2008). These exportins facilitate the nuclear export of a variety of cargoes with some exportins displaying different specificity for individual cargoes. Similar to NLS-mediated import, NES-containing proteins may be exported by a single exportin or may utilize multiple exportins (Okada et al., 2008). For example, the essential yeast exportin, CSE1, or CAS

in vertebrates, has one export cargo, KPNA of the classical nuclear import pathway (Cook and Conti, 2007), while the best characterized exportin, CRM1 (XPO1 or exportin-1), mediates export of a multitude of different classical NES-containing proteins (Cook and Conti, 2007; Shen et al., 2010; Wada et al., 1998). Exportin family members also facilitate the export of tRNAs and pre-miRNAs (Cook and Conti, 2007; Lund et al., 2004). Together, these studies suggest exportins may have differing roles in the export of a wide variety of cargo proteins and RNAs required for proper cell function.

Nucleocytoplasmic shuttling via CRM1-dependent export and KPNA-dependent import coordinate the nuclear steady-state levels of proteins critical to muscle cell biology. For example, the nuclear localization of the transcription factor NF-κB is controlled by the subunit p65 which mediates both import and export of the complex through binding to KPNA/KPNB1 or CRM1, respectively (Micheli et al., 2010; Zerfaoui et al., 2010). The nuclear accumulation of p65 NF-κB suppresses MyoD transcription, therefore modulation of nuclear import or export of p65 could control p65 NF-κB activity, MyoD expression and ultimately myogenesis (Guttridge et al., 2000). The nuclear import and export of the forkhead box transcription factor FOXO3a is critical for skeletal muscle atrophy (Sandri et al., 2004). In C2C12 cells, the nuclear import of Foxo3a was observed upon inhibition of the phosphatidylinositol 3-kinase, PI3K/Akt pathway, while nuclear export of FOXO3a was observed upon activation of the stress-activated protein kinase (SAPK) pathway (Clavel et al., 2010). Control over the cellular localization of FOXO3a by two different signaling pathways may provide global control over atrophy by regulating the transcription of genes, such as Atrogin-1, that are involved in skeletal muscle atrophy (Clavel et al., 2010). Control over the nucleocytoplasmic

shuttling of cargo proteins by different transport pathways may provide global control over gene expression during myogenesis.

1.3 Identifying Classical Nuclear Import-Dependent Cargoes

Identifying the specific cargo proteins that are transported via various nucleocytoplasmic transport pathways is key for understanding the regulatory networks that govern cell function. Here we focus on how cNLS-dependent cargoes are identified since this pathway is the best characterized nuclear transport pathway; however, many of the challenges in cargo identification presented here apply also to other receptor-mediated transport pathways. In *Mus musculus*, 30-55% of nuclear proteins are predicted to depend on classical nuclear import (Marfori et al., 2010). However, only a few proteins with key functional roles in muscle are known to contain a functional cNLS, such as Notch (Huenniger et al., 2010) and NFATc2 (Okamura et al., 2000). While bioinformatics approaches exist to identify classical nuclear import signal sequences within cargoes, these putative cargoes still require functional testing to ensure that such signals actually mediate transport via this pathway.

Putative cNLS motif sequences within proteins can be identified with prediction software (Cokol et al., 2000; Horton et al., 2007; Kosugi et al., 2009; Nguyen Ba et al., 2009). The consensus sequence for the monopartite cNLS has been characterized in both structural and thermodynamic studies where the first residue is a lysine followed by a second and fourth basic residue as follows: K(K/R)X(K/R) (Conti and Kuriyan, 2000; Fontes et al., 2000; Hodel et al., 2001). The consensus sequence for the bipartite cNLS has also been characterized as KRX10-12KRRK (Fontes et al., 2003). Small deviations

from the consensus sequence may increase or decrease KPNA-cargo binding affinity, while large deviations likely result in failed import because KPNA-cargo binding is either too weak or too strong for efficient cargo import and release (Lange et al., 2007). A drawback to cNLS prediction algorithms is that linear sequence is analyzed and these algorithms do not identify non-linear synthetic cNLS signals created through intra or inter-protein interactions. For example, signal transducer and activator of transcription (STAT1) forms a homodimeric complex in which each dimer contributes basic resides to form a functional synthetic cNLS that is not detected by current cNLS algorithms (Fagerlund et al., 2002). While cNLS prediction models identify consensus sequences, functional studies meeting several criteria must be performed before a cNLS is deemed functional (Lange et al., 2007). A cNLS is functional if it is both necessary and sufficient for import of the cargo protein and import of the cargo depends upon the classical nuclear import machinery (Lange et al., 2007).

An alternative approach to identifying cNLS-dependent cargoes is a candidate-based approach which involves transport receptor loss of function experiments, whereby a phenotype observed upon receptor depletion may offer hints to potential cargo. A candidate-based approach may prove difficult since the phenotypes observed during depletion of transport receptors are likely combinatorial due to the altered nuclear transport of many cargo proteins that are involved in regulating a large number of genes. Overall, identifying cargoes dependent upon classical nuclear import receptors will be critical to understanding the role of nucleocytoplasmic transport in regulating cell function and fate in skeletal muscle.

1.4 Remodeling of the Nuclear Transport Machinery

Alterations in global nucleocytoplasmic transport provide another layer of control over gene expression. Global changes in the efficiency or rate of nuclear transport can occur through alterations or remodeling of key components of the nuclear transport machinery. For example, altering the expression or localization of karyopherin transport receptors, Ran and/or Ran-associated proteins, or Nups results in changes in transport efficiency (Hodel et al., 2006; Hodel et al., 2001; Riddick and Macara, 2005; Timney et al., 2006; Wagstaff and Jans, 2009). A muscle cell could alter nucleocytoplasmic transport to adjust for changes in demand for nuclear transport over a wide range of cellular conditions such as cell quiescence, proliferation, differentiation, stress, aging and disease.

Experimental evidence indicates that steady-state levels for different components of the nuclear transport machinery can vary during myogenesis. Microarray analyses suggest that satellite cell entry into the cell cycle is marked by global remodeling of the nuclear transport machinery (Fukada et al., 2007; Pallafacchina et al., 2010). A wide variety of mRNAs that encode components of the nuclear transport machinery, such as nucleoporins and various karyopherin transport receptors, were increased in proliferating satellite cells *in vivo* compared to quiescent satellite cells. This widespread upregulation of the nuclear transport machinery may be functionally required to allow for rapid changes in gene expression associated with the myogenic lineage progression of satellite cells. Nuclear pore composition can influence stem cell differentiation as evidenced from studies of NUP-133-deficient epiblast and embryonic stem cells in mice which differentiated inefficiently along the neural lineage (Lupu et al., 2008).

Nuclear transport machinery remodeling has also been observed during muscle differentiation, however the extent and type of remodeling differs among muscle cell types. The differentiation of mouse embryonic stem cells into a cardiac lineage resulted in the downregulation of karyopherins, exportins, Nups and Ran-related proteins, while an increase in NPC density was observed along with the expansion of individual NPC diameter suggesting an increase in demand for nuclear transport during cardiac differentiation (Perez-Terzic et al., 2007). In contrast, during the differentiation of C2C12s, the steady-state levels of Nup proteins and overall density of NPCs remained constant (D'Angelo et al., 2009). Differences in nuclear transport machinery remodeling appear to be cell type-dependent and suggest that remodeling of the nuclear transport machinery is a key process that controls global nucleocytoplasmic transport during cellular differentiation. Characterizing the functional role of members of the nuclear transport machinery during skeletal muscle proliferation and differentiation will be essential to understanding the role of nucleocytoplasmic transport as a driver of muscle cell differentiation and function.

Remodeling of the nuclear transport machinery has also been observed during cellular response to chemical or mechanical stress in multiple cell types. Remodeling of the nuclear transport machinery to reduce or block transport during cellular stress may allow a cell to globally “pause” gene signaling pathways in order to redirect gene expression to respond to a particular cellular stress. Cellular stress, such as oxidative stress, can inhibit both import and export transport receptors and reduce the levels, localization and post-translation modifications of several Nups involved in nuclear export (Crampton et al., 2009; Kodiha et al., 2008; Miyamoto et al., 2004). In vascular smooth

muscle cells (VSMCs), remodeling of the nuclear import machinery was observed during exposure to ceramide, an antiproliferative sphingolipid implicated in the final stage of atherosclerotic plaque formation. Ceramide treatment of cultured VSMCs resulted in reduced cell proliferation and inhibition of classical nuclear import due to mislocalization of KPNA and CAS to the cytoplasm (Faustino et al., 2008). Nuclear transport machinery remodeling during mechanical stress was also observed during stretching of VSMCs which results in smooth muscle cell hyperplasia and hypertrophy (Richard et al., 2007). Mechanical stretching of VSMCs *in vitro* resulted in an increase in nuclear import and the steady-state protein levels of Nups, along with alterations in MAPK signaling. Further studies are required to determine whether remodeling of the nuclear transport machinery also occurs with oxidative or mechanical stress in skeletal muscle cells and what role it may play in controlling the nuclear localization of critical proteins necessary for cellular responses to these physiologic perturbations.

Aging and disease are also associated with changes in the nuclear transport machinery. In myocardial microvascular endothelial cells and human fibroblasts, a reduction in expression of KPNA and therefore, classical nuclear import occurred with cellular aging (Ahluwalia et al., 2010; Pujol et al., 2002). Another study using Hela cells expressing a mutant form of Lamin A responsible for the premature aging disease, Hutchinson Gilford progeria syndrome, revealed a reduction in nuclear import efficiency along with alterations in the localization of NUP62, NUP153 and the exportin CRM1 (Busch et al., 2009), suggesting that remodeling of the nuclear import machinery may have a role in disease pathology. In contrast, human cardiomyocytes from patients with heart failure, displayed an increase in karyopherin import receptors, exportins, Ran

regulators, and Nups as well as differences in NPC configuration and morphology as compared to healthy cardiomyocytes (Cortes et al., 2010) suggesting that remodeling may occur as a disease response. These findings suggest that changes in the nuclear transport machinery may occur either as a result of aging and disease or may occur in response to disease to restore tissue function. Given the extensive loss of skeletal muscle mass that can occur with aging or disease, further studies are warranted to determine if remodeling of the nuclear transport machinery also occurs with age or disease in skeletal muscle and whether nucleocytoplasmic communication is impaired as a consequence.

1.5 Challenges in Studying Nucleocytoplasmic Transport in Multinucleated Cells

The basic mechanics for nucleocytoplasmic transport of proteins and RNA identified to date and described here have almost exclusively been examined in cells with a single nucleus. Skeletal muscle is the only permanent multinucleated cell type in the body and constitutes ~50% of body mass yet how these cells spatially and temporally regulate and coordinate nucleocytoplasmic transport among hundreds of nuclei is unknown.

Spatial and temporal regulation of nucleocytoplasmic transport between nuclei likely occurs within a single myofiber since transcriptional activity of specific gene loci can differ among nuclei within the same myofiber. Such differences in transcription could arise from specific regional requirements for cell function. Myonuclei at the neuromuscular junction express transcripts for subunits of the acetylcholine receptor at much higher levels than non-synaptic nuclei leading to the accumulation of the acetylcholine receptor protein at the neuromuscular junction (Burden, 1993; Fontaine and

Changeux, 1989; Sanes et al., 1991; Simon et al., 1992) and thereby facilitating coordinated neuronal activation of muscle contraction. In addition, the myonuclei located at the myotendinous junction in stretched myofibers express the transcript for sarcomeric myosin heavy chain at higher levels than other myonuclei (Dix and Eisenberg, 1990), thereby enhancing sarcomere addition and cell growth at the ends of myofibers in response to muscle stretching. Less clear are the reasons why myonuclei distributed along the length of a myofiber and at times even right next to each other exhibit differences in the transcription of both endogenous genes and transgenes (Newlands et al., 1998). Transcriptional differences among nuclei also occur in myotubes *in vitro* (Berman et al., 1990; Su et al., 1995). The molecular mechanisms responsible for such nuclear diversity in transcriptional activity in skeletal muscle are unknown. Differences in transcriptional activity among nuclei in a common cytoplasm also occur in other cell types and organisms. For example, only a subset of nuclei in multinucleated mouse osteoclasts or human placental syncytiotrophoblasts is transcriptionally active (Ellery et al., 2009; Youn et al., 2010). In the binucleate *Tetrahymena thermophila*, the macronucleus is transcriptionally active, whereas the micronucleus is transcriptionally inert (Karrer, 2000). Furthermore, nuclei in the syncytial blastoderm of Drosophila and the syncytial germ line of *C. elegans* are also transcriptionally distinct (Burden, 1993). The molecular mechanisms responsible for the transcriptional differences among nuclei in these other cell types and organisms are not fully elucidated.

Nuclear proteins are localized to some nuclei and not others within a myofiber which further suggests differential nuclear targeting occurs in skeletal muscle. One such

protein is endonuclease G, which is a mitochondrial protein that can translocate to nuclei and induce DNA fragmentation and apoptosis independent of caspase. At the initiation of disuse muscle atrophy, endonuclease G translocates to a subset of myofiber nuclei (Dupont-Versteegden et al., 2006) and may serve as a means to control the loss of myonuclei commonly observed in disuse atrophy without cell death. Other examples of such proteins are specific nuclear envelope proteins which are more highly concentrated in synaptic myonuclei compared to non-synaptic nuclei. These include Syne-1, Syne-2 and nesprin-1α which appear to participate in nuclear localization and/or anchoring (Apel et al., 2000; Grady et al., 2005; Puckelwartz et al., 2010; Zhang et al., 2007). Unequal nuclear localization of proteins is also observed in cultured myotubes. These include transcription factors with important roles in muscle differentiation and growth such as NFAT5 (O'Connor et al., 2007), NFATc1 (Abbott et al., 1998) and MyoD (Ferri et al., 2009), the growth inhibitory protein, myostatin (Artaza et al., 2002) as well as MYO18B (Salamon et al., 2003), an unconventional myosin heavy chain with an unidentified role in muscle physiology. The mechanisms that govern differential protein targeting among neighboring myonuclei *in vivo* and *in vitro* are unknown but may be related to nucleus-specific transport mechanisms and merit further study.

Studies in the binucleate *Tetrahymena thermophila* provide potential clues for how nucleus-specific transport mechanisms may be regulated in multinucleated myofibers. Certain nuclear proteins in *T. thermophila* are selectively accumulated in either macro- or micronuclei (White et al., 1989). Analyses of GFP-labeled karyopherin alpha proteins revealed that nine of the thirteen karyopherin alpha proteins present in this organism localized exclusively to the micronucleus suggesting that nucleus-specific

transport systems must exist (Malone et al., 2008). Further studies demonstrated that the NPCs of macronuclei and micronuclei contain unique subsets of FG-containing nucleoporins which are responsible for this nuclear-selectivity (Iwamoto et al., 2009; Malone et al., 2008). Interestingly, homologs of NUP98 contributed to nuclear-selectivity: two NUP98 homologs localized exclusively to macronuclei, whereas the other two exclusively localized to macronuclei. Specific structural components of the NUP98 homologs were functionally required for the nuclear-selectivity as shown by chimeric protein experiments (Iwamoto et al., 2009). The NUP98 homologs that localized to the macronucleus contained amino acid repeats of GLFG, whereas homologs that localized to the micronucleus lacked GLFG and instead contained novel NIFN repeats. These results suggest that structural alterations of the NPC can contribute to nucleus-specific protein transport in a multinucleated cell. Such structural alterations may modulate the interaction of karyopherin transport receptors with specific components of the NPC and consequently alter nuclear accumulation of proteins. Whether the steady-state levels of KPNAs differ among myonuclei sharing a common cytoplasm is unknown, but differential localization among nuclei would support the hypothesis that specific karyopherin transport receptors may also undergo selective nuclear targeting in skeletal muscle as in the binucleate *Tetrahymena thermophila*. Further studies are required to define the contribution of NPC composition and karyopherin transport receptors to nuclear differences in transcription and protein content in skeletal muscle.

1.6 Summary

Skeletal muscle is a very plastic tissue that undergoes changes in structure, morphology and function during growth and regeneration. Muscle growth and regeneration is dependent on satellite cells that have the ability to proliferate, differentiate and fuse into multinucleated myofibers. Much of our understanding of satellite cell function and muscle regeneration has occurred through the use of rodent models. These models continue to be valuable in understanding how proper gene regulation is achieved during the different stages of muscle growth and regeneration. While the complexities of gene regulation during myogenesis are becoming clearer, the role of nucleocytoplasmic transport in satellite cells and during muscle regeneration is just now emerging. Multinucleated myofibers are faced with unique challenges compared to most other mammalian cell types in controlling the function of hundreds of nuclei in a common cytoplasm. Although a fair bit is known about nuclear envelope proteins in skeletal muscle because of their association with several muscular dystrophies, very little is known about the NPC or karyopherin transport receptors. Further knowledge of the role of the nucleus and the nuclear transport machinery is needed to enhance our understanding of how gene expression is controlled in normal, aged and diseased muscle as well as to provide insight into both satellite cell and myofiber biology.

1.7 Figures

Figure 1.7.1: Skeletal muscle structure

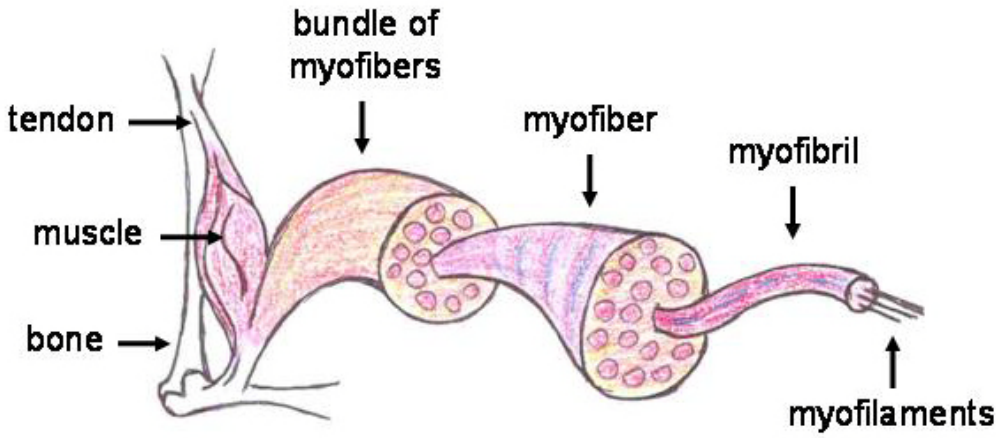
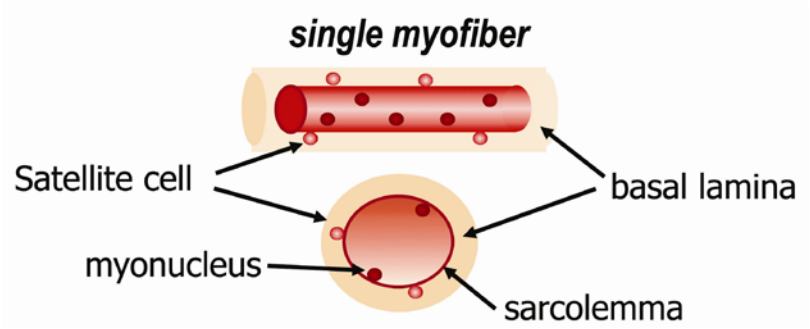


Figure 1.7.1: Skeletal muscle structure

Individual muscle is attached to bone through tendons. Muscle is made up of many bundles of myofibers. Each myofiber is comprised of many myofibrils that contain interlocking filaments which are the parts that contract when a fiber is stimulated.

Figure 1.7.2: Myofiber structure and stages of myogenesis

A.



B.

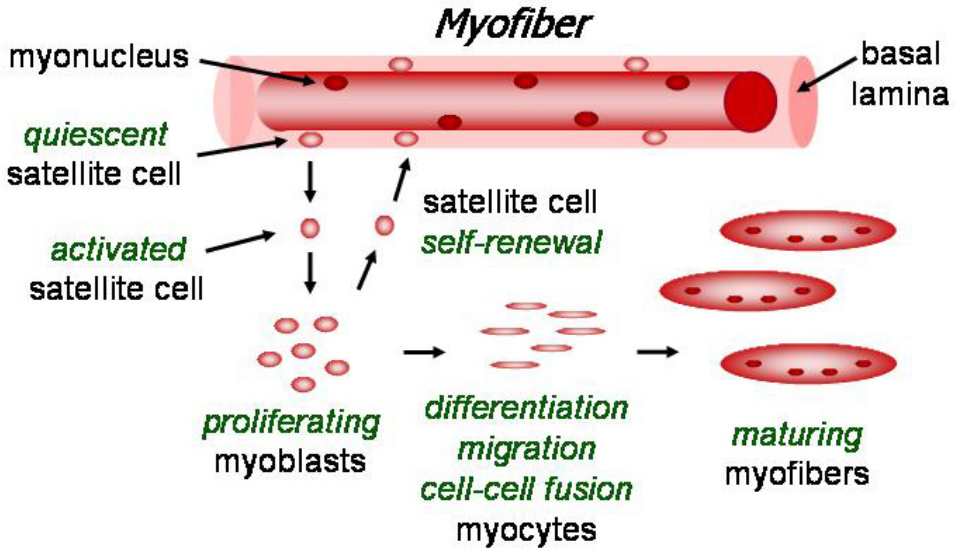


Figure 1.7.2: Myofiber structure and stages of myogenesis

A) Single myofibers contain many myonuclei that line the cytoplasmic side of the sarcolemma or cell membrane. A single myofiber is surrounded by a basement membrane called the basal lamina. Satellite cells are located between the basal lamina and the myofiber sarcolemma. **B)** Quiescent satellite cells activate, proliferate or undergo self-renewal. Myoblasts, the progeny of satellite cells, are capable of either proliferating or differentiating into myocytes that migrate and fuse with each other or existing myofibers to generate mature myofibers.

Figure 1.7.3: Schematic illustrating the relative location of various Nups within the Nuclear Pore Complex

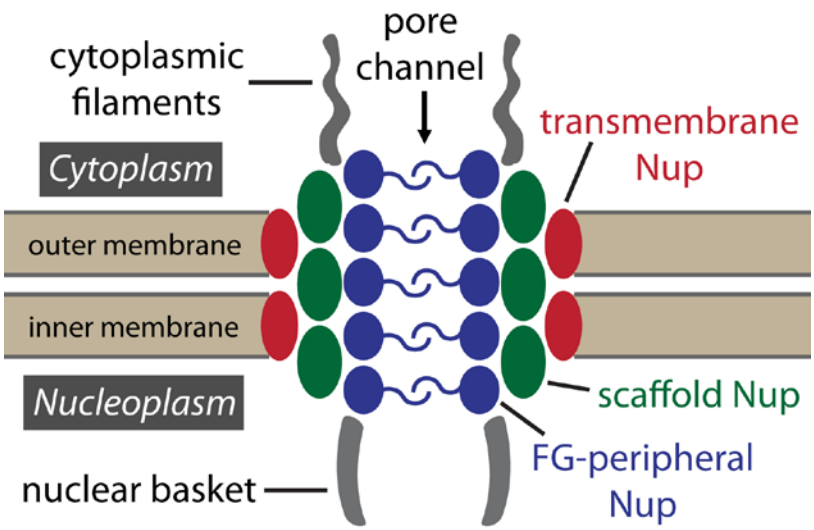


Figure 1.7.3: Schematic illustrating the relative location of various Nups within the Nuclear Pore Complex

The NPC resides within the nuclear envelope, a bilipid membrane comprised of an inner and outer nuclear membrane. The NPC has cytoplasmic filaments that extend into the cytoplasm and a nuclear basket that extends into the nucleoplasm. Peripheral Nups containing phenylalanine-glycine (FG) repeats line the pore channel to function in NPC permeability and the facilitated transport of macromolecules. Transmembrane Nups localize to the nuclear envelope-NPC interface, while scaffold Nups reside between transmembrane and peripheral Nups.

