**Distribution Agreement**

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Adriana Simionescu Bankston Date

Regulation of Myoblast Fusion by Creatine Kinase B and its Interacting Partners

By

Adriana Simionescu Bankston

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Biochemistry, Cell and Developmental Biology

_____

Grace K. Pavlath, Ph.D.

Advisor

_____ _____

Michael H. Koval, Ph.D. Andrew P. Kowalczyk, Ph.D.

Committee Member Committee Member

_____ _____

Asma Nusrat, M.D. Winfield S. Sale, Ph.D.

Committee Member Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.

Dean of the James T. Laney School of Graduate Studies

_____

Date

# Regulation of Myoblast Fusion by Creatine Kinase B and its Interacting Partners

By

Adriana Simionescu Bankston

B.S., Clemson University, 2005

Advisor: Grace K. Pavlath, Ph.D.

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Biochemistry, Cell and Developmental Biology

Graduate Division of Biological and Biomedical Sciences

2013

Abstract

Regulation of Myoblast Fusion by Creatine Kinase B and its Interacting Partners

By Adriana Simionescu Bankston

Myoblast fusion is critical for proper muscle growth and regeneration. During myoblast fusion, the localization of some molecules is spatially restricted, however the exact reason for such localization is unknown. Creatine kinase B (CKB), which replenishes local ATP pools, localizes near myotube ends. To gain insights into the function of CKB at the ends of myotubes, we sought to identify CKB interacting proteins using a yeast-two hybrid screen. We identified molecules with a broad diversity of roles, including actin polymerization. Given the importance of actin polymerization for myoblast fusion, we focused on the interaction between CKB and various actin isoforms, as well as actin regulatory proteins. Using co-immunoprecipitation, we identified α-skeletal-actin and α-cardiac-actin, two predominant skeletal muscle actin isoforms, as novel CKB interacting partners, which also colocalized with CKB in cultured mouse myotubes. Importantly, inhibition of CK activity by cyclocreatine treatment led to depolymerized F-actin in myotubes, as well as reduced myotube size and number, suggesting that CKB may be an essential factor for myotube formation by interacting with and modulating the actin cytoskeleton. However, CKB did not directly interact with α-skeletal-actin by co-sedimentation, indicating that intermediary proteins likely mediate the interaction between CKB and the actin cytoskeleton. CKB could therefore regulate actin dynamics indirectly via actin regulatory proteins, such as the N-BAR domain protein, Bridging integrator 3 (Bin3), which we also identified as a CKB interacting partner. We found that Bin3 regulated myofiber size *in vitro* and *in vivo*, as well as migration of differentiated muscle cells, where it colocalized with F-actin in lamellipodia. In addition, Bin3 formed a complex with Rac1 and Cdc42, Rho GTPases involved in actin polymerization, and regulated their activity in differentiated muscle cells. Overall, these results suggests a Bin3-dependent pathway is a major regulator of actin dynamics in differentiated muscle cells, which may, in turn, be modulated by the ability of CKB to provide ATP for actin polymerization.

# Regulation of Myoblast Fusion by Creatine Kinase B and its Interacting Partners

By

Adriana Simionescu Bankston

B.S., Clemson University, 2005

Advisor: Grace K. Pavlath, Ph.D.

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Biochemistry, Cell and Developmental Biology

Graduate Division of Biological and Biomedical Sciences

2013

**Acknowledgements**

I would like to thank my advisor, Dr. Grace K. Pavlath, for training me to develop into the scientist I am today. The skills I have gained in the Pavlath lab will be invaluable in my scientific career. Thank you for being a great leader, an inspiration, and a great friend. Thank you also to the past and current members of the Pavlath lab for your help and support, and for sharing in my successes. I am grateful to have found such wonderful friends and coworkers among the Pavlath lab members.

I am also appreciative to the many faculty members whom I have worked with at Emory, particularly Anita Corbett, Andrew Kowalczyk, Doug Falls, and Victor Faundez. Thank you to the members of my thesis committee for all their help and support, I am very lucky to have been guided by such a wonderful group of people. I also greatly appreciate the collaborations with the Faundez and Nusrat laboratories.