Figure 1.7.4: Basic model of karyopherin-mediated nuclear import and export pathways

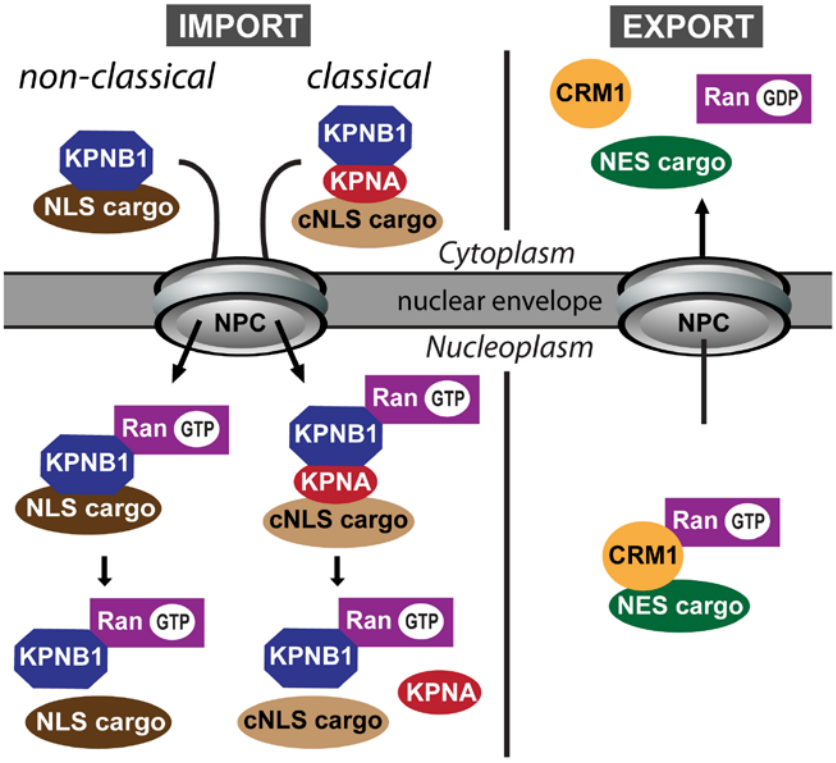


Figure 1.7.4: Basic model of karyopherin-mediated nuclear import and export pathways

The non-classical nuclear import pathway involves nuclear import receptor karyopherin beta1 (KPNB1) which recognizes proteins containing a non-classical nuclear localization signal (NLS). The classical nuclear import pathway is defined by the involvement of karyopherin alpha (KPNA), in complex with karyopherin beta1 (KPNB1), where KPNA recognizes proteins containing a classical nuclear localization signal (cNLS). The nuclear import of a cNLS-containing cargo involves both KPNA and KPNB1 import receptors, since KPNA recognizes the cNLS motif in the cargo protein and then KPNB1 mediates translocation of the import complex through the NPC. Once in the nucleus, Ran-GTP binding to KPNB1 results in the dissociation of the import complex and cargo release into the nucleus. The nuclear export pathway consists of an obligate trimeric complex consisting of the exportin (CRM1 for classical NES-containing cargo), export cargo, and Ran-GTP. Translocation of the complex through the NPC is mediated by interaction between the exportin and FG Nups. Once in the cytoplasm, the hydrolysis of Ran-GTP to Ran-GDP results in the dissociation of the export complex and subsequent cargo release.

Figure 1.7.5: Karyopherin alpha paralogs in different organisms

Subtype	*Homo sapiens*		*Mus musculus*		*Drosophila melanogaster*		*Saccharomyces cerevisiae*	
	Gene name	Importin designation	Gene name	Importin designation	Gene name	Importin designation	Gene name	Importin designation
S	*KPNA1*	alpha5	*Kpna1*	alpha1	*Kap-alpha1*	alpha1	*Srp1*	Kap60
	KPNA5	alpha6	-	-	-	-	-	-
	KPNA6	alpha7	*Kpna6*	alpha6	-	-	-	-
P	*KPNA2*	alpha1	*Kpna2*	alpha2	*Pen*	alpha2	-	-
	KPNA7	alpha7	*Kpna7**	alpha7*	-	-	-	-
Q	*KPNA3*	alpha4	*Kpna3*	alpha3	-	-	-	-
	KPNA4	alpha3	*Kpna4*	alpha4	*Kap-alpha3*	alpha3	-	-

Figure 1.7.5: Karyopherin alpha paralogs in different organisms

KPNA family members are categorized into three subtypes, S, P and Q based on amino acid sequence homology. *Saccharomyces cerevisiae* has a single karyopherin (SRP1), while *Homo sapiens*, *Mus musculus*, and *Drosophila melanogaster* each contain multiple karyopherin paralogs. The gene name and importin alpha designation are given for each species to clarify confusion regarding karyopherin/importin designations between species. A dash (-) indicates the absence of a karyopherin homologue in that species. * Placement of recently discovered murine KPNA7 into subtype P is tentative.

Chapter 2: Introduction

Chapter 2: Introduction

Myogenesis is dependent on satellite cells that have the ability to proliferate, differentiate and fuse into multinucleated myofibers. Changes in gene expression during different stages of myogenesis are due to nuclear proteins entering the nucleus and regulating gene expression. However, the role of nucleocytoplasmic import in regulating gene expression in satellite cells or myofibers, which contain many hundreds of nuclei, is unknown. The subcellular localization of nuclear proteins both in a mono and multinucleated muscle cells must be tightly controlled because defective nuclear import could result in aberrant myogenesis and improper tissue function.

Chapter 4 describes the characterization and functional role of KPNA paralogs during *in vitro* myogenesis. The work in this chapter involved characterizing KPNA paralog expression during myogenesis and investigating whether KPNA paralogs have non-redundant roles during myogenesis. In addition, this work has laid the foundation for understanding how multinucleated cells regulate the differential nuclear import of proteins among myonuclei sharing a common cytoplasm.

To determine whether KPNA paralogs have non-redundant roles during myogenesis, we utilized an established *in vitro* model of myogenesis using primary mouse muscle cells (Rando and Blau, 1994). In this model, precursor mononucleated myoblasts proliferate in high serum-containing media but upon switching to a low mitogen media the cells exit the cell cycle, differentiate into myocytes that migrate and adhere to other myocytes and undergo membrane fusion to form multinucleated nascent myotubes. Further rounds of myocyte fusion with nascent myotubes yield large mature myotubes with many myonuclei. We used this model to analyze classical nuclear import

in muscle cells, specifically the role of different KPNA subtypes represented by KPNA1, KPNA2 and KPNA4. This model offers the advantage that the role of KPNA-mediated nuclear import can be studied both in the context of mono and multinucleated muscle cells. Since our first goal was to test the hypothesis that KPNA paralogs have non-redundant roles during skeletal muscle myogenesis, we examined the expression of individual KPNA paralogs during myogenesis to determine if individual KPNA paralogs are expressed and or if they are expressed at a particular stage of myogenesis, since this might offer a clue to their role in myogenesis. We determined that five mouse karyopherin alpha paralogs are expressed in primary myoblasts *in vitro* and their steady-state levels increase as myoblasts progress through myogenesis to form multinucleated myotubes. In addition, the nuclear localization of KPNA2, but not KPNA1, appeared to differ among nuclei sharing a common cytoplasm, suggesting that KPNA2 may import nuclear proteins differentially among nuclei. To investigate the functional roles of KPNA paralogs, we utilized RNAi to demonstrate that KPNA1 and KPNA2 have differential roles in regulating myoblast proliferation as well as myotube size. In contrast to KPNA1 and KPNA2, knockdown of KPNA4 had no effect on myogenesis. Furthermore, following knockdown of different KPNA paralogs, we detected changes in the steady-state localization of a key cNLS-dependent cargo required for growth of myotubes, Nuclear Factor of Activated T cells, cytoplasmic 2 (NFATc2).

The work presented in Chapter 5, involved characterizing the expression and role of karyopherin alpha1 import receptor, KPNA1, in regulating satellite cell proliferation and satellite cell pool size in skeletal muscle during the basal state (maintenance) and during regeneration (injury). We determined that newly isolated satellite cells express

four karyopherin alpha paralogs, *Kpna2*, *Kpna3*, *Kpna4* and *Kpna6*, but not *Kpna1*. Meanwhile, *Kpna1* is expressed in proliferating satellite cells. Our previous data implicated KPNA1 in negatively regulating myoblast proliferation, therefore, we investigated the role of KPNA1 in satellite cell proliferation during muscle maintenance and regeneration. Analysis of *Kpna1*$^{+/-}$ mice revealed an increase in satellite cell proliferation during muscle maintenance and regeneration. We hypothesize that KPNA1 may function in satellite cells to import nuclear proteins required for the negative regulation of proliferation to prevent over-proliferation and potential exhaustion of the satellite cell pool during muscle regeneration.

The research presented in this dissertation provides evidence for distinct classical nuclear import pathways in skeletal muscle that rely on specific KPNA import receptors. In addition, our research has laid the foundation for understanding how multinucleated cells regulate the differential nuclear import of proteins among myonuclei. We suggest that classical nuclear import may provide a novel regulatory mechanism for both mono and multinucleated muscle cells during the maintenance, formation and growth of multinucleated myofibers. Lastly, our work in skeletal muscle may have implications for the role of classical nuclear import in modulating gene expression and cell fate in other stem cell populations.

Chapter 3: Materials and Methods

Chapter 3: Materials and Methods

Primary muscle cell culture

Primary myoblasts were isolated from the hind limb muscles of adult Balb/c or C57 B/6 mice between 8-12 wk of age. Muscles were excised, separated from excess fat and connective tissue, minced into a coarse slurry, and then digested for 1 hour with 0.1% pronase (Calbiochem) in DMEM at 37°C with mild agitation. After digestion, cultures were mechanically dissociated by repeated trituration followed by filtration through a 100 μm sterile vacuum filter (Millipore). After isolation cells were cultured in growth medium (GM: Ham's F-10, 20% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, 100 U/ml penicillin and 100 μg/ml streptomycin) on collagen coated plates in a humidified 5% CO2 incubator at 37ºC. Primary cultures were enriched for myogenic cells by using the preplating technique and determined to be 97% pure by MyoD immunostaining.

Animal muscle injuries

The *Kpna1* (-/-) knockout mouse line (C57BL/6) has been previously described (Shmidt et al., 2007) and was kindly provided by Dr. Michael Bader (Max-Delbruck-Center for Molecular Medicine, Germany). To analyze muscle growth during regeneration, injury was induced in the TA (tibialis anterior) or the gastrocnemius muscles of wild-type and KPNA1 +/- mice (n=5-6) by injection of 40μL of 1.2% $BaCl_2$ diluted in PBS with a 27g needle along the length of the muscle. The TA was collected at five days post injury, sectioned and stained with hematoxylin and eosin. Histological analyses were performed on sections collected from similar regions of each TA muscle. Images were captured

from each section using Scion Image software (NIH, version 1.63) in order to determine the cross-sectional area (XSA) of 50-100 myofibers per field. All photography was performed on an Axioplan microscope with a 0.3 NA 10X ZEISS Plan-Neofluar objective equipped with a CCD camera (ZEISS). All analysis were performed blinded. Mice were cared for and handled in accordance with the institutional guidelines of Emory University.

Single myofiber isolation

Single myofibers were isolated from gastrocnemius muscles. The gastrocnemius was dissected and digested in DMEM containing 25 mM HEPES and 0.1% collagenase (type I, Worthington) for 90 min with gentle agitation. Single myofibers were extracted individually into 24-well plates pre-coated with 10% growth factor reduced Matrigel (BD Biosciences). Following plating, myofibers were centrifuged at 1100 ×g to facilitate adhesion to the Matrigel and fixed immediately upon plating with 3.75% formaldehyde, and DAPI-stained.

Cell proliferation, differentiation and fusion assays

To analyze cell proliferation *in vitro*, transfected cells were seeded onto 6-well plates (2x105 cells/well), grown for 23 hours and then labeled with 10 μM bromodeoxyuridine (BrdU) (Sigma) for 1 hour. Subsequently, cultures were immunostained for BrdU, as described below, and the percentage of $BrdU^+$ cells was determined using fluorescence microscopy with >700 nuclei analyzed for each condition in three independent experiments.

To analyze differentiation and fusion, transfected cells were seeded on entactin-collagen-laminin (ECL, MilliPore) coated 6-well dishes at a density of 2 x 10^5 cells/well in GM. Cells were allowed to adhere to the dish for approximately 1.5 hr before switching to DM (DM, DMEM, 1% Insulin-Transferring-Selenium-A supplement (Gibco), 100 U/ml penicillin G, 100 µg/ml streptomycin). Upon differentiation for 0, 24 and 48 hours, cells were fixed in 3.7% formaldehyde for 10 min and subsequently immunostained for embryonic myosin heavy chain (eMyHC, F1.652, neat hybridoma supernatant, Developmental Studies Hybridoma Bank) as described below. The differentiation index was determined by counting the number of nuclei in $eMyHC^+$ cells as a percentage of the total nuclei analyzed. The fusion index was calculated by dividing the total number of nuclei in myotubes by the total number of nuclei analyzed. Over >700 nuclei were analyzed for each condition in three independent experiments.

RT-PCR analyses

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. The reverse transcriptase reaction was performed using 2 µg of total RNA/sample using random hexamers and M-MLV reverse transcriptase (Invitrogen). cDNA was amplified using 2.5 µM of each primer and the Expand High Fidelity PCR system (Roche). All KPNA primers spanned intron-exon boundaries to control for genomic contamination and were specific to individual KPNA paralogs as determined by Blast search. In addition, RNA was tested by PCR for DNA contamination. Primer sequences were: KPNA1 (F: 5'-TCCTGCTTTGCGGGCTGTGG-3' and R: 5'-GGGGTGCGATGCTGCTGTCC-3');

KPNA2 (F: 5'-CTGCTGGGCCATTTCCTACCTGA-3' and R: 5'-ACGCGGCCTCCTTCTGTGCTT-3'); KPNA3 (F: 5'-CAAGGGCCGCGATGTGGAGA-3' and R: 5'-CTGATGTGGGGAATGGAGGAGTCG-3'); KPNA4 (F: 5'-GGGCGGTGGGGAGAGTC-3' and R: 5'-TGAGTAGCGGAACCAAGTGAGGAA-3'); KPNA6 (F: 5'-GAGAACATTCTTCGGCTTGG -3' and R: 5'-CCGGAGGCAGACATTATAGC-3'); KPNA7 (F: 5'- TCCAGCTACTTCACTGACCC-3' and R: 5'-GGTACTTATAGTTCTTCAGCCTCT-3'). Amplification cycles for KPNA1 and KPNA2 consisted of 94ºC for 5 minutes, followed by 25 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 30 seconds and a final termination step at 72º C for 5 minutes. Amplification cycles for KPNA3 and KPNA6 consisted of 94ºC for 5 minutes, 26 cycles of 94ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 30 seconds and a final termination step at 72ºC for 5 minutes. Amplification cycles for KPNA4 consisted of 94ºC for 5 minutes, 25 cycles of 94ºC for 30 seconds, 56ºC for 60 seconds, 72ºC for 30 seconds and a final termination step at 72ºC for 5 minutes. Amplification cycles for KPNA7 consisted of 95ºC for 5 minutes, 35 cycles of 94ºC for 30 seconds, 54ºC for 30 seconds, 72ºC for 30 seconds and a final termination step at 72ºC for 4 minutes. As an internal control, 18S cDNA was amplified with QuantumRNA 18S rRNA primers (Ambion). Additional primers used for controls were: Pax7 (F: 5'-CACCCCGGGGACAGAGGAAGAT-3' and R: 5'-GAGAGGGCGGGGTACAAGGAAGAC-3'); CD31 (F: 5'-AGGAGTCAGAACCCATCAGG-3' and R: 5'-GCTACTGGCTTTGGAGATACG-3'). Amplification cycles for Pax7 consisted of 94ºC for 5 minutes, followed by 30 cycles of

94ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 30 seconds and a final termination step at 72º C for 5 minutes. Amplification cycles for CD31 consisted of 94ºC for 5 minutes, followed by 25 cycles of 94ºC for 30 s seconds, 55ºC for 30 seconds, 72ºC for 30 seconds and a final termination step at 72º C for 5 minutes. PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. Two independent experiments were analyzed each in duplicate.

Immunoblotting

To isolate cytosolic and nuclear extracts, myoblasts were trypsinized and centrifuged at 500 x g at 4 ºC for 5 minutes. Cell pellets were washed twice with PBS, resuspended in Nonidet NP-40 lysis buffer (10 mM TrisCl, pH 7.4, 10 mM NaCl2, 3 mM MgCl2, 0.5% (v/v) NP-40), lightly vortexed and incubated on ice for 5 minutes. Nuclei were pelleted by centrifugation at 500 x g at 4ºC for 5 minutes, followed by removal of the supernatant containing the cytoplasmic fraction. Nuclei were resuspended in Nonidet NP-40 lysis buffer by light vortexing, centrifuged at 500 x g at 4ºC for 5 minutes and washed with PBS before being resuspended in RIPA-2 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl2, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) containing protease inhibitors (Mini complete; Roche). Protein concentration was determined using the Bradford assay (Bio-Rad) and 25-60 µg of total protein was separated by SDS-PAGE. Following transfer to a PVDF membrane (Millipore), non-specific binding was blocked with blocking buffer [5% non-fat dry milk in Tris-buffered saline (TBS)] overnight at 4ºC, membranes were incubated in blocking buffer containing anti-KPNA1 (Novus Biologicals), anti-KPNA2 (MBL) or NF-κB p65 subunit [a kind gift from Dr. Denis

Guttridge (Wang et al., 2007)] at 1:10,000 dilution or anti-KPNA4 (MBL) at 1:1,000 dilution, for 1 hour. Blots were washed in TBS containing 0.1% Tween 20 (TBS-T) and then incubated in either HRP-conjugated goat anti-mouse IgG (KPNA1) , HRP-conjugated goat anti-rat IgG (KPNA2 and 4) or HRP-conjugated donkey anti-rabbit IgG (NF-κB p65) (Jackson ImmunoResearch) for 1 hour. After washing with TBS-T, antibody binding was detected using enhanced chemiluminescence. To demonstrate equal protein loading and/or efficient separation of nuclear-cytoplasmic fractions, membranes were reprobed with antibodies against alpha-tubulin (Sigma), EF1-alpha (Upstate), GAPDH (Santa Cruz), or ZC3H14 (Leung et al., 2009). Three independent experiments were performed.

Immunocytochemistry

Immunostaining for KPNA1, KPNA2, and NFATc2 was performed using TSA (Tyramide Signal Amplification) Red or Green (PerkinElmer). Myogenin immunostaining was performed using Vectastain ABC kit (Vector Laboratories Inc.). Cells were fixed with either 3.7% formaldehyde for KPNA immunostaining or methanol for NFATc2 immunostaining for 10 minutes at room temperature and following successive washes with PBS, were incubated in blocking buffer (0.5% BSA, 0.1% Triton X-100 in PBS) containing either 5% donkey serum for KPNAs, 5% goat serum for NFATc2, or TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% blocking reagent) for myogenin for 1 hour. The cells were incubated overnight at 4ºC in 10 µg/ml anti-KPNA1 (Novus), 1µg/ml anti-NFATc2 (Thermo Scientific), 5 µg/ml anti-KPNA2 (MBL), 1:10 dilution anti-myogenin (F5D, Developmental Studies Hybridoma Bank) or appropriate

isotype controls. Cells were washed with PBS containing 0.2% Tween 20 (PBS-T) and incubated with appropriate secondary antibodies (Jackson ImmunoResearch) diluted 1/250 in PBS-T: KPNA1 (biotin-conjugated donkey anti-mouse IgG F(ab')2 fragments), KPNA2 (biotin-conjugated anti-rat IgG F(ab')2 fragments), myogenin (biotin-conjugated anti-mouse IgG F(ab')2 fragments), NFATc2 (biotin-conjugated goat anti-mouse IgG F(ab')2 fragments). After washes with PBS-T, cells immunostained for KPNAs and NFATc2 were incubated in TNB for 1 hour followed by incubation with HRP-conjugated streptavidin diluted 1:200 in TNB for 30 minutes followed by TSA Red or Green diluted 1:200 for 5 minutes. Cells immunostained for myogenin were incubated with Vectastain ABC solution (Vector Laboratories Inc.) after several PBS-T washes, followed by incubation with DAB Fast 3, 3'-Diaminobenzidine (Sigma). Cells immunostained for KPNAs and NFATc2 were counterstained with 25 μM 4'6-diamidino-2-phenylindole (DAPI) in PBS-T to identify nuclei.

Transfection with siRNAs

Primary myoblasts were seeded onto 6-well plates (1x105 cells/well) and transfected with 80 nM control scrambled siRNA or one of two siRNAs targeting the KPNA of interest (Stealth siRNA; Invitrogen) using Lipofectamine 2000 (Invitrogen), with up to three serial transfections performed 24-48 hours apart. Myoblasts were cultured for 24-48 hours after the last transfection to ensure optimal protein knockdown before performing experiments. To assess knockdown, RT-PCR and immunoblotting was performed using RNA isolated after 24-48 hrs in DM. siRNAs used are as follows:

KPNA1-1, (5'-3') GCCUGUUGGACAAUCUCUAAUAUUA

KPNA1-2, (5’-3’) ACAAAUCUCUGAAUCCUGAUGAGAU

KPNA2-1, (5’-3’) AACGCGGCGUCACCGGGAAAUUUAA

KPNA2-2, (5’-3’) UCCUAGAAGCUUCACAAGUUGGGGA

KPNA4-1, (5’-3’) GACUCCGAUAUAGAUGGUGAUUAUA

KPNA4-2, (5’-3’) GCAGUUCAAGCUGCUAGGAAGCUUU.

Control scrambled siRNA was obtained from Invitrogen (Stealth RNAi Medium GC negative control).

Cell migration assays

Migration of myocytes was quantified using time-lapse microscopy. Primary myoblasts transfected with either control scrambled siRNA or KPNA2 siRNA were seeded onto 100 mm plates at low density and switched to DM for 24 hours to generate myocytes in the absence of myotube formation. Images were acquired using a Axiovert 200M microscope with a 0.30 NA 10X Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and camera (QImaging) with OpenLab 5.50 (Improvision) every five minutes for three hours. Cell velocities were calculated in μm/hr using ImageJ software by tracking the paths of approximately 20 mononucleated cells in each of three independent experiments.

Cell-cell adhesion assays

Cell-cell adhesion was analyzed by incubating myocytes in suspension while gently rocking at 37ºC. Duplicate aliquots of cells were taken at 20 minute intervals for 60 minutes and the numbers of single cells and cells in clusters were determined by phase-contrast microscopy for each condition in three independent experiments. Trypan blue

staining was used at 0 and 60 minutes to determine cell viability which was not significant between control and siRNA conditions. All adhesion assays were performed blinded.

Flow cytometry

The Nestin-GFP transgenic mouse line (C57BL/6), which expresses GFP in quiescent satellite cells, has been previously described (Day et al., 2007; Mignone et al., 2004) and was kindly provided by Dr. Zipora Yablonka-Reuveni (University of Washington). Mononucleated cells were isolated from the hindlimb muscles of five 8-12 week old Nestin-GFP mice with Pronase digestion as described above in the section primary muscle cell culture. Upon isolation, mononucleated cells were immunostained with an APC-conjugated CD31 antibody followed by fluorescence activated cell sorting (FACS) as a control for endothelial expressing cells. Isolation of GFP^+ cells was performed with the assistance of the Emory University Flow Cytometry Core Facility. Total RNA isolated from GFP^+ cells (PicoPure RNA isolation kit, Arcturus) was used for RT-PCR as described above.

To analyze satellite cell proliferation during regeneration, the gastrocnemius muscles of *Kpna1*$^{+/-}$ and *Kpna1*$^{+/+}$ mice were induced to regenerate by $BaCl_2$ injection and given twice daily injections of BrdU (100μg/gm weight) following the day of muscle injury. To ensure BrdU delivery into mice, drinking water containing BrdU (0.8mg/ml) was provided to mice daily. Three days post injury, muscles were harvested and mononucleated cells were isolated as described above with the addition of a Percoll gradient to further purify the collected cell population. Cells were immunostained using

FITC-conjugated α-CD45 (1:100; BD Biosciences), FITC-α-CD31 (1:100; eBiosciences), and PE-conjugated α-alpha7-integrin (1:200; gift of Fabio Rossi, University of British Columbia). Cells were immunostained for BrdU using BrdU Flow kit (BD Biosciences). The percentage of α7-integrin^{+}CD31^{-}CD45^{-} BrdU^{+} cells was determined via FACSCalibur (Becton-Dickinson). The addition of propidium iodide to the collected cells allowed for the detection of dead cells and their removal from flow analysis. Data analysis was performed using FlowJo v. 6.2.1 (TreeStar, Inc.).

Image preparation

Confocal images for KPNA immunocytochemistry were acquired using a Zeiss LSM510 META microscope with a 40X objective with LSM510 software (Carl Zeiss MicroImaging, Inc., Germany). Multi-channel z-stack fluorescent images through DAPI stained nuclei were summed using Image J software version 10.2 and globally processed for size, brightness and contrast using Photoshop 11.0 (Adobe). Other images for KPNA immunocytochemistry were acquired using a Axioplan microscope with a 0.5 NA 20X Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and charge-coupled device camera (Carl Zeiss MicroImaging, Inc.) with Scion Image 1.63 (Scion Corp.). Images taken for proliferation, differentiation and fusion assays were acquired using a Axiovert 200M microscope with a 0.30 NA 10X or 20X Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.) and camera (QImaging) with OpenLab 5.50 (Improvision). All images were globally processed for size, brightness and contrast using Photoshop CS3 (Adobe).

Statistical analyses

To determine significance between two groups, comparisons were made using unpaired Student's unpaired t-tests. Analyses of multiple groups were performed using a one-way ANOVA followed by Dunnett's test for multiple group comparisons using Sigma Stat (version 2.03). For all statistical tests, $p<0.05$ was accepted for significance.

Chapter 4: Distinct Roles for Karyopherin Alpha Import Receptors during Myogenesis

A portion of this chapter is published as:

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Contributions:

Adriana Simionescu (Figure 4.4.9), Christine A. Griffin (4.4.10)

Chapter 4: Distinct Roles for Karyopherin Alpha Import Receptors during Myogenesis

4.1 Introduction

Skeletal muscle function is dependent upon bundles of multinucleated cells called myofibers. These myofibers contain many hundreds of nuclei spread across the length of the cell in a common cytoplasm. Each myonucleus is believed to express protein products for a defined volume of surrounding cytoplasm called the myonuclear domain (Allen et al., 1999). How a myofiber with hundreds of nuclei coordinates and regulates the transport of macromolecules into and out of these nuclei is unknown. Spatial and temporal regulation of nucleocytoplasmic transport into individual nuclei must occur within a single myofiber since transcriptional activity of a specific gene locus can differ among nuclei within the same myofiber (Newlands et al., 1998)

The classical nuclear import pathway is one mechanism for facilitated nuclear protein import in eukaryotic cells and is dependent upon a cNLS within a cargo that is recognized by the cNLS receptor karyopherin alpha (Lange et al., 2007). Proteins that contain a cNLS are imported into the nucleus by a heterodimeric receptor consisting of the classical nuclear import receptor karyopherin alpha (KPNA) and the pore targeting subunit karyopherin beta1 (KPNB1) (Figure 1.2.3) (Lange et al., 2007). Once in the nucleus, the cNLS-containing protein is released and both KPNA and KPNB1 are recycled separately back to the cytoplasm for another round of import (Hood and Silver, 1998; Kutay et al., 1997).

The role of classical nuclear import in regulating nucleocytoplasmic import and therefore gene expression in skeletal muscle is unknown. Six KPNA paralogs exist in

mouse: KPNA1, KPNA2, KPNA3, KPNA4, KPNA6 and KPNA7. These KPNA paralogs differ both in expression in various tissues and in binding affinity for specific cNLS cargoes. In addition, different KPNA paralogs have been implicated in cell fate decisions during development in non-muscle tissues (Geles and Adam, 2001; Kohler et al., 2002; Mason et al., 2002; Mason et al., 2003; Yasuhara et al., 2007). Understanding how nucleocytoplasmic transport is regulated in muscle cells will lead to a greater understanding of how external signals are sensed by muscle cells and translated into changes in gene expression necessary for tissue homeostasis.

Using primary mouse muscle cells, we determined that five *Kpna* paralogs are expressed in myoblasts and their levels increase as cells differentiate. To investigate the functional role of KPNAs during myogenesis, we conducted RNAi-mediated knockdown of KPNA1, KPNA2 or KPNA4. Myoblasts treated with KPNA1 siRNA displayed an increase in proliferation. During differentiation, we detected a decrease in myotube size in cells treated with KPNA2 siRNA. Through the use of RNAi, we demonstrate that KPNA1 and KPNA2 have differential roles in regulating myoblast proliferation as well as myotube size. Furthermore, we detect changes in the steady-state localization of a key cNLS-dependent cargo required for growth of myotubes, Nuclear Factor of Activated T cells, cytoplasmic 2 (NFATc2). In contrast to KPNA1 and KPNA2, knockdown of KPNA4 has no effect on myogenesis. This chapter provides evidence for distinct classical nuclear import pathways in skeletal muscle that rely on specific KPNA import receptors. We suggest that classical nuclear import may provide a novel regulatory mechanism during the formation and growth of multinucleated cells.

4.2 Results

Five karyopherin alpha paralogs and karyopherin beta1 are expressed during myogenesis

To determine whether the key transport receptors required for classical nuclear transport are expressed during myogenesis, we analyzed highly purified primary mouse muscle cells using RT-PCR and immunoblotting. RT-PCR was performed on total RNA isolated at 0, 24 and 48 hours of differentiation using primers specific for each of the six karyopherin alpha paralogs. The 0-hour timepoint represents proliferating myoblasts, the 24-hour timepoint represents a mix of unfused myocytes and small multinucleated nascent myotubes, and the 48-hour timepoint represents mainly large mature myotubes (Figure 4.4.1.A). mRNA for five KPNA paralogs was detected at all timepoints, with increased amounts detected in differentiated cultures (Figure 4.4.1.B), while mRNA for KPNA7, an ovary-specific paralog (Hu et al., 2010), was not detected at any timepoint (Figure 4.4.1.C). To analyze the steady-state levels of KPNA and KPNB1 proteins, immunoblotting was performed to detect KPNB1 and one representative KPNA paralog for each subtype: KPNA1 (Subtype S), KPNA2 (Subtype P) and KPNA4 (Subtype Q). These particular KPNA paralogs were chosen for analysis because these are the only ones for which monoclonal antibodies exist that have been verified to be paralog-specific (Kamikubo et al., 2004; Tachibana et al., 2008). Protein lysates were collected at 0, 24 and 48 hours of differentiation and immunoblotting was performed. KPNA1, KPNA2 and KPNA4 were present at all timepoints with protein levels increasing with differentiation, consistent with the increase in steady-state levels observed for their respective mRNA

transcripts (Figure 4.4.1.B). In contrast, the steady-state levels of KPNB1 protein remained unchanged during myogenesis (Figure 4.4.1.B). Thus, the key transport receptors required for classical nuclear import are expressed throughout myogenesis. The increased steady-state levels of KPNA paralogs during myogenesis suggests a global increase in the demand for nuclear protein import during myogenesis.

To complement immunoblotting analysis, we examined the expression of KPNA1 and KPNA2 at the single cell level using immunocytochemistry. KPNA1 and KPNA2 were detected in all cells at 0, 24 and 48 hours during myogenesis (Figure 4.4.2.A). The fluorescence intensities of KPNA1 and KPNA2 increased during myogenesis consistent with the increases detected in the steady-state levels of protein and mRNA. Neither KPNA1 nor KPNA2 showed gross change in intracellular localization over the course of muscle differentiation (Figure 4.4.2.A). However, KPNA2 appeared to be more highly concentrated in nuclei compared to KPNA1 at all stages examined, suggesting their roles in muscle cells may differ. Interestingly, the fluorescence intensity of KPNA2 differed among individual myonuclei within a myotube, suggesting that KPNA2-dependent import could vary among myonuclei sharing a common cytoplasm (Figure 4.4.2.B, C). In contrast, no significant difference in the fluorescence intensity of KPNA1 was observed among nuclei within a single myotube (Figure 4.4.2.A, B).

KPNA paralogs differentially alter the steady-state localization of NFATc2

To investigate the role of KPNA nuclear import receptors in nucleocytoplasmic import in muscle cells, we analyzed the steady-state localization of several cNLS proteins in myoblasts or myotubes upon depletion of individual KPNA paralogs. We chose

proteins on the basis of two or more of the following criteria: known or predicted cNLS, nuclear localization dependent on classical nuclear import, or known role in myogenesis. The nuclear localization of the following proteins was studied: the muscle-specific transcription factor myogenin (Buckingham et al., 2003), the general transcription factors NF-κB (Bakkar and Guttridge, 2010)and NFATc2 (Rao et al., 1997). We predict that a cNLS protein that requires KPNA paralogs to access the nucleus would have an altered steady-state nuclear localization upon depletion of particular KPNAs. Since a cNLS-containing protein may depend solely on a specific KPNA paralog to access the nucleus or may exploit multiple paralogs with one paralog being preferred (Kohler et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007), we performed RNAi-mediated knockdown of a representative member of each KPNA subtype Myoblasts were transfected with either control siRNA or siRNA targeting the KPNA of interest and significant knockdown of each paralog was obtained (Figure 4.4.3.A). The double band detected for KPNA2 in myoblast lysates at this exposure has been observed in different mouse and human tissues and is likely due to post-translational modification or alternative mRNA splicing (Kamei et al., 1999; Kohler et al., 1997). Off target effects against other KPNA paralogs during RNAi experiments were not detected as neither the steady-state level of protein nor mRNA of non-target KPNAs was altered (Figure 4.4.3.B). Following transfection, cells were either analyzed as myoblasts or induced to differentiate for 24 or 48 hours into myotubes depending on the particular cargo protein under investigation. The steady-state nuclear localization of candidate proteins was analyzed by immunocytochemistry of fixed cells or by immunoblotting of fractionated cells. No significant change in nuclear localization was observed for myogenin (Figure

4.4.4.A) or NF-κB (Figure 4.4.4.B). The lack of change in nuclear localization for these proteins with knockdown of a single KPNA may be due to redundancy among KPNA proteins for these particular cargoes as recently shown for the intracellular domain of Notch for which knockdown of three different KPNAs was required to maximally decrease transcriptional activity (Huenniger et al., 2010). In contrast to our results with myogenin or NF-κB, differences were noted with NFATc2, a protein whose cellular localization and role during myogenesis have been well characterized (Abbott et al., 1998; Horsley et al., 2001) and which is solely dependent on a characterized cNLS for nuclear import (Okamura et al., 2000; Torgerson et al., 1998). Following knockdown of KPNA1, KPNA2 or KPNA4, myotubes were immunostained with an antibody specific to NFATc2 (Tone et al., 2008) and counterstained with DAPI to locate nuclei. The fluorescence intensity of NFATc2 in nuclei was increased in both KPNA2 and KPNA4 siRNA-treated cultures compared to control cultures, with the most significant increase observed in cells depleted of KPNA2 (Figure 4.4.5). In contrast, the steady-state localization of NFATc2 in myotubes depleted of KPNA1 was similar to cells treated with control siRNA. Differences in NFATc2 steady-state nuclear localization in myotubes knocked down for KPNA1, KPNA2 or KPNA4 reveal specific roles for these KPNA paralogs in modulating the intracellular localization of NFATc2.

KPNA1 and KPNA2 regulate myoblast proliferation

The formation of multinucleated myotubes during myogenesis depends on an adequate pool of myoblasts which proliferate, differentiate and undergo cell-cell fusion. To investigate the role of KPNA paralogs in myoblast proliferation, we performed RNAi-

mediated knockdown of KPNA1, KPNA2 or KPNA4. Cultures treated with each siRNA were labeled with bromodeoxyuridine (BrdU), a marker of S phase, and immunostained for BrdU to determine the percentage of cells actively replicating DNA (Figure 4.4.6.A). Knockdown of KPNA1 significantly increased the number of $BrdU^+$ cells by ~25% versus control siRNA, whereas KPNA2 siRNA-treated myoblasts displayed a ~25% decrease in proliferation. In contrast, no significant difference was observed in cells treated with KPNA4 (Figure 4.4.6.B). These data indicate that KPNA paralogs have non-redundant roles in regulating proliferation in primary muscle cells. Furthermore, KPNA1 and KPNA2 may have specific roles in the nucleocytoplasmic import of cNLS proteins required for the negative or positive regulation of proliferation, respectively.

KPNA2 regulates myotube size

To investigate the role of KPNA paralogs during myoblast differentiation and fusion to form multinucleated myotubes, we performed RNAi-mediated knockdown of KPNA1, KPNA2 or KPNA4 in myoblasts and then induced the cells to differentiate for 24 and 48 hours. The 24-hour timepoint, representing a mix of unfused myocytes and small multinucleated nascent myotubes, was used to assess early stages of differentiation and fusion, while the 48-hour timepoint representing mainly large mature myotubes was used to assess completion of myogenic fusion. Cultures treated with KPNA2 siRNA contained thinner myotubes than cultures treated with control siRNA at both 24 and 48 hours (Figure 4.4.7.A). Since thin myotubes can correlate with differences in myonuclear number, cultures were immunostained for embryonic myosin heavy chain (eMyHC), which stains only the cytoplasm thereby facilitating the counting of nuclei to quantify the

fusion index (Horsley et al., 2001). The fusion index indicates the percentage of cells that have fused to form multinucleated myotubes. A significant decrease in fusion was observed in KPNA2 siRNA-treated cultures at both 24 and 48 hours (Figure 4.4.7.B), whereas, no significant difference in fusion was observed between KPNA1 or KPNA4 siRNA-treated cultures and control siRNA-treated cultures at either 24 or 48 hours (Figure 4.4.8.A). Overall, these data indicate that KPNA2 has a distinct role in regulating myotube formation during myogenesis.

Myoblast proliferation and differentiation are upstream of myotube formation during myogenesis and defects in either could result in reduced myotube formation. However, the fusion defect observed for KPNA2 is not due to differences in cell number, since control siRNA and KPNA2 siRNA-treated cultures were plated at the same cell density and immediately induced to differentiate such that proliferation was not a factor. To investigate defects in myoblast differentiation, we determined the differentiation index, which indicates the percentage of cells that have differentiated as determined by immunostaining for the differentiation marker eMyHC (Horsley et al., 2001). We did not observe a defect in differentiation in KPNA2 siRNA-treated cultures at 24 or 48 hours; therefore, the fusion defect observed upon KPNA2 knockdown is not due to a defect in differentiation (Figure 4.4.8.B). We also did not detect differentiation defects in KPNA1 or KPNA4 RNAi-treated cultures (Figure 4.4.8.B). These data suggest these particular KPNA paralogs either do not import cargo proteins required for differentiation or they have redundant roles in this process. However, the fusion defect observed solely in KPNA2 siRNA-treated cultures reveals a specific role for KPNA2 in importing cNLS proteins involved in signaling pathways that regulate myotube formation and growth.

KPNA2 regulates myocyte migration

Migration and cell-cell adhesion are essential to myogenesis, where myoblasts exit the cell cycle, differentiate into myocytes that migrate, adhere to other myocytes and undergo membrane fusion to form multinucleated myotubes (Griffin et al., 2009; Jansen and Pavlath, 2006). Since altered cell adhesion could contribute to the reduction in myotube size upon depletion of KPNA2, we utilized a suspension-based adhesion assay (Griffin et al., 2009), which allows analysis of cell-cell adhesion without the confounding effects of cell migration. Myocytes treated with control siRNA or KPNA2 siRNA were suspended in media and aliquots were removed over a 60 minute timecourse to count the number of adhered or unadhered cells using phase contrast microscopy. No significant differences in adhesion, as measured by the percentage of single cells (Figure 4.4.9.A) or clusters (Figure 4.4.9.B), were noted between control siRNA and KPNA2 siRNA-treated cultures. Thus, KPNA2 does not appear to have a significant role in regulating cell-cell adhesion. Subsequently, we used time-lapse microscopy to examine whether the reduction in myotube size upon depletion of KPNA2 could be due to altered myocyte motility. Myocytes treated with KPNA2 siRNA migrated shorter distances than myocytes treated with control siRNA (Figure 4.4.10.A). In addition, KPNA2 siRNA-treated cultures contained more slowly migrating cells than those treated with control siRNA (Figure 4.4.10.B) with the mean velocity decreased by ~25% compared to control (Figure 4.4.10.C). Our data suggest that defects in myocyte migration may contribute to the fusion defect observed upon KPNA2 knockdown and that KPNA2 may import cNLS-containing proteins that regulate cell migration.

4.3 Discussion

Control of gene expression during myogenesis is dependent upon nuclear proteins that require facilitated nuclear import. We determined that skeletal muscle utilizes the well characterized classical nuclear import pathway which is a prevalent mechanism for facilitated nuclear protein import (Lange et al., 2007; Marfori et al., 2010). Myoblasts and myotubes express the key transport receptors that are required for classical nuclear import, with non-redundant roles for specific KPNAs in regulating distinct import pathways involved in myoblast proliferation and myotube growth.

We show that five karyopherin alpha paralogs are expressed in primary mouse myoblasts and their steady-state levels increase in myotubes, suggesting a global increase in demand for classical nuclear import during myogenesis. These results differ from studies of differentiation in other cell types such as the cell line HL-60, keratinocytes, sperm and embryonic stem cells in which the steady-state levels of some KPNAs increase with differentiation whereas others decrease (Hogarth et al., 2006; Kamei et al., 1999; Kohler et al., 1997; Kohler et al., 2002; Loveland et al., 2005; Okada et al., 2008; Suzuki et al., 2008; Umegaki et al., 2007; Yasuhara et al., 2009; Yasuhara et al., 2007). Such KPNA subtype switching during differentiation has been proposed to allow for differential nuclear import of transcription factors critical in either maintaining the undifferentiated state or inducing the differentiated state (Yasuhara et al., 2007). While no KPNA subtype switching occurs during muscle differentiation *in vitro*, as in monocyte cell differentiation (Nitahara-Kasahara et al., 2007), differential nuclear import of cNLS proteins required for various stages of myogenesis could still occur as individual KPNA paralogs may have different access to or affinities for cNLS proteins. Our results suggest

that KPNA subtype switching is not a universal mechanism for modulating nuclear import of cargo proteins during cellular differentiation.

Three lines of evidence indicate that specific KPNA paralogs have non-redundant roles in muscle cells. First, differences were observed in the nuclear localization of NFATc2 upon knockdown of individual KPNAs, suggesting that KPNAs have distinct roles in regulating the steady-state localization of NFATc2. Altered nuclear localization was observed with knockdown of KPNA2 and KPNA4, but not KPNA1. The increase in NFATc2 nuclear steady-state localization observed upon depletion of KPNA2 or KPNA4 could be indirect, perhaps due to changes in the import of other cNLS proteins that have a role in regulating the nuclear import or export of NFATc2. Consistent with our results showing increased nuclear localization of NFATc2 upon knockdown of single KPNAs, Notch transcriptional activity was increased with knockdown of a single KPNA in muscle cells, but not with triple KPNA knockdown (Huenniger et al., 2010). Together these results illustrate the complexities in how nucleocytoplasmic transport of individual proteins is regulated.

A second line of evidence for non-redundant roles of KPNA paralogs during myogenesis is highlighted by the fact that knockdown of KPNA1 led to enhanced proliferation of myoblasts, whereas KPNA2 knockdown decreased myoblast proliferation and no change was observed with KPNA4 knockdown. These data suggest specific roles for KPNA1 and KPNA2 in importing cNLS-containing proteins involved in distinct signaling pathways that regulate myoblast proliferation. In contrast to our results, a study in Hela cells revealed that five of the seven human KPNA paralogs are required for cell proliferation, with a 25-80% decrease noted in cell number depending on the specific

KPNA paralog analyzed (Quensel et al., 2004) However, our data provide the first evidence that a KPNA paralog, specifically KPNA1, functions in a signaling pathway that negatively regulates proliferation. Overall, our results using muscle cells reveal that individual KPNAs participate in distinct import pathways that positively or negatively regulate myoblast proliferation, a crucial step for generating adequate cell numbers for proper formation and growth of multinucleated myotubes.

An alternate explanation for the phenotypes we observe during knockdown of KPNA1 or KPNA2 is that depletion of specific paralogs simply decreases the total pool of cellular KPNA. While we can not formally rule out this possibility, our finding that depletion of KPNA1 increases cell proliferation whereas the depletion of KPNA2 decreases cell proliferation argues against a general effect on the overall pool of cellular KPNA. If the effects observed were simply due to a decrease in the overall pool of functional KPNA, one would expect that depletion of any KPNA paralog would alter cell function in the same manner, but potentially to different extents. Our finding that depletion of either KPNA1 or KPNA2 results in opposing proliferation phenotypes is counter to effects being due to general loss of KPNA and is more consistent with at least some paralog-specific function in classical nuclear protein import.

Finally, further non-redundancy among KPNA1, KPNA2 and KPNA4 was also noted as KPNA2 displayed a distinct role in regulating myotube size, since small, thin myotubes with reduced nuclear number were observed only upon depletion of KPNA2. The small myotubes observed with reduced KPNA2 are somewhat similar to the small myotube phenotype observed upon loss of NFATc2 (Horsley et al., 2001). However, upon KPNA2 knockdown, we observed an increase in the steady-state nuclear

localization of NFATc2, suggesting that the phenotype we observed upon KPNA2 depletion is not due to reduced nuclear NFATc2, but possibly to altered nuclear import of multiple cNLS proteins involved in regulating both myotube formation and growth. While our results indicate that a KPNA2-dependent pathway does not play a significant role in cell-cell adhesion, KPNA2 may import cNLS cargo proteins that regulate expression of structural or signaling proteins with a role in myocyte migration, or possibly cell-cell fusion, during myogenesis. Indeed, the reduction in cell velocity observed upon KPNA2 knockdown could contribute to the small myotube phenotype since decreased cell velocity would hinder myotube formation (Bae et al., 2008; Jansen and Pavlath, 2006). Similar to our results, decreased migration was observed upon depletion of KPNA2 in a lung cancer cell line (Wang et al., 2010). Whether similar KPNA2-dependent signaling pathways function in other cell types that migrate or undergo cell-cell fusion will require further study.

In summary, the key transport receptors KPNA and KPNB1 that are required for classical nuclear import are expressed during *in vitro* myogenesis. KPNA paralogs display non-redundant roles in regulating distinct pathways involved in myoblast proliferation and myotube formation. These studies suggest that KPNA1 and KPNA2 transport critical cargo proteins required for different stages of myogenesis and that classical nuclear import likely has a key role in controlling gene expression in skeletal muscle.