I thank my parents for the sacrifices they made to allow me to pursue my education, as well as for their guidance, love and encouragement throughout the years. Thank you also to my grandmother, uncle and aunt, for always encouraging me to push forward. Thank you to my in-laws and brother-in-law, and the rest of the Bankston family, for your continuous love and support. To my graduate school friends, as well as old friends, thank you for being there for me during this entire process.

Finally, I am grateful to my wonderful husband, Andrew, who taught me to persevere when things seemed hopeless, never give up and always hope for the best. I would not be here today without your continuous love, patience, and understanding. Your belief in my abilities as a person and as a scientist has allowed me to accomplish greater things than I ever thought possible.

## Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

Acta1 - Alpha-actin-1

Arhgap23 - Rho GTPase-activating protein 23

Arp2/3 - Actin-related protein-2/3

BAR - Bin-Amphiphysin-Rvs

BAR-PH - BAR-Pleckstrin Homology

Bin1/2/3 - Bridging integrator 1/2/3

Cdc42 - Cell division control protein 42

CKB - Creatine kinase, Brain

CKM - Creatine kinase, Muscle

CSA - Cross-sectional area

DAPI - 4’,6-diamidino-2-phenylindole

DM - Differentiation media

ECL - Entactin-collagen IV-laminin

eMyHc - Embryonic myosin heavy chain

F-actin - Filamentous actin

F-BAR - Fes/CIP4 Homology-BAR/FCH-BAR

FGF - Fibroblast growth factor

FITC - Fluorescein isothiocyanate

G-actin - Globular actin

GAP - GTPase-activating protein

GEF - Guanine nucleotide exchange factor

Ggnbp2 - Gametogenetin binding protein 2

GM - Growth media

GRAF1 - GTPase regulator associated with focal adhesion kinase-1

HA - Human influenza hemagglutinin

Hob1/3p - Homolog of Bin1/3 protein

Hsbp3 - Heat shock 27 kD protein 3

I-BAR - Inverse-BAR/IMD-BAR/IRSp53-MIM Homology domain

KO - Knockout

Mbnl3 - Muscleblind-like 3 (Drosophila)

Mc - Myocyte

MOR23 - Mouse odorant receptor 23

MR - Mannose receptor

Mt - Myotube

N-BAR - N-terminal amphipathic helix-BAR

N-WASP - Neural Wiskott-Aldrich syndrome protein

Nap1 - Nck-associated protein 1

PAK1 - p21-activated protein kinase 1

PBD - p21-binding domain

PCr - Phosphocreatine

PX-BAR - PhoX-BAR

Rac1 - Ras-related C3 botulinum toxin substrate 1

RV - Retrovirus

Rvs161/167p - Reduced viability upon starvation 161/167 protein

Sept8 - Septin-8

SH3 - Src homology 3

TA - Tibialis anterior

Tmed10 - Transmembrane emp24-like trafficking protein 10 (yeast)

TSA - Tyramide signal amplification

VASP - Vasodilator-stimulated phosphoprotein

WASP - Wiskott-Aldrich syndrome protein

WAVE - WASP-family verprolin-homologous protein

WT - Wild-type

X-gal - 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

# Chapter 1: Introduction

## Chapter 1: Introduction

Skeletal muscle myogenesis is fundamental to muscle growth and regeneration. During myogenesis, satellite cells, the adult muscle stem cells, are mitotically quiescent. In response to trauma or muscle injury, however, satellite cells proliferate, giving rise to progeny myoblasts. Myoblasts then differentiate into myocytes, which migrate, adhere and fuse to one another or to existing myofibers to restore the muscle tissue architecture (Relaix and Zammit 2012). *In vitro* studies have allowed researchers to mimic to muscle regeneration process in the absence of other cell types. In this context, satellite cells proliferate, differentiate, migrate, adhere to one another and fuse, giving rise to nascent myotubes *in vitro*, which are small and have few nuclei; after subsequent rounds of fusion, mature myotubes form, which are large and have many nuclei (Abmayr and Pavlath 2012). Migration and fusion, two major points of focus in this dissertation, are essential for both muscle growth and repair. Thus, identifying the molecules that regulate these two processes can provide insights into the fundamental mechanisms of myogenesis, as well as provide avenues for therapeutic strategies to improve muscle growth and regeneration following injury.