4.4 Figures

Figure 4.4.1: Components of the classical nuclear import system are expressed during myogenesis

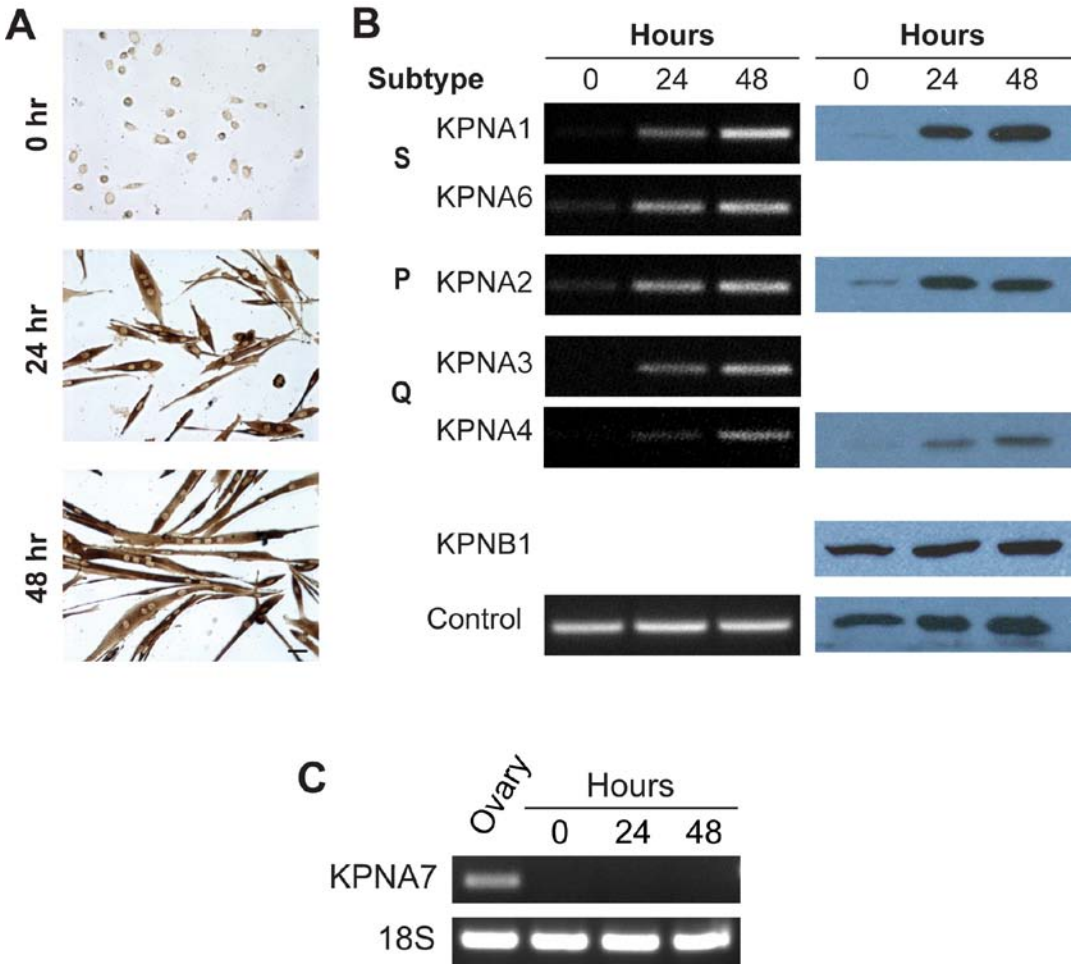

Figure 4.4.1: Components of the classical nuclear import system are expressed during myogenesis

(A) Primary mouse myoblasts were differentiated for 0, 24 and 48 hours and immunostained for embryonic myosin heavy chain, eMyHC, a marker of differentiation (bar, 50μm). **(B)** Total RNA or protein was isolated from primary myoblasts at 0, 24 and 48 hours of differentiation or RNA isolated from mouse ovary **(C)**. RT-PCR was performed using primers specific for each karyopherin alpha paralog and 18S rRNA as an internal cDNA control **(B, C)**. Immunoblotting was performed with antibodies specific for each KPNA paralog or KPNB1 with GAPDH antibody as a loading control. The steady-state levels of karyopherin alpha paralogs increased during myogenesis, whereas mRNA for KPNA7, an ovary-specific paralog, was not detected at any timepoint but was detected in mouse ovary. KPNB1 levels did not change during differentiation.

Figure 4.4.2: KPNA1 and KPNA2 nuclear steady-state levels differ during myogenesis

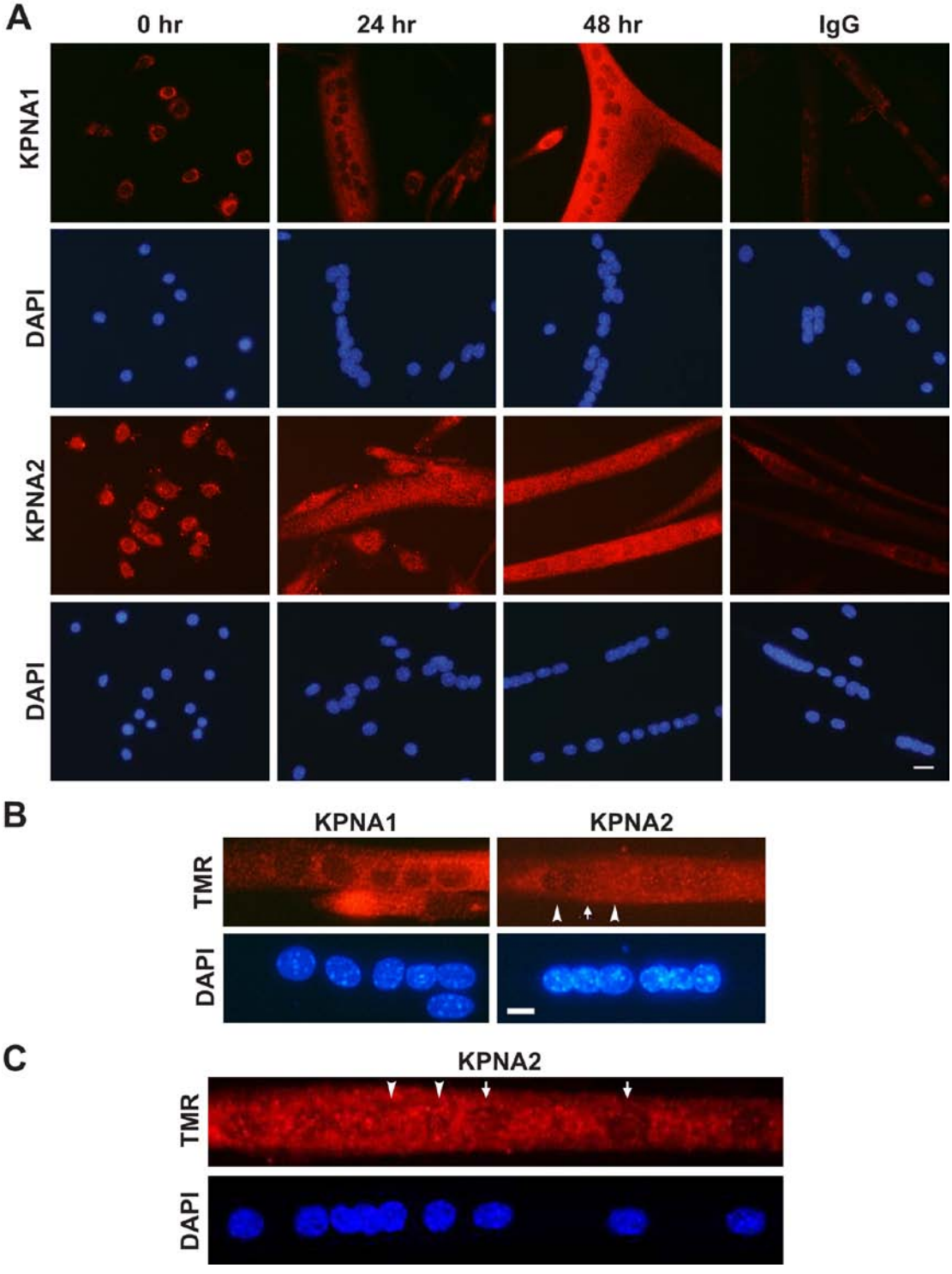

Figure 4.4.2: KPNA1 and KPNA2 nuclear steady-state levels differ during myogenesis

KPNA1 and KPNA2 nuclear steady-state levels differ during myogenesis. **(A)** Representative fluorescent images of primary myoblasts differentiated for 0, 24 and 48 hours and immunostained for either KPNA1 or KPNA2 and counterstained with DAPI (bar, 50μm). KPNA1 and KPNA2 were detected in all cells with greater nuclear fluorescence observed for KPNA2 at all stages of myogenesis. **(B)** Representative fluorescent images of myotubes at 48 hours immunostained for either KPNA1 or KPNA2 (TMR, tetramethylrhodamine) and counterstained with DAPI (bar, 10μm). KPNA2 appeared to be more highly concentrated in some myonuclei (arrowhead) compared to other myonuclei (arrow), whereas no significant difference in the fluorescence intensity of KPNA1 was observed among nuclei. **(C)** Confocal z-stacked images of myotubes at 48 hours immunostained for KPNA2 (TMR, tetramethylrhodamine) and counterstained with DAPI (bar, 10μm). Fluorescence was captured in successive confocal slices through DAPI stained nuclei to compare KPNA2 immunostaining among nuclei. Confocal microscopy confirmed that KPNA2 was increased in some myonuclei (arrowhead) compared to other myonuclei (arrow).

Figure 4.4.3: siRNA mediated knockdown efficiency of KPNA1, KPNA2, KPNA4

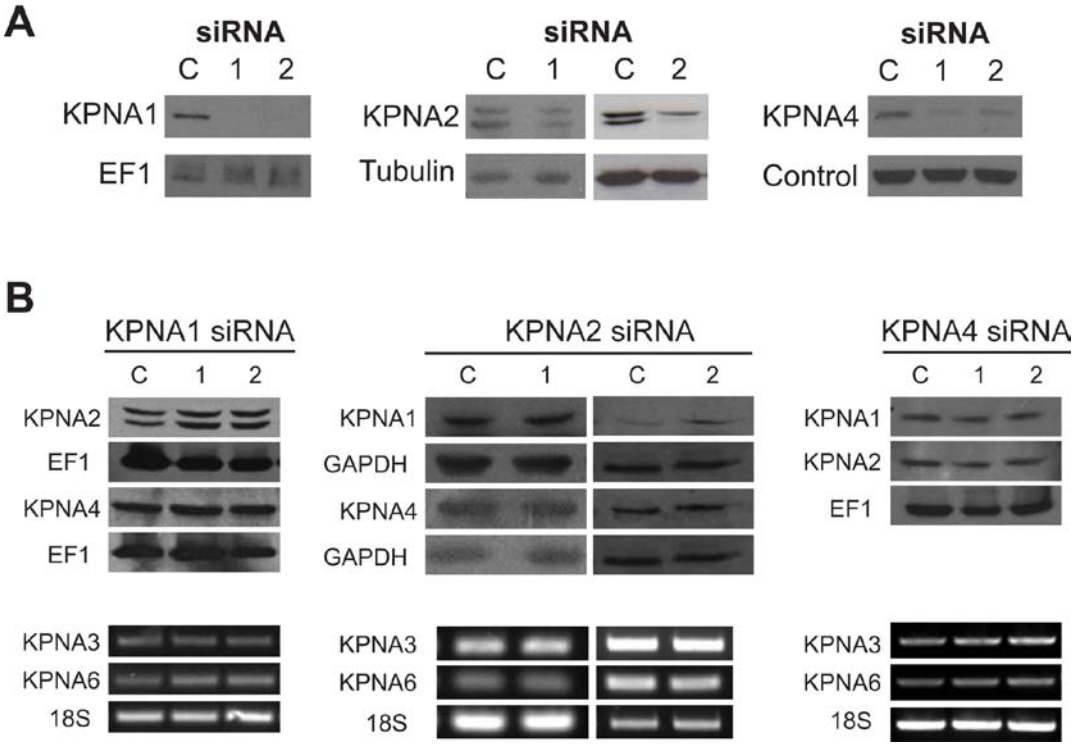

Figure 4.4.3: siRNA mediated knockdown efficiency of KPNA1, KPNA2, KPNA4

(A) Primary myoblasts were transfected with either control scrambled siRNA or siRNAs directed against KPNA1, KPNA2 or KPNA4. Immunoblotting was performed with antibodies specific for each KPNA to determine the efficiency of knockdown. Antibodies targeting EF1-alpha, alpha-tubulin or a non-specific band (control) obtained during KPNA4 immunoblotting were used as a loading control. **(B)** siRNAs against KPNA1, KPNA2 and KPNA4 do not alter protein or mRNA levels of non-targeted paralogs. Primary myoblasts were transfected with either control scrambled siRNA or one of two siRNAs (1 or 2) against KPNA1, KPNA2 or KPNA4. Protein expression was determined via immunoblotting with antibodies specific for KPNA1, KPNA2 or KPNA4 using EF1-alpha, alpha-tubulin or GAPDH antibody as a loading control. Total RNA was isolated and RT-PCR was performed using primers specific for KPNA3 and KPNA6 with 18S rRNA as an internal cDNA control. Neither the protein nor mRNA levels of non-targeted KPNA paralogs was altered following KPNA1, KPNA2 or KPNA4 knockdown.

Figure 4.4.4: The steady-state localization of myogenin or NF-κB p65 is not significantly altered after knockdown of KPNA1, KPNA2 or KPNA4

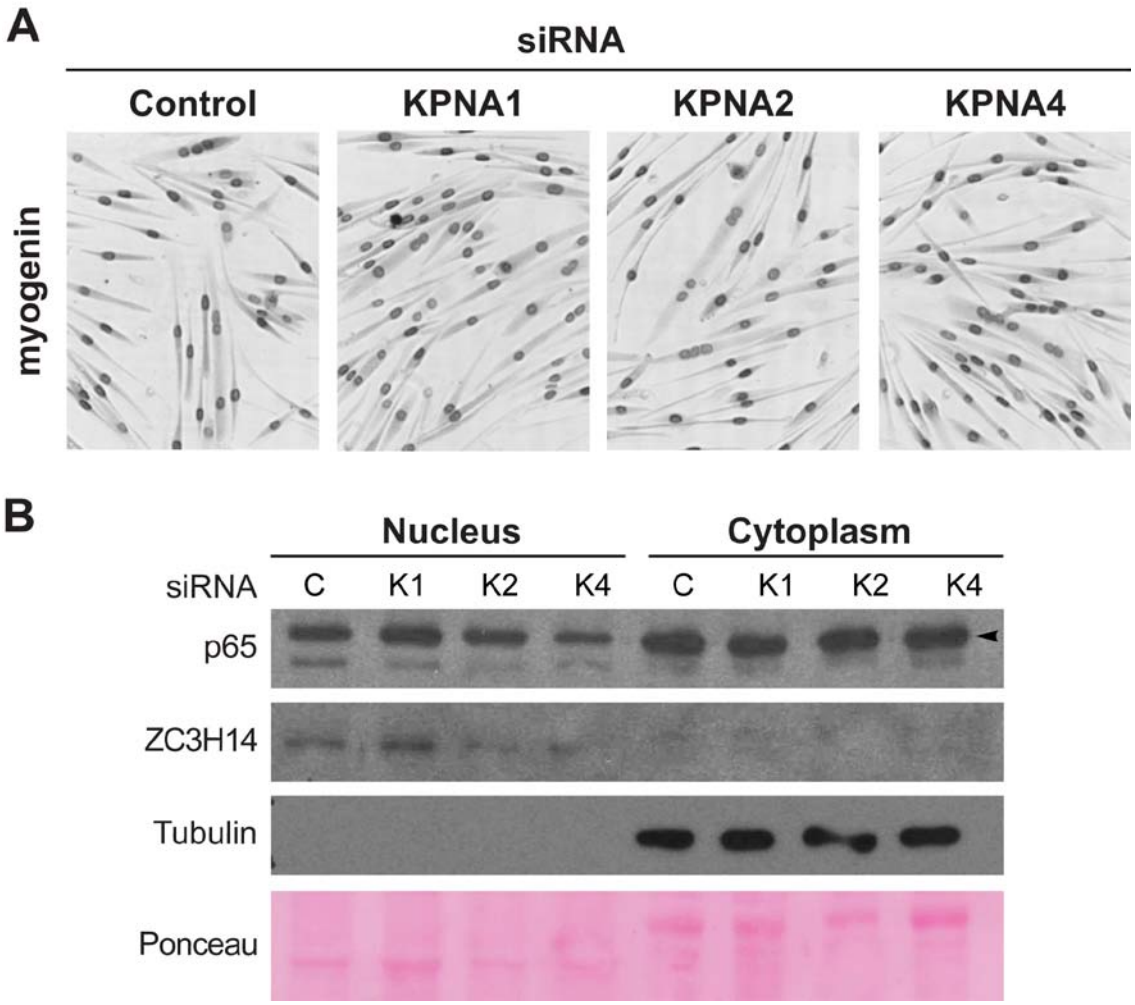

Figure 4.4.4: The steady-state localization of myogenin or NF-κB p65 is not significantly altered after knockdown of KPNA1, KPNA2 or KPNA4

Primary myoblasts were transfected with either control scrambled siRNA or siRNAs against KPNA1, KPNA2 or KPNA4. **(A)** Cells were differentiated for 24 hours and immunostained for myogenin. The nuclear localization of myogenin was not altered following knockdown of individual KPNA paralogs. **(B)** Nuclear and cytoplasmic extracts were isolated from myoblasts following a 1-hour treatment with 20 ng/mL of TNF-alpha to induce NF-κB p65 nuclear translocation. Immunoblotting was performed with antibodies against NF-κB p65 (arrowhead denotes p65), as well as ZC3H14 and alpha-tubulin as a control for nuclear or cytoplasmic protein contamination, respectively. Ponceau staining is shown as a loading control. The steady-state levels of NF-κB p65 in the nucleus and cytoplasm were not altered upon knockdown of individual KPNA paralogs.

Figure 4.4.5: The steady-state localization of NFATc2 is altered following knockdown of KPNA1, KPNA2 or KPNA4

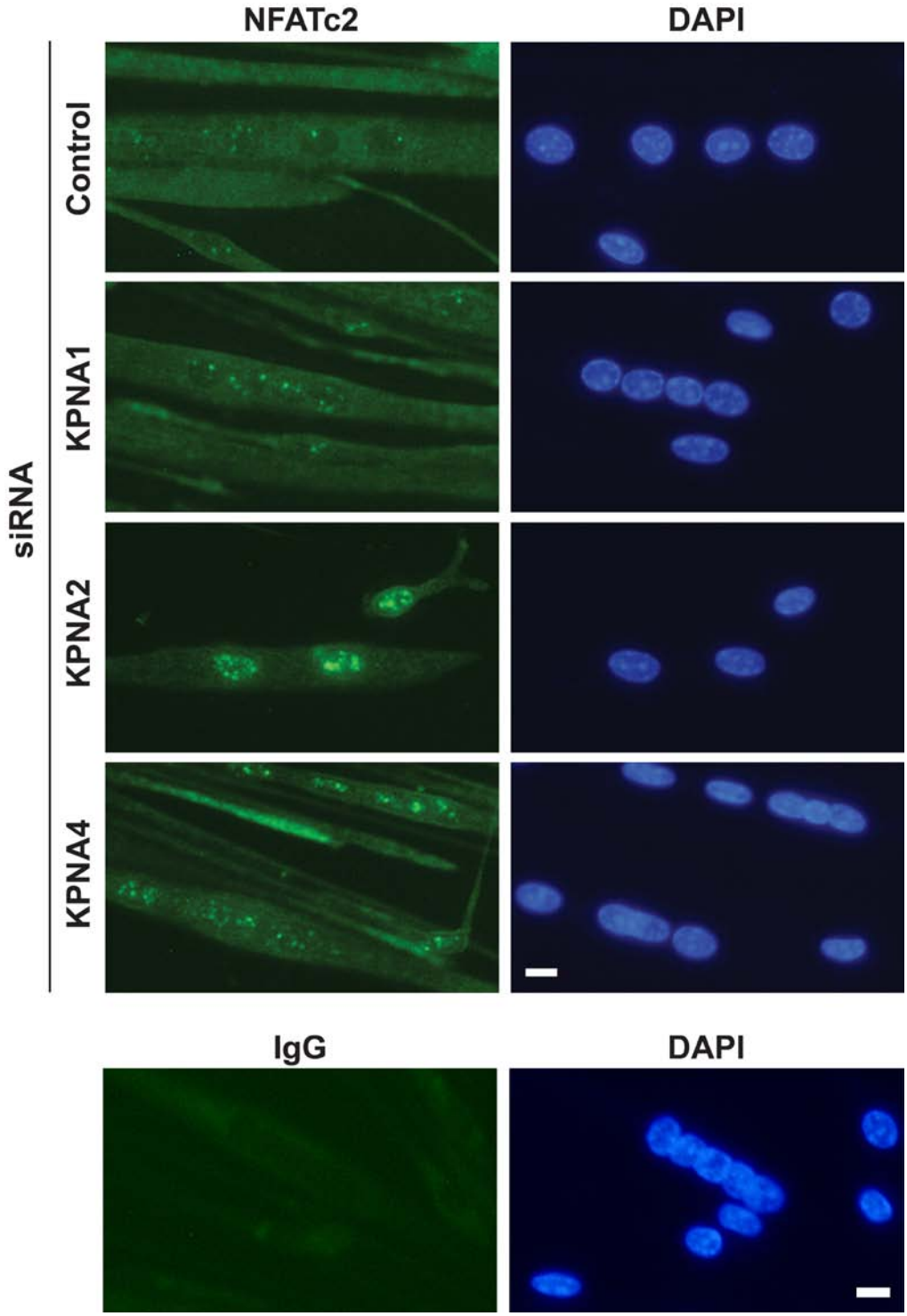

Figure 4.4.5: The steady-state localization of NFATc2 is altered following knockdown of KPNA1, KPNA2 or KPNA4

Representative fluorescent images of primary myoblasts transfected with different siRNAs. Myoblasts were differentiated for 48 hours and immunostained using antibodies against NFATc2 or IgG isotype antibodies and counterstained with DAPI (bar, 25μm). NFACTc2 was increased in the nuclei of cells treated with KPNA2 and KPNA4 siRNA compared to control with the greatest increase observed with KPNA2 siRNA; no differences were observed with KPNA1 siRNA.