Myotube formation requires extensive membrane and actin dynamics. In this dissertation, we concentrated on identifying novel regulators of myotube formation among molecules controlling actin dynamics. Therefore, **the central goal of this dissertation was to discover novel proteins involved in myotube formation through regulation of actin polymerization.** Actin polymerization, which is crucial for myotube formation, is an ATP-dependent process (Lodish et al. 2000). Creatine kinase (CK) enzymes replenish the local ATP pool (Wyss and Kaddurah-Daouk 2000). Previous data

show CK may be important for myotube formation (O'Connor et al. 2008). To define the mechanisms by which CK may contribute to myotube formation, **our first goal was to find novel CK interacting proteins**. We hypothesized that CK may provide ATP for actin polymerization during myotube formation. However, a direct connection between CK and actin dynamics during myogenesis has not been previously established. Our data identify α-skeletal-actin and α-cardiac-actin, two predominant skeletal muscle-specific actin isoforms, as novel binding partners of the cytosolic CK enzyme brain-type creatine kinase (CKB) (Simionescu-Bankston, et al., unpublished). Additionally, we show the activity of cytosolic CK enzymes is necessary for regulating actin polymerization in muscle cells (Simionescu-Bankston, et al., unpublished). **Chapter 4 outlines CKB as a regulator of ATP-dependent actin polymerization during myotube formation**.

During our studies of CKB interacting proteins, we identified the N-BAR domain protein Bridging integrator 3 (Bin3). BAR domain proteins regulate membrane and actin dynamics (Frost et al. 2009, Ren et al. 2006), likely ATP-dependent processes. Thus, CKB could provide ATP for Bin3-dependent actin regulation during myotube formation. Therefore, **our second goal was to identify the role of Bin3 in myogenesis**. We show Bin3 is a novel regulator of myofiber size and branching *in vivo* (Simionescu-Bankston et al. 2013). Although the exact mechanism regulating myofiber branching is unknown, migration and fusion are likely to contribute. Our *in vitro* studies further suggested that Bin3 is involved in migration of differentiated muscle cells, as well as in myotube formation (Simionescu-Bankston et al. 2013). Thus, **our third goal was to determine the mechanism of Bin3 action in muscle cells**. Our findings indicated Bin3 is involved in both muscle cell migration and formation of lamellipodia, which are actin-based

protrusions associated with motility (Simionescu-Bankston et al. 2013). Importantly, our results further suggest a Bin3-dependent pathway is a novel regulator of Ras-related C3 botulinum toxin substrate 1 (Rac1) and Cell division control protein 42 (Cdc42) activity in differentiated muscle cells (Simionescu-Bankston et al. 2013).As Rac1 and Cdc42 are small GTPases associated with myotube formation and actin polymerization, Bin3 may drive actin-dependent processes during myogenesis in a small GTPase-dependent manner. **Chapter 5 outlines Bin3 as a regulator of myotube formation by controlling actin polymerization via small GTPases in muscle cells.**

The research presented in this dissertation identifies CKB and its interacting partners, α-skeletal-actin, α-cardiac-actin and Bin3, as novel regulators of myotube formation. These findings suggest CKB may provide ATP for α-skeletal-actin, α-cardiac-actin and/or Bin3-dependent regulation of muscle cell migration and fusion. Overall, our data provide insights into the regulation of myotube formation via a CKB-dependent pathway. This work could have important implications in finding therapies for muscle diseases where myotube formation is greatly impaired.

# Chapter 2: Background and Significance

**A portion of this chapter is published as:**

**Simionescu**, **A.** and Pavlath, G.K. (2013) Molecular mechanisms of myoblast fusion across species. *Adv. Exp. Med. Biol.* 713:113-35.