Figure 4.4.6: KPNA paralogs have distinct roles in myoblast proliferation

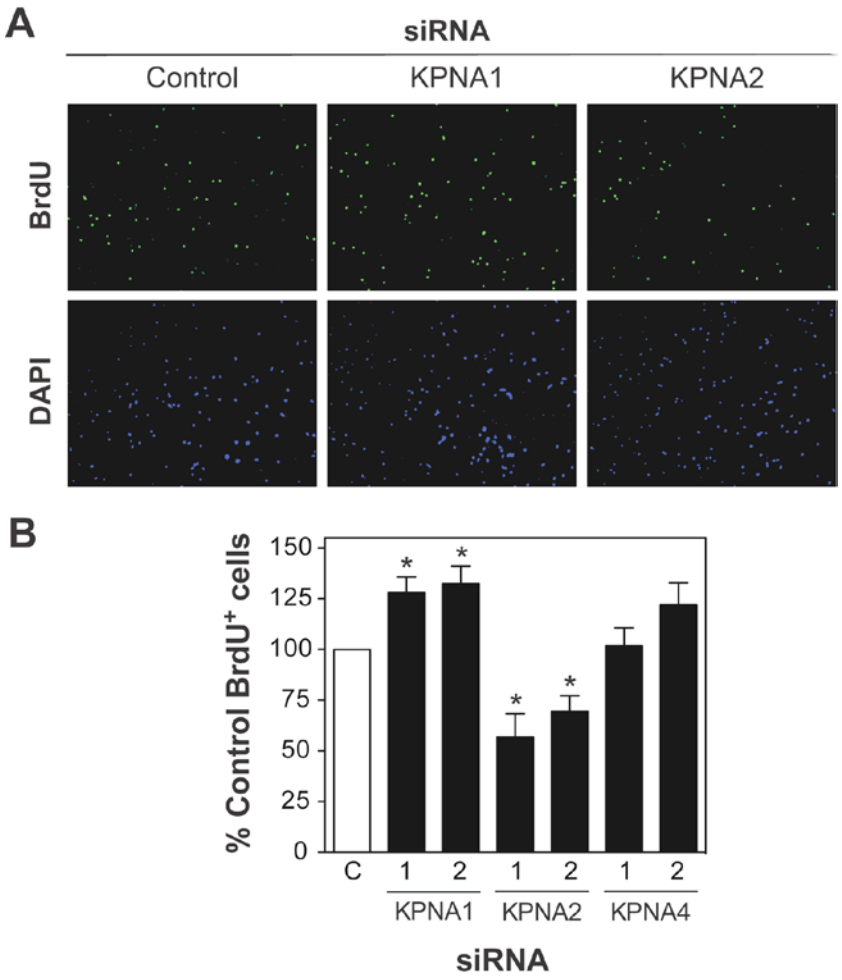

Figure 4.4.6: KPNA paralogs have distinct roles in myoblast proliferation

(A) Primary myoblasts were transfected with either control scrambled siRNA or siRNAs (1 or 2) directed against KPNA1, KPNA2 or KPNA4. Cultures were labeled with BrdU and subsequently immunostained. Representative fluorescent images of BrdU immunostaining and nuclear counterstaining with DAPI are shown (bar, 100μm). **(B)** Knockdown of KPNA1 significantly increased the percentage of $BrdU^+$ cells while loss of KPNA2 significantly reduced the percentage of $BrdU^+$ cells. No significant difference was observed in cells treated with KPNA4 siRNA. Data are mean ± SEM from three independent experiments, *$p<0.05$.

Figure 4.4.7: KPNA2 knockdown decreases myotube size

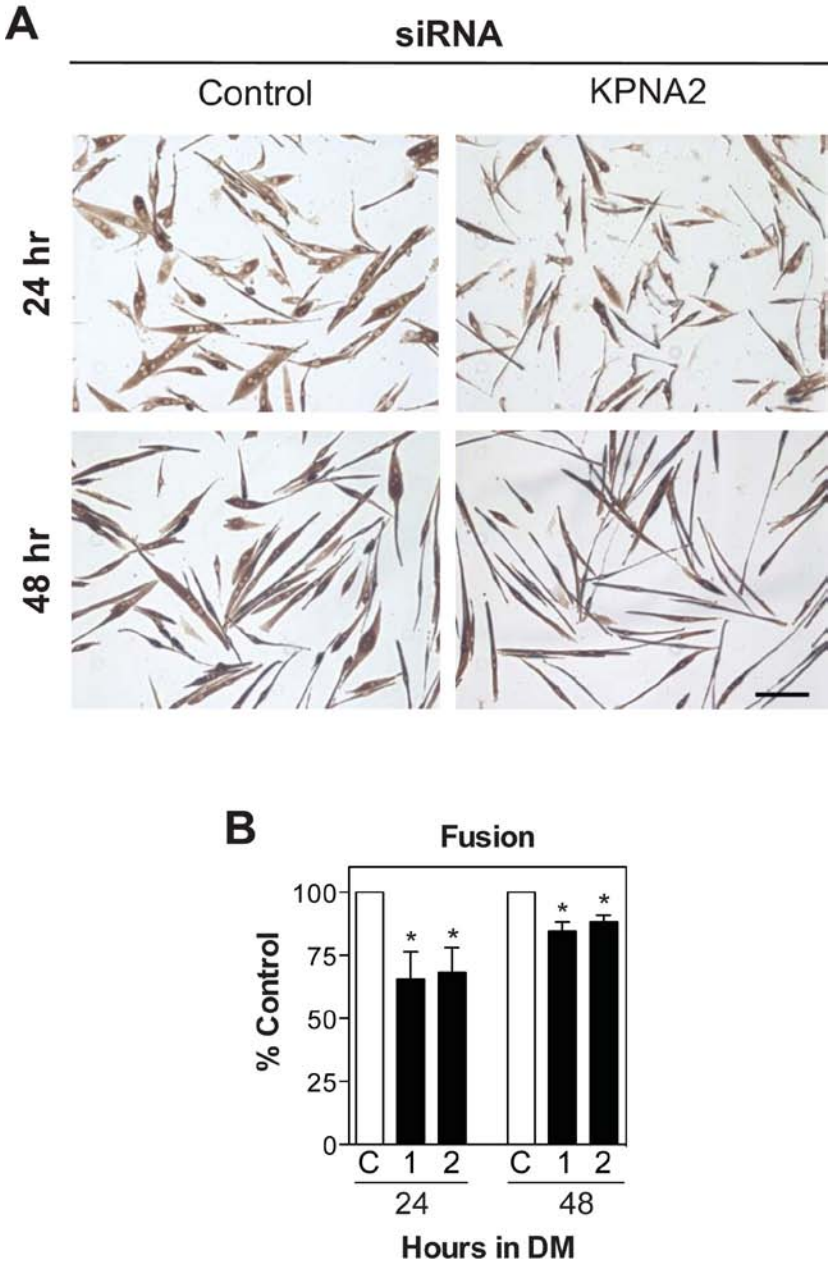

Figure 4.4.7: KPNA2 knockdown decreases myotube size

(A) Primary myoblasts were transfected with either control scrambled siRNA or one of two siRNAs (1 or 2) against KPNA2. Cells were differentiated for either 24 or 48 hours in differentiation media (DM) and immunostained for eMyHC, (bar, 50μm). **(B)** The fusion index was significantly decreased with KPNA2 siRNAs at both 24 and 48 hours of differentiation.

Figure 4.4.8: Differentiation and/or fusion are not significantly altered following knockdown of specific KPNAs

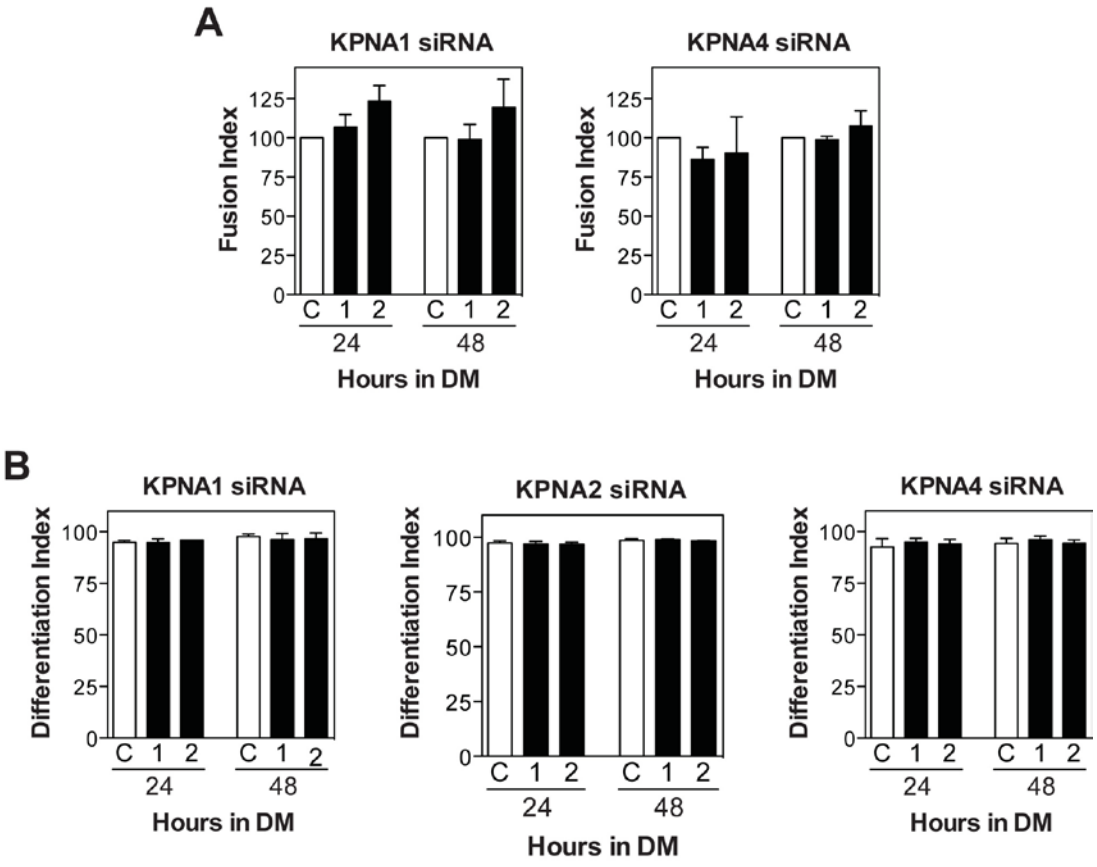

Figure 4.4.8: Differentiation and/or fusion are not significantly altered following knockdown of specific KPNAs

Primary myoblasts were transfected with either control scrambled siRNA or one of two siRNAs (1 or 2) against KPNA1, KPNA2 or KPNA4. Cells were differentiated for either 24 or 48 hours in Differentiation Media (DM) and immunostained for embryonic myosin heavy chain, a marker of differentiation. **(A)** No significant difference in the fusion index was observed upon KPNA1 or KPNA4 knockdown. **(B)** No significant difference in the differentiation index was observed upon KPNA1, KPNA2 or KPNA4 knockdown. Data are mean ± SEM from three independent experiments, $p<0.05$.

Figure 4.4.9: Cell-cell adhesion is not altered following knockdown of KPNA2

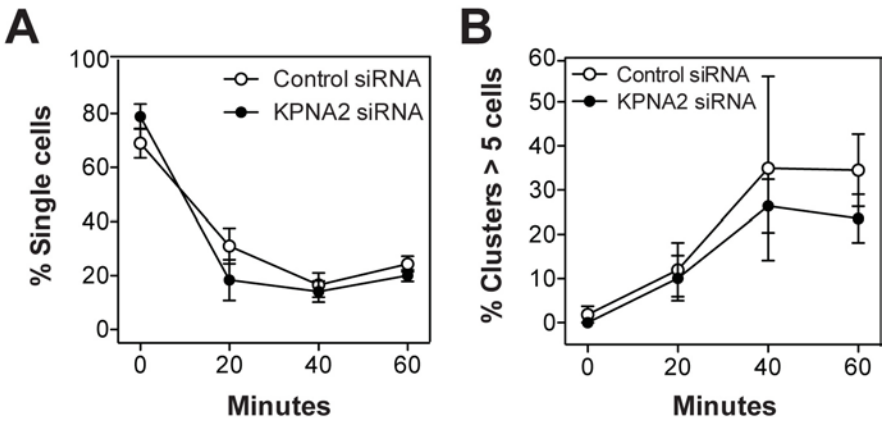

Figure 4.4.9: Cell-cell adhesion is not altered following knockdown of KPNA2

(A) A suspension-based assay was used to examine cell-cell adhesion over a 60-minute time course. No significant difference in either the percentage of single cells or **(B)** the percentage of cell clusters with >5 cells was observed with KPNA2 knockdown. Data are mean ± SEM from three independent experiments.

Figure 4.4.10: KPNA2 knockdown decreases muscle cell migration

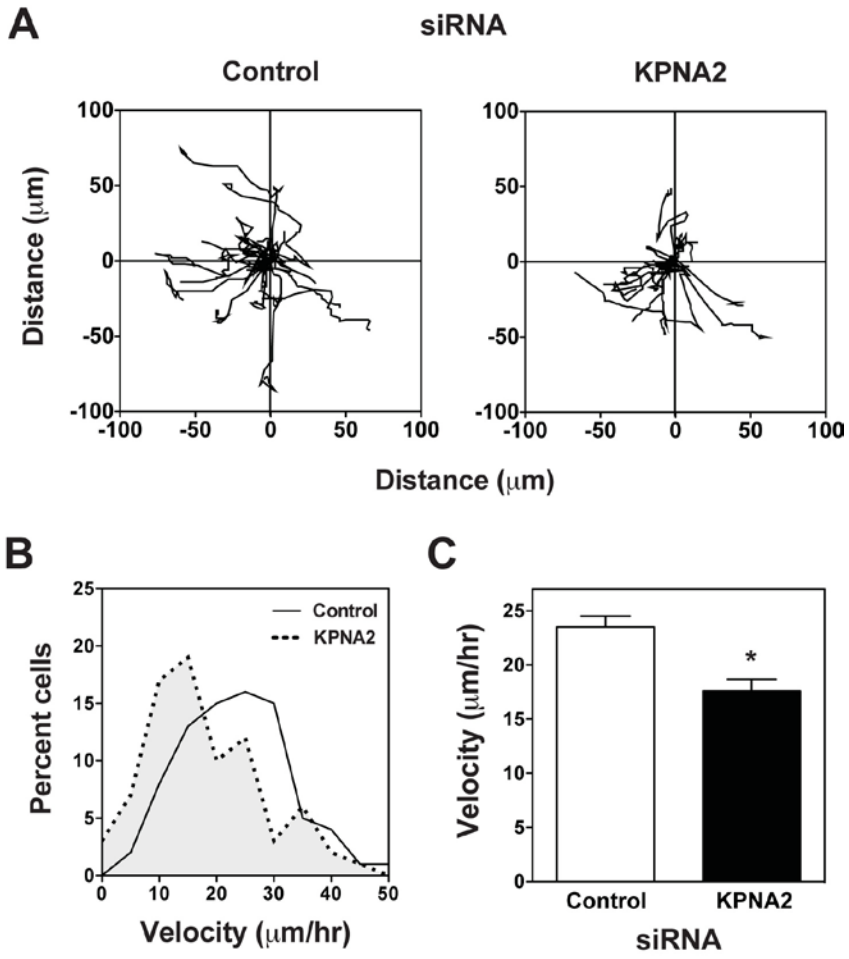

Figure 4.4.10: KPNA2 knockdown decreases muscle cell migration

(A) The migration of myocytes transfected with either control scrambled siRNA or siRNA against KPNA2 were analyzed using time-lapse microscopy. Twenty individual cell traces are shown for each siRNA condition. Myocytes treated with KPNA2 siRNA migrated shorter distances than control myocytes. **(B)** Histogram of the distribution of velocities for control or KPNA2 siRNA treated myocytes. Cells depleted of KPNA2 migrated more slowly than control myocytes. Data are mean ± SEM of 80 cells (20 cells from each of four independent isolates). **(C)** The mean velocity of migration was reduced in KPNA2 siRNA treated cells compared to control. Data are mean ± SEM of 80 cells (20 cells from each of four independent isolates), *$p<0.05$.

Chapter 5: The Role of Karyopherin Alpha1 in Satellite Cell Function

Contributions:

Matthew E. Randolph, D.M.V. (Figures 5.4.3 and 5.4.5),

Jennifer Parks (Figures 5.4.6).

Chapter 5: The Role of Karyopherin Alpha1 in Satellite Cell Function

5.1 Introduction

A large number of adult tissues depend on resident stem cells for proper function and maintenance throughout the adult lifespan. Satellite cell or muscle stem cells are essential to adult myogenesis because they are responsible for generating large numbers of progenitors that are required for muscle maintenance, growth and repair. In addition, the satellite cell pool or the total number of satellite cells within muscle must be maintained throughout the adult lifespan for proper muscle function. Therefore, exhaustion of the satellite cell pool either through loss of proliferative capacity or reduction in total cell number can result in loss of muscle function, mobility or death. While satellite cell proliferation is required to generate progenitors, satellite cell proliferation must also be negatively controlled because satellite cells must exit the cell cycle to become quiescent to replenish the satellite cell pool or to differentiate and fuse into multinucleated myofibers (Charge and Rudnicki, 2004). Satellite cells exhibit a decrease in proliferative potential and total number during aging as well as in skeletal muscle diseases such as Duchenne Muscle Dystrophy where diseased muscle is continuously undergoing regeneration (Blau et al., 1983a; Blau et al., 1983b; Quinn et al., 1984; Sacco et al., 2010; Webster and Blau, 1990). Likewise, other stem cell populations, such as neural and hematopoietic stem cells, require regulation of proliferation for proper stem cell pool maintenance during aging (Janzen et al., 2006; Li, 2011; Yun et al., 2004). However, the molecular mechanisms that control satellite cell proliferation or satellite cell pool size are poorly understood. Understanding how satellite

cell proliferation and pool size is controlled during myogenesis could lead to better understanding of how proliferation is regulated in aging or diseased muscle and could have implications for other stem cell populations.

Changes in gene expression during satellite cell activation, proliferation and differentiation are due in part to the facilitated nuclear import of proteins, such as transcription factors, that function in the nucleus to regulate gene expression. However, little is known regarding the facilitated nucleocytoplasmic transport of nuclear factors in satellite cells. Microarray analyses suggest that satellite cell entry into the cell cycle is marked by changes in the nuclear transport machinery (Fukada et al., 2007; Pallafacchina et al., 2010). Multiple mRNAs that encode components of the nuclear transport machinery, such as nucleoporins and karyopherin transport receptors, were increased in proliferating satellite cells *in vivo* compared to quiescent satellite cells. The upregulation of the nuclear transport machinery may be functionally required to allow for rapid changes in gene expression associated with satellite cell activation and proliferation. Satellite cells express a wide variety of nuclear factors such as transcription factors that are critical to satellite cell function, such as Pax7, which has a role in muscle cell lineage determination (Lepper et al., 2009). Meanwhile subpopulations of satellite cells require the muscle-specific transcription factor MyoD for normal skeletal muscle development (Gensch et al., 2008; Haldar et al., 2008; Rudnicki et al., 1993). The Notch intracellular domain, a non-muscle specific factor dependent upon classical nuclear import, has been shown to have critical role in promoting satellite cell proliferation in a subpopulation of satellite cells (Conboy and Rando, 2002). Understanding how the facilitated nuclear import of nuclear factors, such as Pax7, MyoD and the Notch intracellular domain, is

controlled by the nuclear transport machinery would lead to better understanding of how gene expression is regulated in satellite cells.

Our previous studies in Chapter 4 indicate that classical nuclear import receptor KPNA1 has a role in negatively regulating myoblast proliferation *in vitro*, therefore, we hypothesized that KPNA1 has a similar role in negatively regulating satellite cell proliferation *in vivo*. KPNA1 nuclear import receptor may import cNLS cargoes involved in regulating proliferation, thereby enabling satellite cells to exit the cell cycle to either replenish the satellite cell pool or to differentiate and participate in muscle growth or regeneration.

We determined that freshly isolated satellite cells express four karyopherin alpha paralogs: *Kpna2*, *Kpna3*, *Kpna4* and *Kpna6*. While *Kpna1* was not detected in newly isolated satellite cells, *Kpna1* was detected during the early stages of satellite cell proliferation *in vitro*. To investigate the functional role of KPNA1 and classical nuclear import in regulating satellite cell proliferation, we performed *in vitro* proliferation assays using satellite cells isolated from *Kpna1*$^{-/-}$ and *Kpna1*$^{+/-}$ mice. Both *Kpna1*$^{-/-}$ and *Kpna1*$^{+/-}$ cultures displayed an increase in cell proliferation. Through the use of flow cytometry and *Kpna1*$^{+/-}$ mice, we determined that KPNA1 has a role in *Kpna1* $^{+/-}$ mice in regulating satellite cell proliferation *in vivo* during muscle maintenance and regeneration, while analysis of muscle morphology of *Kpna1* $^{+/-}$ mice revealed a potential increase in both myofiber size and the percentage of fibers with multiple centrally located nuclei compared to wild type mice. In summary, this chapter provides evidence of a role for classical nuclear import and KPNA1 in regulating satellite cell proliferation in skeletal muscle. We suggest that KPNA1 may provide a global regulatory mechanism to

maintain proper satellite cell pool size by importing cNLS-containing cargoes involved in regulating cell proliferation.

5.2 Results

Differential expression of karyopherin alpha paralogs in satellite cells

To determine whether the karyopherin alpha classical nuclear import receptors are expressed in satellite cells, we isolated hindlimb satellite cells from Nestin-GFP mice using an established fluorescence-activated cell sorting (FACS) protocol (Day et al., 2007). Quiescent satellite cells in the Nestin-GFP mouse express GFP from a Nestin promoter, while proliferating progeny of satellite cells do not express GFP. Satellite cells comprise approximately 1-5 % of skeletal muscle nuclei in rodents and humans (Hawke and Garry, 2001), therefore, the FACS protocol allows for enrichment of satellite cells in order to perform RT-PCR. Since endothelial cells of the Nestin-GFP mouse also express GFP (Day et al., 2007), we sorted against CD31, an endothelial cell-surface marker, to remove these cells from the GFP^+ population. Pre-sort and post-sort flow plots demonstrate the purity of the GFP^+ population derived from cell sorting which was ~91% (Figure 5.4.1.A). RT-PCR was performed on total RNA isolated from GFP^+ sorted cells with primers specific for each of the five karyopherin alpha paralogs in mouse and 18S rRNA as an internal cDNA control. In addition, RT-PCR was performed using primers for *Pax7* to confirm the myogenicity of the isolated population and primers for *CD31* to confirm the absence of GFP^+ endothelial cells. mRNA was detected for *Pax7* in the isolated population and in primary mouse myoblasts (Figure 5.4.1.B). Meanwhile, *CD31* mRNA was not detected in the isolated population, while *CD31* mRNA was detected in samples derived from the endothelial cell line MS1 (Figure 5.4.1.B). mRNA was detected for *Kpna2*, *Kpna3*, *Kpna4* and *Kpna6*, while mRNA was not detected for *Kpna1*

(Figure 5.4.1.B). As expected, mRNA for all paralogs, including *Kpna1*, was detected in cultured primary myoblasts. Thus, four of the karyopherin alpha import receptors, which are utilized in classical nuclear import, are expressed in freshly isolated satellite cells. These results suggest that classical nuclear import may be utilized by satellite cells to regulate the nuclear import of factors involved in satellite cell function. *Kpna1* was not detected in newly isolated satellite cells, suggesting that KPNA1 may not be required in quiescent satellite cells to facilitate the nuclear import of cNLS cargoes involved in satellite cell quiescence. However, *Kpna1* was detected in myoblasts which suggest that *Kpna1* expression is induced in proliferating satellite cells.

KPNA1 regulates proliferation in satellite cells

Satellite cell proliferation must be tightly controlled because satellite cells must exit the cell cycle to become quiescent to replenish the satellite cell pool or to differentiate and fuse into multinucleated myofibers. Since *Kpna1* is expressed in proliferating satellite cells and previous studies in Chapter 4 revealed a role for KPNA1 in negatively regulating myoblast proliferation, we investigated the role of KPNA1 in regulating satellite cell proliferation using $Kpna1^{+/+}$, $Kpna1^{+/-}$ and $Kpna1^{-/-}$ mice. $Kpna1^{+/-}$ and $Kpna1^{-/-}$ mice appear normal and grow into adulthood (Shmidt et al., 2007). We initially determined the steady-state mRNA and protein levels for *Kpna1* in satellite cells derived from $Kpna1^{+/+}$, $Kpna1^{+/-}$ and $Kpna1^{-/-}$ mice. RT-PCR was performed on total RNA isolated from cultured satellite cells at 96 hours post-isolation since this timepoint represents the early stages of satellite cell proliferation. RT-PCR was performed using primers specific for mouse *Kpna1* and 18S rRNA. *Kpna1* mRNA was

detected in *Kpna1*$^{+/+}$, but not in *Kpna1*$^{+/-}$ or *Kpna1*$^{-/-}$ cultures (Figure 5.4.2.A). To determine KPNA1 steady-state protein levels, immunoblotting was performed on cell lysates isolated from cultured satellite cells at 96 hours post-isolation using antibodies specific for KPNA1 and EF1alpha as a control for equal protein loading. Consistent with *Kpna1* mRNA expression, KPNA1 protein was detected in *Kpna1*$^{+/+}$, but not in *Kpna1*$^{+/-}$ or *Kpna1*$^{-/-}$ satellite cells (Figure 5.4.2.B). The reduced steady-state levels of *Kpna1* mRNA and protein in *Kpna1*$^{+/-}$ satellite cells may be due to KPNA1 involvement in importing nuclear factors involved in *Kpna1* gene expression. Indeed, authors of a study investigating the role of KPNA1 during mouse neurogenesis suggested that KPNA1 may import cNLS proteins involved in a feedback loop involving *Kpna1* gene expression (Yasuhara et al., 2007). Alternatively, reduced levels of KPNA1 could also be due to maternal or paternal-specific allele expression (Knight, 2004). Regardless, KPNA1 steady-state protein levels are similar between *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ cells (5.4.2.B). Thus, *Kpna1* is expressed in proliferating satellite cells.

To investigate the functional role of KPNA1 in regulating proliferation in satellite cells, we performed proliferation assays with cultured satellite cells derived from *Kpna1*$^{+/+}$, *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ mice. Mononucleated cells were isolated from the hindlimb muscles of *Kpna1*$^{+/+}$, *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ mice and cultured for 96 hours. At 96 hours post isolation, satellite cell cultures were labeled with bromodeoxyuridine (BrdU) for one hour, followed by immunostaining for BrdU to determine the percentage of BrdU^{+} cells. *Kpna1*$^{+/-}$ cultures displayed a ~75% increase in the number of BrdU^{+} cells compared to wild type, while *Kpna1*$^{-/-}$ cultures had a ~90% increase in the number of BrdU^{+} cells (Figure 5.4.2.B). In Chapter 4 studies, cultured myoblasts treated with

KPNA1 siRNA displayed a less dramatic phenotype with only a ~25% increase in proliferation compared with the ~75-90% increase observed in proliferating satellite cells from *Kpna1* mutant mice, however, the less dramatic phenotype may be due to inefficient knockdown of KPNA1 by RNAi. Overall, the increase in proliferation observed in the $Kpna1^{+/-}$ and $Kpna1^{-/-}$ culture suggest a role for KPNA1 in import of cNLS-containing cargoes involved in regulating satellite cell proliferation, similar to the role of KPNA1 in regulating myoblast proliferation. KPNA1 may have a critical role in importing cNLS-containing cargoes involved in negatively regulating cell proliferation to enable replenishment of the satellite cell pool and differentiation of progenitors to contribute to muscle growth.

KPNA1 regulates proliferation and satellite cell pool size during muscle maintenance and regeneration

Since our studies of KPNA1 in satellite cells *in vitro* indicate a role for KPNA1 in negatively regulating satellite cell proliferation, we examined the proliferation status and relative size of the satellite cell pool in $Kpna1^{+/+}$ and $Kpna1^{+/-}$ muscles *in vivo* under basal conditions (muscle maintenance) using flow cytometry. $Kpna1^{+/-}$ mice were used for experiments because the proliferation defects observed in satellite cells were similar between the heterozygous and the null conditions and a larger number of $Kpna1^{+/-}$ mice were available than $Kpna1^{-/-}$. To determine the proliferation status of satellite cells *in vivo*, $Kpna1^{+/-}$ and $Kpna1^{+/+}$ mice were given daily injections of bromodeoxyuridine (BrdU), to label cells in S phase, for two days before skeletal muscle isolation. Following isolation of mononucleated cells from hindlimb muscle, flow cytometry was

performed using antibodies against BrdU to identify the proliferating population and cell surface markers CD31, CD45 and Alpha7 Integrin to identify the satellite cell population ($CD31^-$, $CD45^-$ and Alpha7 $Integrin^+$) (Figure 5.4.3.A) (Kafadar et al., 2009). The percentage of proliferating satellite cells in *Kpna1*$^{+/-}$ muscles was approximately 2.5-fold higher compared with *Kpna1*$^{+/+}$ muscles (Figure 5.4.3.B). However, we did not observe any significant difference in total number of satellite cells in *Kpna1*$^{+/-}$ muscles compared with *Kpna1*$^{+/+}$ muscles (Figure 5.4.3.C). The increase in proliferation of satellite cells in *Kpna1*$^{+/-}$ muscles during basal conditions supports the notion that KPNA1 may have a role in regulating proliferation *in vivo* where KPNA1 may import cNLS-containing proteins involved in negatively regulating satellite cell proliferation during normal muscle maintenance.

A basal level of satellite cell activation and proliferation occurs during normal myonuclear turnover in skeletal muscle (Schmalbruch and Lewis, 2000). However, during muscle regeneration, a larger number of satellite cells reenter the cell cycle and proliferate to generate progenitors that either replenish the satellite cell pool or undergo differentiation to repair damaged muscle (Figure 5.4.4). Our data examining muscle maintenance in *Kpna1*$^{+/-}$ mice revealed an increase in proliferation, a phenotype which more closely resembles satellite cell behavior in regenerating muscle. To determine whether KPNA1 also regulates satellite cell proliferation during muscle regeneration, we examined the proliferation status and the size of the satellite cell pool in *Kpna1*$^{+/-}$ and *Kpna1*$^{+/+}$ mice after muscle injury. We induced regeneration of the gastrocnemius muscle with $BaCl_2$ followed by daily BrdU injections, muscle isolation at three days post injury and flow cytometry as described above (Figure 5.4.3.A). The percentage of

proliferating satellite cells in *Kpna1*$^{+/-}$ muscles was approximately 1.5-fold higher compared with *Kpna1*$^{+/+}$ muscles (Figure 5.4.5.B). In addition, we observed a ~20% decrease in the total number of satellite cells in *Kpna1*$^{+/-}$ compared with *Kpna1*$^{+/+}$ muscles (Figure 5.4.5.C). Taken together, these data suggest that KPNA1 negatively regulates satellite cell proliferation during skeletal muscle maintenance and regeneration. In addition, the reduction in total satellite cells in regenerating muscle suggest a population of satellite cells may exit the cell cycle, differentiate and fuse into multinucleated myofibers and therefore not be detected in our flow cytometry analysis.

KPNA1 regulates myofiber size during muscle regeneration

An increase in satellite cell proliferation could result in an increase in the number of progenitors initially available for myofiber formation during regeneration. To determine whether reduced levels of KPNA1 affect myofiber formation *in vivo* during muscle regeneration, we analyzed the myofiber morphology of regenerating *Kpna1*$^{+/-}$ muscle using histological methods. We induced regeneration of the TA (tibialis anterior) muscle of *Kpna1*$^{+/-}$ or *Kpna1*$^{+/+}$ mice with $BaCl_2$ and isolated muscles five days post injury at a time when proliferation and cell-cell fusion is occurring during muscle regeneration. Histological analysis of the *Kpna1*$^{+/-}$ muscles revealed a shift in the distribution of myofiber cross-sectional areas (XSA) to myofibers with slightly larger area compared to *Kpna1*$^{+/+}$ muscles at five days post-injury (Figure 5.4.6.A). A hallmark of regenerating muscle is the appearance of centrally located nuclei within myofibers due to the recent fusion of satellite cells. Since myofiber area can correlate with the number of myonuclei that myofibers contain, we investigated whether *Kpna1*$^{+/-}$ muscles had an

increase in myofibers with multiple centrally located nuclei compared with *Kpna1*$^{+/+}$ muscles using histological methods. We observed an increase in the percentage of myofibers with multiple centrally located nuclei in regenerating *Kpna1*$^{+/-}$ muscles compared to *Kpna1*$^{+/+}$ muscles (Figure 5.4.6.B, C). The slight increase in myofiber size and the percentage of multiple centrally located nuclei in regenerating *Kpna1*$^{+/-}$ muscles may be the result of an increase in the number of progenitors fusing into myofibers consistent with the decrease in total satellite cell numbers in regenerating *Kpna1*$^{+/-}$ muscles compared to *Kpna1*$^{+/+}$ muscles.

5.3 Discussion

Control of gene expression in satellite cells is due in part to the nucleocytoplasmic import of nuclear proteins that require facilitated nuclear import. We determined that quiescent satellite cells express several karyopherin alpha paralogs of the classical nuclear import pathway and we demonstrated that KPNA1 is required to regulate satellite cell proliferation both *in vitro* and *in vivo*.

We determined that freshly isolated satellite cells express four karyopherin alpha import receptors, *Kpna2*, *Kpna3*, *Kpna4* and *Kpna6*, but not *Kpna1* suggesting that *Kpna1* is not required in quiescent satellite cells. These results are consistent with expression of karyopherin paralogs in other progenitor cell populations such as sperm and embryonic stem cells in which some paralogs are present whereas others are induced during cell differentiation (Hogarth et al., 2006; Yasuhara et al., 2007). Interestingly, *Kpna1* mRNA and protein were induced in proliferating satellite cells suggesting that KPNA1 may have a role in importing cNLS-containing proteins during cell proliferation. Our detection of *Kpna2* and *Kpna3* mRNA in satellite cells is consistent with microarray studies where both *Kpna2* and *Kpna3* mRNA were detected in both quiescent and proliferating satellite cells (Fukada et al., 2007; Pallafacchina et al., 2010). However, the characterization of KPNA steady-state protein expression and cellular localization in both quiescent and proliferating satellite cells will need to be performed if the functional role of classical nuclear import and karyopherin import receptors are to be further investigated.

We investigated the functional role of KPNA1 during satellite cell proliferation *in vitro*, using cells isolated from a $Kpna1^{+/-}$ and $Kpna1^{-/-}$ mice, thereby confirming the

proliferation defects we observed following KPNA1 knockdown in cultured myoblasts in Chapter 4. *Kpna1*$^{+/-}$ satellite cells displayed a ~75% increase in proliferation compared to *Kpna1*$^{+/+}$ cells, while *Kpna1*$^{-/-}$ satellite cells displayed a ~90% increase compared to *Kpna1*$^{+/+}$. Therefore, KPNA1 may have a role in importing cNLS-cargoes required for regulating satellite cell proliferation similar to its role in myoblasts, however, the cNLS-containing cargo or cargoes responsible for altering satellite cell proliferation and/or pool size are unknown. The cell cycle protein p27^{Kip1} has been implicated in the negative regulation of proliferation in satellite cells and hematopoietic stem cells (Fero et al., 1996; Kiyokawa et al., 1996; Spangenburg et al., 2002). p27^{Kip1} interacts with karyopherin alpha import receptors (Shin et al., 2005), however, whether KPNA paralogs are solely responsible for the import of p27^{Kip1} is unknown. Further studies are required to determine whether failure of p27^{Kip1} nuclear import is responsible for the phenotype observed in *Kpna1*$^{-/-}$ mutant satellite cells.

KPNA1 may import a different subset of cNLS proteins in satellite cells than in myoblasts since different stages of myogenesis may express different subsets of cNLS-containing proteins. In addition, control of cell proliferation may vary as muscle cells progress through the myogenic program. An appropriate level of proliferation of satellite cells must occur to generate adequate precursors for muscle repair, however, uncontrolled proliferation that prevents cell cycle exit could deplete the satellite cell pool thereby preventing proper repair during subsequent rounds of muscle regeneration. Lastly, while our *in vitro* studies suggest that KPNA2 may have a role in positively regulating myoblast proliferation, whether KPNA2 has a role in positively regulating satellite cell proliferation will require further investigation.

Additional evidence for the role of KPNA1 in regulating satellite cell proliferation comes from our studies of *Kpna1*$^{+/-}$ and *Kpna1*$^{+/+}$ mice during muscle maintenance *in vivo.* In basal *Kpna1*$^{+/-}$ muscles we observed a 2.5-fold increase in proliferating satellite cells compared to *Kpna1*$^{+/+}$ muscles. While we would predict an increase in total satellite cell number in the *Kpna1*$^{+/-}$ muscles due to enhanced proliferation, we did not observe a significant change in the total number of satellite cells in basal *Kpna1*$^{+/-}$ muscles compared to *Kpna1*$^{+/+}$ muscles. The fate of these proliferating cells in the basal muscle is unclear. These cells may be fusing into myofibers or undergoing apoptosis and therefore undetectable in our flow cytometry analysis. In addition, questions remain as to whether *Kpna1*$^{+/-}$ satellite cells are capable of self-renewal since we did not observe an increase in total satellite cells numbers.

During muscle regeneration, we observed a 1.5-fold increase in the percentage of proliferating satellite cells in *Kpna1*$^{+/-}$ compared to *Kpna1*$^{+/+}$ muscles. In addition, we observed a ~20% reduction in the total number of satellite cells in *Kpna1*$^{+/-}$ muscle compared to *Kpna1*$^{+/+}$ muscles. The increase in proliferation of the satellite cells in basal *Kpna1*$^{+/-}$ muscle could generate progenitors that upon muscle injury are primed to exit the cell cycle, differentiate and fuse into myofibers. Indeed, the increase in myofiber size and number of centrally-located myonuclei per myofiber in *Kpna1*$^{+/-}$ muscles compared *Kpna1*$^{+/+}$ muscles during regeneration could explain the decrease in total satellite cells number in *Kpna1*$^{+/-}$ regenerating muscle. Overall, satellite cell lineage tracing experiments will be required to confirm whether progenitors are fusing into myofibers in *Kpna1*$^{+/-}$ muscle during muscle maintenance or regeneration. Overall, additional flow cytometry and morphological analysis will be required to fully characterize the *Kpna1*$^{+/-}$

proliferation phenotype and to determine the fate of *Kpna1*$^{+/-}$ satellite cells during muscle maintenance and regeneration. Taken together these data suggest KPNA1 has a role in negatively regulating proliferation in both basal and regenerating muscle.

The satellite cell pool or the total number of satellite cells within muscle must be maintained throughout the adult lifespan for proper muscle function. During aging, the quiescent satellite pool exhibits a normal decrease in total cell number and proliferative capacity which could reduce the efficiency of muscle regeneration (Blau et al., 1983a; Quinn et al., 1984). Whether the enhanced proliferation phenotype observed in *Kpna1*$^{+/-}$ satellite cells alters the size of the satellite cell pool or the efficiency of muscle regeneration in aging muscle is unknown. If *Kpna1*$^{+/-}$ satellite cells are capable of self-renewal, the enhanced proliferation phenotype could result in an increase in the total number of quiescent satellite cells and regeneration efficiency during aging compared to wild type muscle. Alternatively, if the *Kpna1*$^{+/-}$ satellite cells are not capable of self-renewal, exhaustion of the satellite cell pool could occur with aging which would decrease regeneration efficiency compared to normal aging muscle. While we uncovered a role for *Kpna1*$^{+/-}$ in satellite cell proliferation, further investigation of the role of KPNA1 in self-renewal will be required to fully understand the impact of the enhanced proliferation phenotype observed in *Kpna1*$^{+/-}$ muscle during maintenance and/or regeneration.

The proliferation status and total satellite cell numbers between the *Kpna1*$^{+/-}$ and *Kpna1*$^{+/+}$ muscles during muscle maintenance and regeneration could be due to defects in non-muscle cell types that require KPNA1 for proper function. Whether non-muscle cell types in *Kpna1*$^{+/-}$ muscle display proliferation defects is unclear. However, data from

Chapter 4 suggest the proliferation phenotype observed in *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ satellite cells is primarily a cell intrinsic effect since a similar proliferation defect was observed in highly pure cultures of myoblasts following KPNA1 knockdown via RNAi. Regardless, further studies of KPNA1 in satellite cells will require the use of a floxed KPNA1 mouse (Moriyama et al., 2011) crossed with a satellite cell-specific cre recombinase expressing mouse (Nishijo et al., 2009) to eliminate confounding effects from non-muscle cell types whose behavior may be altered by loss of or reduced levels of KPNA1.

In summary, these studies suggest that classical nuclear import and KPNA1 may function in satellite cells to import nuclear factors involved in regulating satellite cell proliferation to prevent over-proliferation and potential exhaustion of the satellite cell pool during myogenesis. We propose that KPNA1 expression is induced in satellite cells to control satellite cell proliferation during muscle maintenance and regeneration. Uncovering the role of KPNA1 in regulating satellite cell proliferation is only the first step to understanding the critical role of classical nuclear import in satellite cell biology. In addition, we suggest that classical nuclear import may provide a regulatory mechanism for the nuclear import of a multitude of cNLS-containing proteins involved in satellite cell function.

5.4 Figures

Figure 5.4.1: Differential expression of karyopherin alpha paralogs in satellite cells

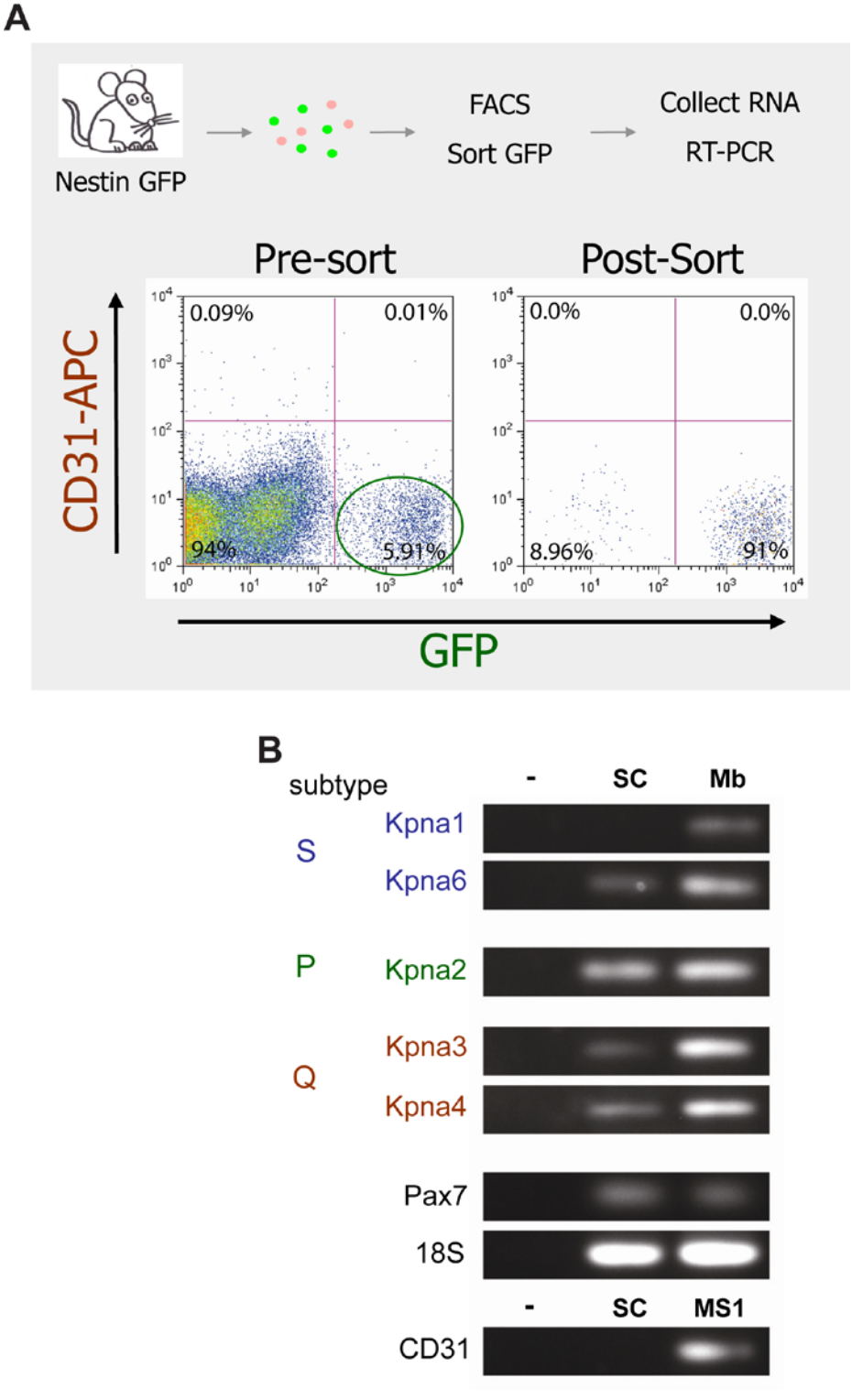

Figure 5.4.1: Differential expression of karyopherin alpha paralogs in satellite cells

(A) Isolation of satellite cells. Satellite cells in the Nestin GFP-mouse express GFP which allows for isolation of these cells from hindlimb muscle. Mononucleated cells were collected from dissociated Nestin-GFP hindlimb muscles and used in fluorescence activated cell sorting (FACS) to isolate GFP^+ cells. Following isolation of mononucleated cells from muscle, flow cytometry was performed using antibodies against cell surface marker CD31, an endothelial cell-surface marker, to remove these cells from the GFP^+ population since endothelial cells in the Nestin-GFP mice also express GFP. Pre-sort and post-sort flow plots demonstrate the targeted population ($CD31^-$ and GFP^+ cells within the circled area) and the purity (91%) of the satellite cells derived from cell sorting. **(B) Four *Kpna* paralogs are expressed in satellite cells, while *Kpna1* was not detected.** RT-PCR was performed on satellite cells (SC) and myoblasts (Mb) using *Kpna* paralog-specific primers along with Pax7 to verify myogenicity and 18S rRNA as an internal cDNA loading control. A negative control (-) PCR was also performed with *Kpna* primers without cDNA. RT-PCR was performed on satellite cells and endothelial cells (MS1) using CD31 primers to confirm the sorted cells did not express the endothelial cell marker CD31. Satellite cells from five Nestin-GFP mice were pooled for RT-PCR analysis in two independent experiments.

Figure 5.4.2: KPNA1 regulates satellite cell proliferation

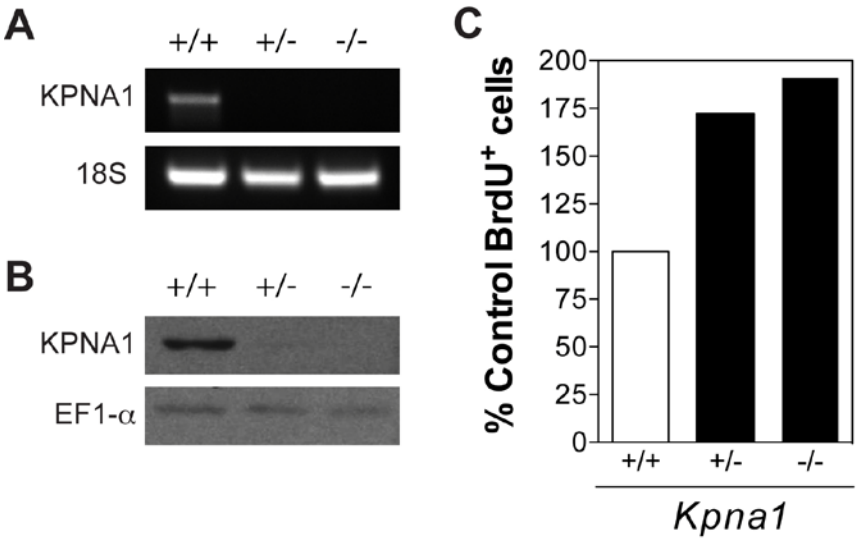

Figure 5.4.2: KPNA1 regulates satellite cell proliferation

(A, B) Total RNA and protein were collected in tandem from pooled satellite cell cultures from *Kpna1* $^{+/+}$, *Kpna1*$^{+/-}$ or *Kpna1*$^{-/-}$ mice at 96 hours post isolation. Pooled satellite cell cultures were derived from two 8-12 week old mice. **(A)** RT-PCR was performed using primers specific for *Kpna1* and 18S rRNA as an internal cDNA control. *Kpna1* mRNA was detected in wild type, but not in *Kpna1*$^{-/-}$ satellite cell cultures, meanwhile *Kpna1* mRNA was barely detected in *Kpna1*$^{+/-}$ satellite cells. **(B)** Immunoblotting was performed with antibodies specific for KPNA1 or EF1α as a protein loading control. KPNA1 protein was detected in wild type, but not in *Kpna1*$^{-/-}$ or *Kpna1*$^{+/-}$ satellite cells. **(C)** Satellite cell cultures from *Kpna1*$^{+/+}$, *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ mice were pulsed for one hour with bromodeoxyuridine (BrdU) at 96 hours post isolation. Cultures were immunostained for BrdU and counterstained with DAPI. To determine the percentage of BrdU^{+} cells in *Kpna1*$^{+/+}$, *Kpna1*$^{+/-}$ and *Kpna1*$^{-/-}$ cultures, the number of BrdU^{+} cells was divided by total satellite cells as determined by fluorescence microscopy. A total of nine fields (>500 nuclei) were analyzed for each condition in two independent experiments using 8-12 week old mice.

Figure 5.4.3: Satellite cell proliferation and number in *Kpna1*$^{+/-}$ mice during muscle maintenance

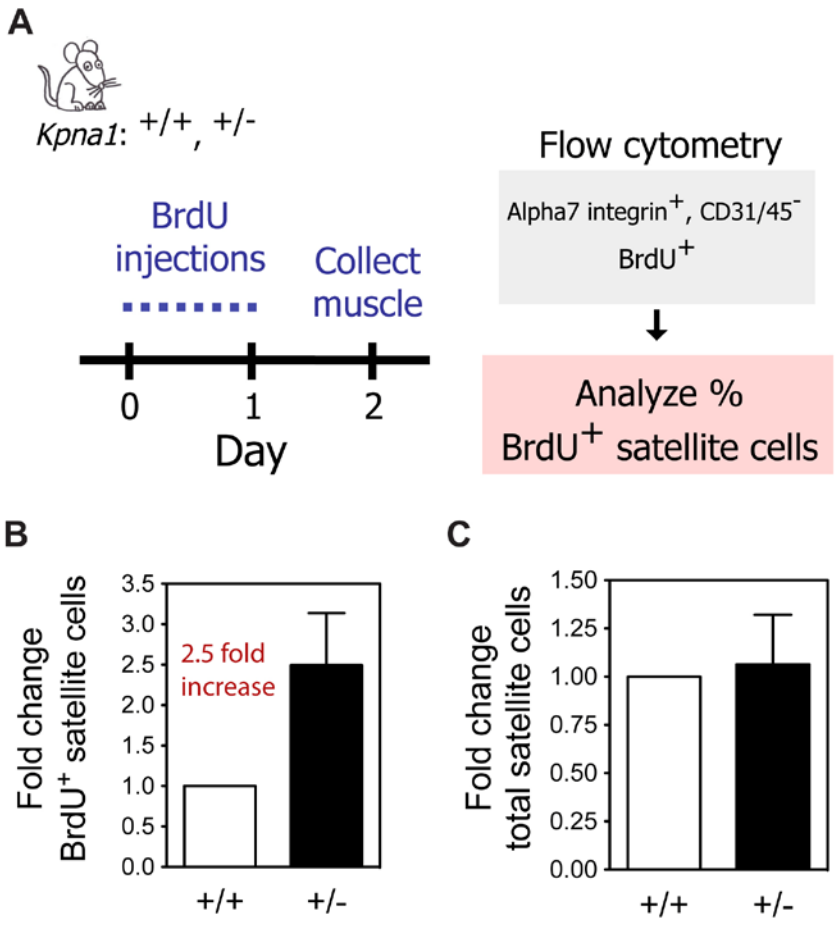

Figure 5.4.3: Satellite cell proliferation and number in *Kpna1*$^{+/-}$ mice during muscle maintenance

(A) Experimental schematic to analyze satellite cell number and proliferation in *Kpna1* *Kpna1*$^{+/-}$ mice. Mononucleated cells were isolated from the hindlimb muscles of *Kpna1*$^{+/-}$ or *Kpna1*$^{+/+}$ mice following two days of peritoneal injections with BrdU. Mononucleated cells were immunostained with antibodies against the cell markers CD31, CD45 and Alpha7 Integrin, along with antibodies to BrdU to analyze proliferating satellite cells via flow cytometry. **(B)** A 2.5-fold increase in the percentage of BrdU^{+} satellite cells was observed in *Kpna1*$^{+/-}$ muscles compared with *Kpna1*$^{+/+}$ muscles. **(C)** No significant difference in the total number of satellite cells was observed in *Kpna1*$^{+/-}$ muscles compared with *Kpna1*$^{+/+}$ muscles. Satellite cells from four *Kpna1* $^{+/+}$ or *Kpna1*$^{+/-}$ mice were pooled for flow cytometry analysis. Data are mean ± SEM from three independent experiments.

Figure 5.4.4: Satellite cell function during muscle maintenance and regeneration

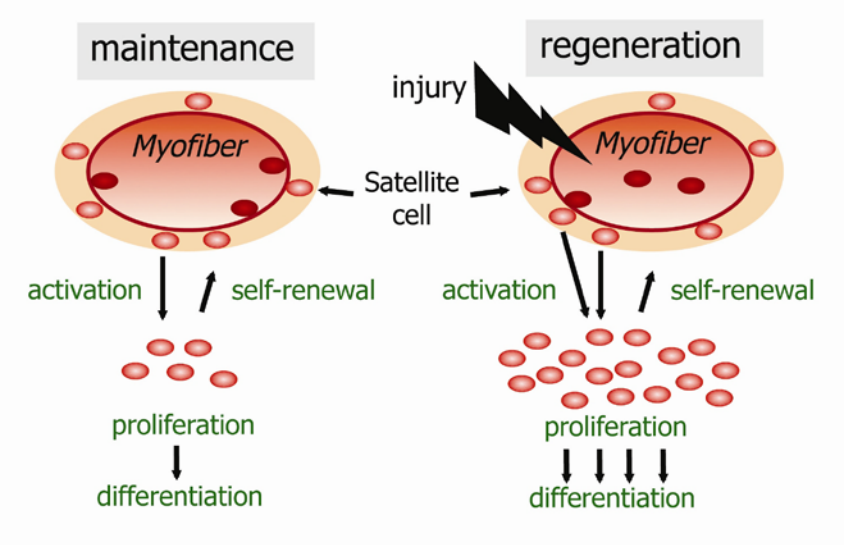

Figure 5.4.4: Satellite cell function during muscle maintenance and regeneration

Satellite cells are located at the periphery of myofibers in skeletal muscle and are collectively referred to as the satellite cell pool. During muscle maintenance a small percentage of quiescent satellite cells activate, proliferate and either undergo self-renewal to replenish the satellite cell pool or differentiate to participate in muscle maintenance. Muscle regeneration due to injury or growth activates a large number of quiescent satellite cells to undergo proliferation and self-renewal after which a large number of cells undergo differentiation to repair injured myofibers.

Figure 5.4.5: Satellite cell proliferation and number in *Kpna1*$^{+/-}$ mice during muscle regeneration

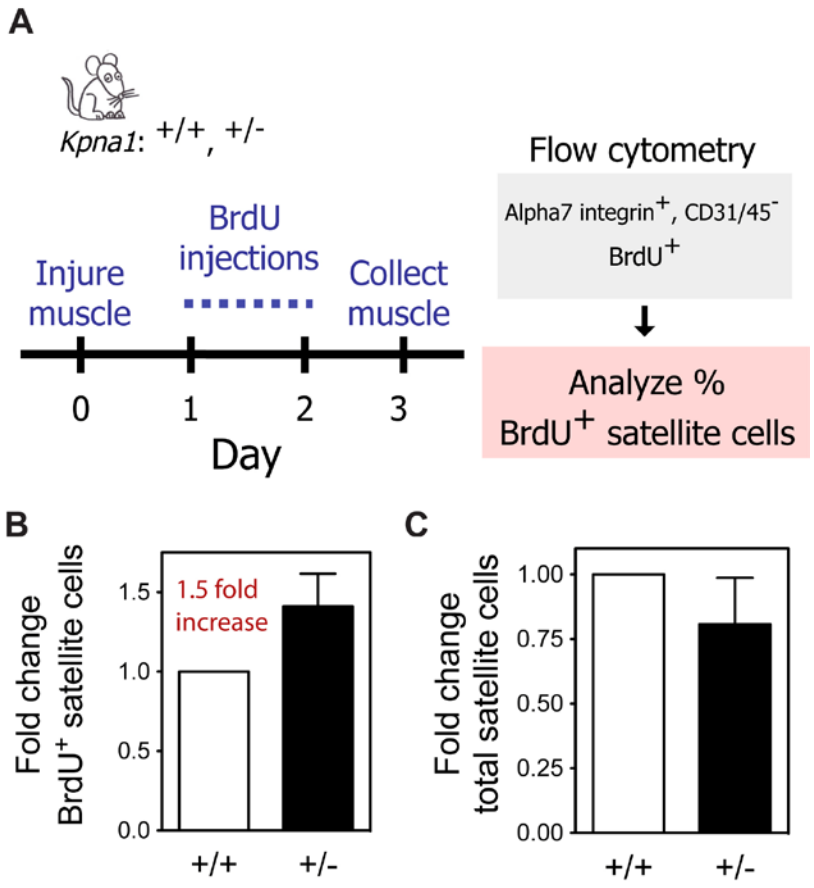

Figure 5.4.5: Satellite cell proliferation and number in *Kpna1*$^{+/-}$ mice during muscle regeneration

(A) Experimental schematic to analyze satellite cell number and proliferation in *Kpna1*$^{+/-}$ mice. Mononucleated cells were isolated from regenerating gastrocnemius muscle from *Kpna1*$^{+/-}$ or *Kpna1*$^{+/+}$ mice at 72 hours post injury with $BaCl_2$ and following two days of peritoneal injections with BrdU. Mononucleated cells were immunostained with antibodies against the cell markers CD31, CD45 and Alpha7 Integrin, along with antibodies to BrdU to analyze proliferating satellite cells via flow cytometry. **(B)** A 1.5-fold increase in the percentage of BrdU^{+} satellite cells was observed in *Kpna1*$^{+/-}$ muscles compared with *Kpna1*$^{+/+}$ muscles. **(C)** A ~20% decrease in the total number of satellite cells was observed in *Kpna1*$^{+/-}$ muscles compared with *Kpna1*$^{+/+}$ muscles. Satellite cells from four *Kpna1*$^{+/+}$ or *Kpna1*$^{+/-}$ mice were pooled at 72 hours post injury for flow cytometry analysis. Data are mean ± SEM from three independent experiments.

Figure 5.4.6: *Kpna1*$^{+/-}$ mice display a slight increase in myofiber size and centrally-located nuclei in myofibers during muscle regeneration

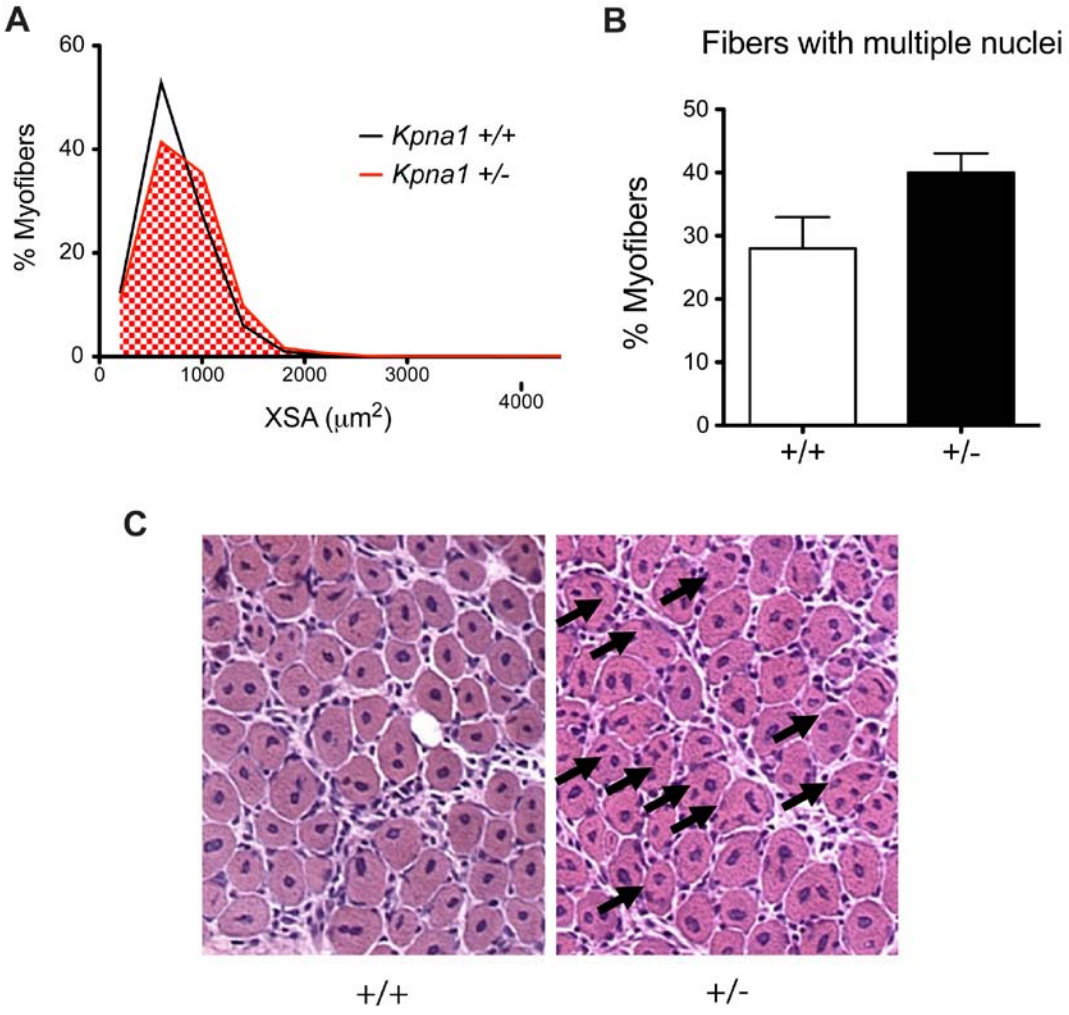

Figure 5.4.6: *Kpna1*$^{+/-}$ mice display a slight increase in myofiber cross-sectional area and more nuclei in myofibers during muscle regeneration

(A) A histogram shows the distribution of myofiber cross-sectional areas of muscles in *Kpna1*$^{+/-}$ and *Kpna1*$^{+/+}$ muscles. A slight shift to the right (larger myofiber XSA) was observed in *Kpna1*$^{+/+}$ muscles compared to *Kpna1*$^{+/+}$ muscles. **(B)** A ~40% increase in the number of myofibers with multiple centrally located nuclei was observed in regenerating *Kpna1*$^{+/-}$ (40%) muscles compared with *Kpna1*$^{+/+}$ muscles (28%). (C) *Kpna1*$^{+/+}$ and *Kpna1*$^{+/-}$ muscles stained with hematoxylin and eosin. Arrows indicate examples of centrally located nuclei in regenerating *Kpna1*$^{+/-}$ muscles. *Kpna1*$^{+/-}$ mice n=5, 1758 fibers; *Kpna1*$^{+/+}$ mice n=4, 1637 fibers.

Chapter 6: Discussion

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Chapter 6: Discussion

6.1 Introduction

Adult myogenesis is dependent on satellite cells that have the ability to proliferate, differentiate and fuse into multinucleated cells called myofibers. Satellite cells and myofibers require the coordinate activation and repression of a multitude of genes to maintain muscle function. Changes in gene expression during myogenesis involve the nuclear import of proteins, such as transcription factors, that enter the nucleus to function. While 35-55% of nuclear proteins are predicted to depend on classical nuclear import in *Mus musculus* (Marfori et al., 2010), what role classical nuclear import has in mono and multinucleated muscle cells in transporting nuclear proteins was unclear. The goal of this dissertation was to uncover the role of classical nuclear import and classical nuclear import receptors during skeletal myogenesis.

The first part of this dissertation focused on investigating the role of classical nuclear import and karyopherin alpha import receptors during myogenesis utilizing an *in vitro* myogenesis model system. The first step involved determining whether karyopherin alpha and karyopherin beta1 import receptors were expressed in skeletal muscle as these proteins are required for the classical nuclear import pathway. Data presented in Chapter 4 demonstrate that five of the six karyopherin alpha import receptors and karyopherin beta1 are expressed in mouse skeletal muscle *in vitro*. Chapter 4 studies also revealed that classical nuclear import is required during myogenesis and that two KPNAs have paralog-specific roles during myogenesis. We identified paralog-specific roles for KPNA1 and KPNA2 in myoblast proliferation, while KPNA2 alone had a role in myotube growth (Figure 6.7.1). These studies identify distinct transport

pathways that rely on specific KPNAs were individual paralogs may differentially import a subset of cNLS-containing proteins during myogenesis (Figure 6.7.2).

The second part of this dissertation focused on uncovering the role of classical nuclear import, in particular the KPNA1 import receptor, in satellite cell function. Chapter 5 studies determined that satellite cells express four karyopherin alpha paralogs, *Kpna2, Kpna3, Kpna4* and *Kpna6,* while *Kpna1* is expressed later in proliferating satellite cells. We determined a role for KPNA1 in satellite cells during muscle maintenance and regeneration *in vivo* in regulating satellite cell proliferation. Furthermore, the increase in satellite cell proliferation may result in an increase in myofiber size and the number of centrally located myonuclei observed within myofibers during muscle regeneration. Overall, the work presented in this dissertation identifies classical nuclear import and karyopherin alpha import receptors as having critical roles in regulating satellite cell function and myogenesis through the import of nuclear factors.

6.2 The Existence of Multiple Karyopherin Alpha Paralogs in Skeletal Muscle

We established that five karyopherin alpha paralogs are expressed by primary mouse myoblasts *in vitro* and that their steady-state levels increase during myogenesis which could suggest an increase in demand for classical nuclear import during differentiation. We would predict that KPNB1 steady-state levels would also increase with the changing demand for classical nuclear import during myogenesis, however, the steady-state levels of KPNB1 did not increase during myogenesis. Although KPNB1 is required for classical nuclear import, KPNB1 also functions as an import receptor in non-classical nuclear import pathways, therefore KPNB1 steady-state expression is not a

reliable indicator for surveying demand for classical nuclear import (Cook et al., 2007). Determining whether there is an increase in demand for classical nuclear import during myogenesis presents a great challenge because it would require quantitating the total number of cNLS-containing proteins as well as the total number of import events. Alternatively, global changes in the efficiency or rate of nuclear transport could be inferred through changes in the steady-state levels of key components of the nuclear transport machinery such as Ran and/or Ran-associated proteins or Nups within the NPC. In addition, an increase in NPC density and diameter could suggest an increase in demand for nuclear transport. During the differentiation of C2C12s, the steady-state levels of Nup proteins and overall density of NPCs remained constant (D'Angelo et al., 2009), however, whether this occurs during muscle cell differentiation *in vivo* is unknown.

We examined the cellular localization of KPNA1 and KPNA2 in mono and multinucleated muscle cells *in vitro* were KPNA1 and KPNA2 were detected in all muscle cells during myogenesis. While neither KPNA1 nor KPNA2 showed gross changes in intracellular localization over the course of muscle differentiation, KPNA2 appeared to be more highly concentrated in nuclei compared to KPNA1 at all stages of myogenesis, suggesting their roles in muscle cells may differ. Furthermore, we observed an increase in the fluorescence intensity of KPNA2 in certain nuclei within a myotube which suggests that KPNA2-dependent protein import may differ among individual nuclei within a common cytoplasm. Karyopherins localize to different nuclei in the binucleate *Tetrahymena thermophila* (Malone et al., 2008). Differences in KPNA paralog localization could allow for the differential import of proteins to individual

myonuclei within a myofiber and may contribute to transcriptional differences among nuclei within a myofiber (Newlands et al., 1998), such as nuclei localized at the neuromuscular synapse (Mejat et al., 2003) or myotendinous junction (Dix and Eisenberg, 1990). Questions remain as to whether classical nuclear import to different nuclei within a multinucleated myofiber varies depending on nuclear location within a myofiber or on the physiologic state of the muscle.

We examined the localization of the transcription factor NFATc2 in myotubes, following knockdown of individual KPNA paralogs since NFATc2 is dependent on classical nuclear import and has a known role in myogenesis. We predicted that there would be a decrease in NFATc2 nuclear localization following knockdown of individual KPNAs, however, surprisingly, we observed an increase in the nuclear localization of NFATc2 upon knockdown of KPNA2 and KPNA4, but not KPNA1. Although the defects in NFATc2 localization could be indirect due to the reduced import of other factors regulating NFATc2 localization, these data suggest that KPNAs have distinct roles in regulating the steady-state localization of NFATc2. Differences in the cellular localization of NFATc2 following knockdown of individual KPNA paralogs supports the notion that KPNA paralogs have paralog-specific roles during myogenesis. Overall, our study of cNLS-cargo NFATc2 illustrates the complexity of how nucleocytoplasmic transport of individual proteins is regulated in multinucleated muscle cells.

We examined the localization of myogenic regulatory factor myogenin and NF-κB subunit p65, in myotubes and mononucleated cells respectively, following knockdown of individual KPNA paralogs since these factors are dependent on classical nuclear import and have known roles in myogenesis. We did not observe a difference in

cellular localization of these cNLS cargoes, however, these proteins may enter the nucleus at specific times or under special physiological conditions or their export may be increased following RNAi. Functional assays directly studying cargo protein transport need to be performed to determine whether a cargo's cNLS sequence is functional as well as whether the cNLS is both necessary and sufficient for import of the cargo by the classical nuclear import machinery. Site mutagenesis studies should be performed to confirm the functionality of a cNLS sequence within a cargo protein. Meanwhile, *in vitro* transport assays can be performed to study whether a protein depends on classical nuclear import. In this assay, digitonin-permeabilized cells with intact nuclei are reconstituted with exogenous cytosol that may contain different combinations of import machinery proteins, such as different KPNA paralogs, along with the cargo protein of interest to determine the requirements for nuclear localization. Lastly, while our nuclear protein localization studies were performed using cultured muscle cells, future studies will need to be performed *in vivo* to determine the role of classical nuclear import receptors in nascent and mature myofibers. Questions remain as to how classical nuclear import and cNLS-dependent cargoes regulate the dynamic changes a multinucleated myofiber undergoes *in vivo* in response to skeletal muscle adaptation, aging and disease.

Increasing evidence suggests that classical nuclear import has a critical role in controlling the nuclear import of key proteins involved in cell proliferation and differentiation in a large number of non-muscle cell types and tissues (Kohler et al., 1999; Quensel et al., 2004; Talcott and Moore, 2000; Yasuhara et al., 2007). Furthermore, studies using genetic model organisms support the notion that individual KPNA paralogs have evolved distinct functions in different cell types during development (Geles and

Adam, 2001; Mason et al., 2002; Mason et al., 2003; Ratan et al., 2008). In Chapter 4, we provide evidence for KPNA paralog-specific roles in myoblasts where knockdown of KPNA1 led to enhanced proliferation of myoblasts, whereas KPNA2 knockdown decreased myoblast proliferation and no change was observed with KPNA4 knockdown. Meanwhile, we did not observe a defect in cell differentiation upon depletion of KPNA1, KPNA2 or KPNA4 suggesting that these paralogs have redundant roles in or are not required for differentiation. Overall, these studies implicate KPNA1 and KPNA2 in the import of cNLS-containing proteins involved in distinct signaling pathways that regulate myoblast proliferation. Contrary to these findings, in Hela cells, RNAi-mediated knockdown of each KPNA, including KPNA1, resulted in a decrease in cell proliferation, which suggests that the role of individual KPNAs in cell proliferation may differ between cell types (Kohler et al., 2002). Our results might suggest that KPNA1 imports a negative regulator of proliferation and KPNA2 imports a positive regulator of proliferation during myogenesis. However, the proliferation defects observed in both cases are likely the net effect of loss of import of both positive and negative regulators of proliferation following knockdown of either KPNA1 or KPNA2. KPNAs import regulators of proliferation, such as p21, Rb and $p27^{Kip1}$ in other cell types (Harper et al., 1993; Hu et al., 2005; Shin et al., 2005). Further studies are required to determine which cNLS-containing cargoes are responsible for the proliferation defects observed. Regardless, our data provide the first evidence that a KPNA paralog, specifically KPNA1, functions in a signaling pathway that negatively regulates proliferation. Overall, our results using muscle cells reveal that individual KPNAs participate in the positive or negative regulation of myoblast proliferation, a crucial step for generating adequate cell

numbers for proper formation and growth of multinucleated myotubes. Contrary to the results obtained *in vitro*, a recent study examining a *Kpna1* null mice revealed normal development of brain and other tissues, however, KPNA1 may have a different role in proliferation in the brain or compensation by other KPNAs may have occurred during development since the steady-state levels of KPNA4 were increased in this mouse model (Shmidt et al., 2007).

Further evidence for the paralog-specific roles of KPNAs in skeletal muscle comes from our studies of myogenesis *in vitro*. Myogenesis depends upon differentiation, migration, adhesion and fusion of myocytes with each other or existing myofibers *in vivo* or myotubes *in vitro*. While we did not observe a role for KPNA paralogs in regulating cell differentiation, we determined that classical nuclear import is required for proper myotube formation *in vitro*. In particular, KPNA2, but not KPNA1 or KPNA4, resulted in reduced myotube size and decreased ability of muscle cells to migrate. The small myotube phenotype observed following knockdown of KPNA2 may be due to the reduced import of multiple cNLS-containing proteins involved in regulating cell migration. Indeed, a similar migration defect was also observed upon loss of KPNA2 in a lung cancer cell line (Wang et al., 2010). Whether KPNA2 is required in other non-muscle cell populations for migration is unknown. Defects in cell-cell adhesion and fusion can also lead to reduced myotube size, however, we did not observe a defect in cell-cell adhesion following knockdown of KPNA2. Meanwhile, further studies are required to determine whether KPNA2 is also involved in regulating cell-cell fusion. While we studied the role of KPNA2 in regulating myocyte migration, whether KPNA2 is required *in vivo* for proper muscle cell migration is unknown. Satellite cell migration

studies *in vivo* following muscle transplants suggest satellite cells migrate from donor muscle grafts into host muscle to participate in muscle regeneration (Jockusch and Voigt, 2003; Schultz et al., 1988). Experiments to assess cell migration *in vivo* are technically challenging because it is difficult to visualize living cells within skeletal muscle. Recent advances in real-time imaging of satellite cell migration in rat skeletal muscle involve satellite cell-specific antibodies conjugated to quantum dots that unlike fluorescent proteins or dyes display intense brightness and stability allowing for enhanced single-cell visualization (Ishido and Kasuga, 2011). This new method could be used to assess the migration of satellite cells lacking KPNA2 in skeletal muscle. Uncovering the role of classical nuclear import in regulating satellite cell migration *in vivo* could further our understanding satellite cell migration in skeletal muscle homeostasis, regeneration and disease. In addition, furthering our knowledge of classical nuclear import could provide targets to enhance the migration of transplanted satellite cells which could increase the efficiency of satellite cell-based transplantation therapies.

While we studied the role of KPNA1, KPNA2 and KPNA4 in skeletal muscle, the role of KPNA3 and KPNA7 has not been investigated. One drawback for studying KPNA3 and KPNA7 is that antibodies recognizing these paralogs have not been generated to date. The development of a KPNA3 or KPNA6-specific antibody presents a challenge because these paralogs have a high level of protein sequence similarity with KPNA4 and KPNA1, respectively. In summary, the functional studies presented within the first part of this dissertation suggest that myoblast proliferation and myotube growth rely on specific KPNA paralogs to regulate the nuclear import of key factors involved in proliferation and myotube growth during myogenesis (Figures 6.7.1 and 6.7.2).

6.3 Classical Nuclear Import and Karyopherin Alphas Function in Satellite Cells

We established that quiescent satellite cells express four karyopherin alpha import receptors, *Kpna2, Kpna3, Kpna4* and *Kpna6,* while *Kpna1* is not expressed suggesting that *Kpna1* is not required in quiescent satellite cells. However, *Kpna1* mRNA and protein is expressed in proliferating satellite cells suggesting a role for KPNA1 in importing cNLS-containing proteins during satellite cell proliferation. The presence of multiple karyopherin paralogs in satellite cells suggests that, similar to myoblasts, individual KPNAs may have paralog-specific roles in classical nuclear import thereby regulating satellite cell function. Muscle regeneration requires a rapid response from satellite cells which need to activate, proliferate and differentiate to repair or grow muscle. Classical nuclear import may allow for a rapid response to muscle injury where the nuclear import of existing transcriptions factors from the cytoplasm would allow for more timely changes in satellite cell gene expression than the generation of new transcription factors.

Our detection of *Kpna2* and *Kpna3* mRNA in satellite cells is consistent with microarray studies investigating gene expression in satellite cells (Fukada et al., 2007; Pallafacchina et al., 2010). However, our study contrasts with expression of KPNAs in other progenitor cell populations such as sperm and neuronal progenitors where not all paralogs are present and the steady-state levels of particular KPNA paralogs increase, while that of others decrease during lineage progression (Hogarth et al., 2006; Yasuhara et al., 2007). While we characterized the steady-state mRNA levels of the *Kpna* paralogs in satellite cells, the presence and cellular localization of KPNA proteins in satellite cells

will need to be confirmed before further functional studies are performed. The detection of KPNA in single satellite cells would also reveal whether KPNA paralogs are expressed in a subset of satellite cells. The satellite cell pool is a heterogeneous population of cells with several proteins such as the Notch intracellular domain and its antagonist Numb being present in a subpopulation of satellite cells (Conboy and Rando, 2002). The heterogeneity of satellite cells is thought to correlate with their cell function and fate during myogenesis, such as whether a cell returns to quiescence or continues down the myogenic lineage to participate in myogenesis. Expression of individual KPNA paralogs in a subset of satellite cells could contribute to the functional heterogeneity of the satellite cell pool where specific paralogs import cargoes that dictate cell function. While we investigated the role of KPNA1 in satellite cell proliferation, further studies will be required to characterize the role of KPNA paralogs in regulating satellite cell fate.

The satellite cell is the primary cell required for generating skeletal muscle, the proliferation status of the satellite cell pool must be controlled throughout the adult lifespan to reserve progenitors for ongoing rounds of muscle growth and regeneration. Our studies of KPNA1 in satellite cells suggest that classical nuclear import may function in satellite cells to import nuclear factors involved in regulating satellite cell proliferation to prevent over-proliferation and potential exhaustion of the satellite cell pool during myogenesis (Figure 6.7.3). However, whether *Kpna1* mutant satellite cells are returning to quiescence both in young and aging skeletal muscle will need to be explored further. The enhanced proliferation phenotype we observed with *Kpna1*$^{+/-}$ and *Kpna1*$^{+/-}$ satellite cells is not a common phenotype in the skeletal muscle field. The enhanced proliferation phenotype could result in an accelerated depletion of the satellite cell pool that is often

observed in muscle dystrophies or with advanced age (Blau et al., 1983a; Blau et al., 1983b; Quinn et al., 1984; Webster and Blau, 1990). The reason for the decrease in satellite cell pool size during aging is poorly understood. In addition, the functional capacity of satellite cells is also diminished with age and several hypotheses exist to explain this including satellite cell replicative senescence and/or failure of the satellite cell niche to provide proper signals (Conboy et al., 2005; Decary et al., 1997; Gopinath and Rando, 2008; Kuang et al., 2008; Sacco et al., 2010). Understanding the role of classical nuclear import in satellite cell proliferation could shed light on the mechanisms involved in satellite cell pool exhaustion.

The regulation of proliferation in other stem cell populations, such as neural and hematopoietic stem cells is required for proper tissue function (Janzen et al., 2006; Li, 2011; Myatt and Lam, 2007; Yun et al., 2004). For example, hematopoietic progenitors in the spleen of $p27^{Kip1}$ null mice display enhanced proliferation resulting in hyperplasia of many hematopoietic associated organs (Fero et al., 1996; Kiyokawa et al., 1996) suggesting that $p27^{Kip1}$ has an essential role in negatively regulating precursor pool proliferation. While $p27^{Kip1}$ has been shown to interact with the nuclear import machinery to access the nucleus (Shin et al., 2005), it is unclear whether the reduced import of $p27^{Kip1}$ is responsible for the enhanced proliferation phenotype we observe in *Kpna1* mutant satellite cells. While the data presented in Chapter 5 suggest that the enhanced proliferation defect observed in $Kpna1^{+/-}$ muscle results in a surplus of progenitors that fuse into myofibers, satellite cell lineage tracing will need to be performed to definitively determine the fate of these satellite cells. In addition, whether cells are returning to quiescence over the course of muscle aging also requires further

investigation. Further studies of classical nuclear import and the nuclear import receptor KPNA1 in satellite cells cold offer critical insight into the regulation of proliferation in both muscle and non-muscle stem cell populations.

Whether multiple KPNA paralogs have a role in regulating satellite cell proliferation is unknown. Our *in vitro* studies in myoblasts suggest that KPNA2 has a role in positively regulating myoblast proliferation; however, further studies will be required to determine if KPNA2 has a role in positively regulating satellite cell proliferation. Lastly, identifying the cNLS-containing proteins responsible for the enhanced proliferation phenotype observed in *Kpna1*$^{+/-}$ satellite cells will be challenging and critical to fully understanding the role of KPNA1 in satellite cells. In summary, we demonstrate that classical nuclear import, in particular the KPNA1 import receptor, functions in satellite cells to negatively regulate proliferation to prevent over-proliferation and potential exhaustion of the satellite cell pool during muscle maintenance and regeneration.

6.4 Identifying Classical Nuclear Import Cargoes during Myogenesis

Myogenesis requires the activation and repression of many genes during the transition from quiescent satellite cell to mature multinucleated myofiber. Further advances in our understanding of how gene regulation is controlled in satellite cells and during myogenesis will better enable us to treat aging and diseased muscle. Myogenesis requires both muscle-specific and non-muscle regulatory factors that activate myogenic regulatory cascades in response to extrinsic factors such as growth factors and other signaling molecules (Kuang et al., 2008). Identifying the cNLS-containing proteins

responsible for the phenotypes observed upon depletion of KPNA1 or KPNA2 during myogenesis presents a great challenge. While redundancy between KPNA paralogs likely exists in importing many nuclear proteins, several proteins such as the transcription factor STAT1 and the RanGEF protein RCC1 are dependent on a single KPNA or two KPNAs for nuclear import (Fagerlund et al., 2002; Kohler et al., 1999). Whether specific cNLS-containing cargoes in skeletal muscle rely on several or a single karyopherin alpha paralogs for nuclear import will require further investigation. The proliferation and migration phenotypes we observed are likely due to the altered nuclear import of many cNLS-containing proteins involved in regulating a multitude of genes required for proper myogenesis. While a few nuclear proteins required for myogenesis have a characterized functional cNLS, such as Notch (Huenniger et al., 2010), and NFATc2 (Okamura et al., 2000), the vast majority of cNLS proteins have not been analyzed for their dependency upon specific KPNAs for nuclear import. In *Mus musculus*, ~35-55% of nuclear proteins are predicted to use classical nuclear import (Marfori et al., 2010), however, the contribution of the "cNLS importome" to the total nuclear proteome in muscle or other mammalian cells is unknown. Cataloging the "cNLS importome" for skeletal muscle and other cell types will be critical to revealing the prevalence of classical nuclear import and defining the role of individual KPNA paralogs in regulating cell fate and function.

Therapeutic Strategies

Therapeutic strategies to treat failing muscle could extend lifespan or alleviate medical conditions associated with disease or aging. Age-related changes in the nuclear transport machinery have been observed in myocardial microvascular endothelial cells

and human fibroblasts where a reduction in KPNA expression resulted in a decrease in classical nuclear import (Ahluwalia et al., 2010; Pujol et al., 2002). A global decrease in classical nuclear import and possible alteration in gene expression could be partially responsible for the decrease in regenerative capacity that occurs in many tissues during aging. Better understanding the contribution of classical nuclear import in the regulation of gene expression in skeletal muscle could provide new targets to control or halt the loss of muscle mass that occurs with aging.

Therapeutic strategies involving satellite cell transplantation to treat muscle dystrophies are inefficient due to problems associated with host immune rejection and the altered function of transplanted satellite cells such as loss of myogenic potential, poor cell migration and cell survival (Kuang and Rudnicki, 2008). The genetic reprogramming that occurs during satellite cell proliferation, migration and survival likely involve the nuclear import of proteins that depend on facilitated nuclear import. However, the number of classical nuclear import-dependent cargoes required for proper satellite cell function is unknown. The genetic reprogramming of fibroblasts to iPS cells (induced pluripotent stem cells) can occur with the ectopic expression of only four transcription factors, Oct3/4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006), all of which utilize karyopherin nuclear transport receptors to access the nucleus (Gontan et al., 2009; Li et al., 2008; Makkerh et al., 1996; Quadrini and Bieker, 2002; Shields and Yang, 1997; Welch et al., 1999; Yasuhara et al., 2007). However, the targeting of cNLS-containing transcription factors in satellite cells to reprogram satellite cells to enhance their proliferative or self-renewal capacity has not been studied. Understanding the role of classical nuclear import in regulating satellite cell gene expression could allow for the

genetic reprogramming of satellite cells to enhance their capacity to regenerate diseased or aging muscle.

Satellite cell transplant strategies involving the injection of a larger number of satellite cells is thought to decrease the efficiency of transplantation because a larger number of cells depletes the availability of oxygen and nutrients (Kuang and Rudnicki, 2008). A solution to this problem would be to transplant a smaller number of cells that have an enhanced proliferation and self-renewal capacity which would result in a large number of progeny to participate in regeneration as well as replenishment of the satellite cell pool (Kuang and Rudnicki, 2008). Further characterizing the role of import receptor KPNA1 in the enhanced proliferation phenotype of *Kpna*$^{+/-}$ *and Kpna1*$^{-/-}$ satellite cells could provide targets to improve satellite cell transplantation efficiency via the enhanced proliferation capacity of transplanted cells. In addition, uncovering the role of KPNA1 in satellite cell self-renewal could also provide targets to enhance the self-renewal capacity of transplanted cells. A combination of an enhanced proliferation and self-renewal could make for a "super" satellite cell that upon transplantation could efficiently generate a large number of progeny for muscle regeneration while efficiently replenishing the satellite cell pool. Overall, uncovering the role of classical nuclear import may provide molecular targets that could allow for improvement in satellite cell-based therapies to treat muscular dystrophies or prevent the loss of muscle mass during aging.

Summary

The work presented in this dissertation demonstrates that classical nuclear import is a critical regulatory pathway in skeletal muscle. Karyopherin alpha paralogs and

karyopherin beta1 which are required for the classical nuclear import pathway are expressed in satellite cells and during myogenesis. In addition, we have elucidated paralog-specific roles for KPNA1 and KPNA2 during myogenesis, where KPNA1 and KPNA2 have differing roles in myoblast proliferation, while KPNA2 alone regulates myotube growth (Figure 6.7.1). Lastly, we identified a functional role for KPNA1 in regulating satellite cell proliferation (Figure 6.7.3). The work presented here lays the groundwork for unraveling the further complexities relating to classical nuclear import that exist both in satellite cells and in multinucleated myofibers.

6.7 Figures

Figure 6.7.1: KPNA1 and KPNA2 have paralog-specific roles during myogenesis

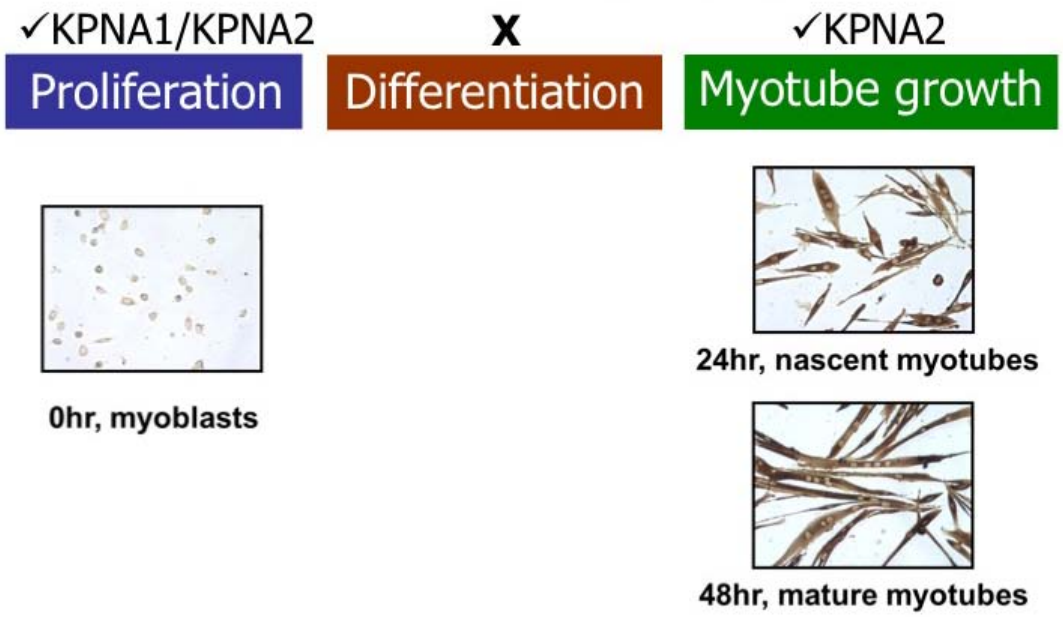

Figure 6.7.1: KPNA1 and KPNA2 have paralog-specific roles during myogenesis

KPNA paralogs display paralog-specific roles in regulating distinct pathways involved in myoblast proliferation and myotube formation, but not differentiation. These studies suggest that KPNA1 and KPNA2 transport critical cargo proteins required for different stages of myogenesis represented in phase images of 0, 24 and 48 hours of differentiation and that classical nuclear import likely has a key role in controlling gene expression in skeletal muscle.

Figure 6.7.2: KPNA1 and KPNA2 may differentially import a subset of cNLS-containing proteins during myogenesis

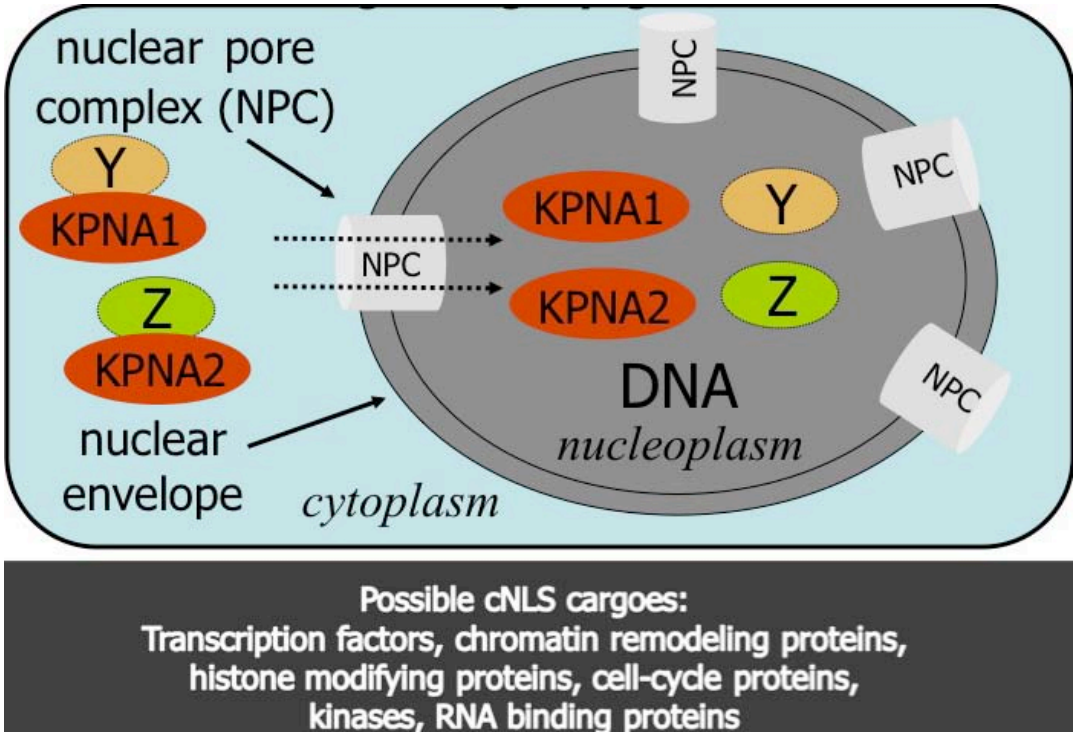

Figure 6.7.2: KPNA1 and KPNA2 may differentially import a subset of cNLS-containing proteins during myogenesis

Different cNLS-containing cargoes (X and Y) may rely on specific KPNAs, such as KPNA1 or KPNA2, for facilitated import from the cytoplasm to the nucleus, through nuclear pore complexes embedded within the nuclear envelope during myogenesis. A variety of possible cNLS cargoes may depend on individual karyopherin import receptors for import during myogenesis.

Figure 6.7.3: KPNA1 expression is induced in satellite cells to control cell proliferation during muscle maintenance and regeneration

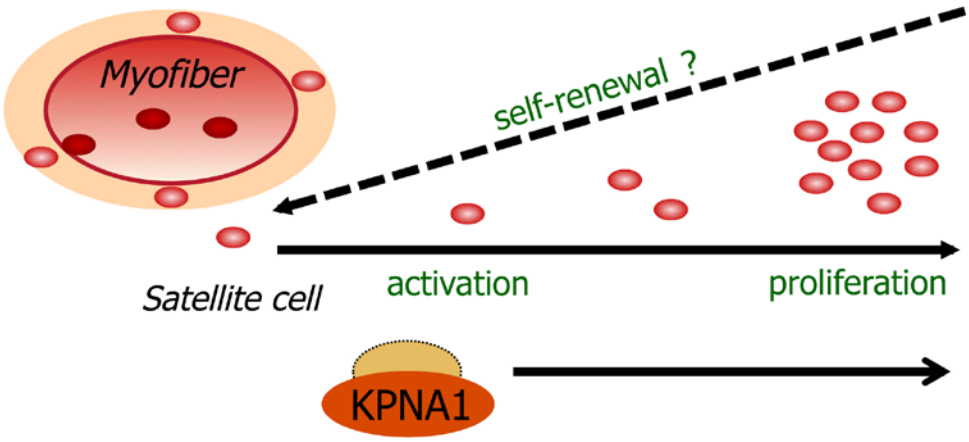

Figure 6.7.3: KPNA1 expression is induced in satellite cells to control cell proliferation during muscle maintenance and regeneration

Satellite cells are located at the periphery of myofibers in skeletal muscle. During muscle maintenance or regeneration, satellite cells activate, proliferate or undergo self-renewal to replenish the satellite cell pool. KPNA1 expression is induced in satellite cells to control cell proliferation during muscle maintenance and regeneration. Whether KPNA1 has a role in regulating satellite cell self-renewal is unknown.

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