# Chapter 2: Background and Significance

## 2.1 Skeletal Muscle

Skeletal muscle is a complex and highly organized tissue that comprises 40-50% of total body weight, and is critical for locomotion, breathing and posture (Powers and Howley 1997). Skeletal muscle is susceptible to injury after direct trauma, or resulting from neurological dysfunction or innate genetic defects. These injuries may lead to loss of muscle mass, locomotive deficiency, and in the worst cases lethality. However, adult skeletal muscle possesses remarkable regenerative capacity. Upon muscle injury, a set of cellular responses is activated, resulting in the regeneration of a well-innervated, fully vascularized, and contractile muscle apparatus (Charge and Rudnicki 2004). Thus, understanding the mechanisms of skeletal muscle repair is critical for developing therapeutic strategies for muscle diseases.

### 2.1.1 Skeletal Muscle Structure and Function

Each skeletal muscle is tethered to skeletal bone and surrounded by a layer of connective tissue called the epimysium. Muscles are composed of fascicles/bundles of muscle fibers, or myofibers, which are multinucleated muscle cells (Fig. 2.6.1). A tough connective tissue layer known as the perimysium surrounds each fasciculus, and it is through this layer that nerves and blood vessels are found. While the nerves are involved in muscle contraction as described later, the extensive vascularization of the muscle tissue provides essential nutrients for muscle function. Each muscle fiber is surrounded by a plasma membrane, the sarcolemma, largely composed of phospholipids and some cholesterol. Outside the sarcolemma surrounding each myofiber is the endomysium, also

called the basal lamina (Fig. 2.6.1), another layer of connective tissue acting as a scaffold for muscle fiber formation and recovery from injury. The role of these connective tissue layers is transmission of force from the muscle fibers to the tendons (Chromiak and Antonio 2008), transforming myofiber contraction into movement.

Muscle contraction is initiated by motor neurons extending outward from the spinal cord and innervating individual muscle fibers at the neuromuscular junction, followed by acetylcholine (Ach) stimulation of the muscle fiber to depolarize (Chromiak and Antonio 2008), a signal to start the contractile process. Importantly, $Na^+$ and $K^+$ channels enable to sarcolemma to conduct a change in electric potential and therefore propagate an action potential down the muscle fiber for contraction (Chromiak and Antonio 2008). Each myofiber expresses characteristic molecules for contractile function, such as different myosin heavy chain (MHC) isoforms and metabolic enzymes, however the precise mechanisms defining myofiber contractile properties remain to be defined (Wigmore and Evans 2002). Individual skeletal muscles are composed of a mixture of myofibers with different physiological properties, ranging from a slow-contracting/ fatigue-resistant type to a fast-contracting/non-fatigue-resistant type (Charge and Rudnicki 2004, Chromiak and Antonio 2008), and the proportion of each fiber type within a muscle determines its overall contractile property.

Myofibers are composed of hundreds of parallel myofibrils, which are long, cylindrical filaments extending the length of the muscle fiber. Myofibrils are the largest functional unit of the myofiber, and are composed of sarcomeres, lined up head-to-head in the myofibril. The sarcomere is the smallest functional unit of the myofiber, composed of actin (part of the thin filament) and myosin (major component of thick filaments), as

well as many cytoskeletal proteins. The highly organized arrangement of thick and thin filaments gives myofibers their striated appearance of dark and light bands. Sarcomeres, arranged in parallel in adjacent myofibrils, extent from one Z line to another, and the number of sarcomeres within a muscle fiber is related to the capacity of the myofiber to produce force (Chromiak and Antonio 2008). Overall, the functional properties of skeletal muscle depend on the maintenance of a complex framework of myofibers, motor neurons, blood vessels, and extracellular tissue matrix.

### 2.1.2 Skeletal Muscle Regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei (Decary et al. 1997). In a normal adult rat muscle, no more than 1-2% of myonuclei are replaced every week (Schmalbruch and Lewis 2000). However, in response to severe damage, skeletal muscle regenerates, giving rise to a large number of new myofibers within a few days (Bintliff and Walker 1960, LeGros Clark 1946). Since myofibers contain post-mitotic myonuclei, nuclei from outside the myofiber are necessary for muscle growth and repair (Allen et al. 1999). Satellite cells, the adult muscle stem cells, repair the muscle after injury (Relaix and Zammit 2012). After their initial identification in 1961 (Katz 1961), satellite cells were given their name due to the close association with the periphery of the frog myofiber (Mauro 1961). Satellite cells are primarily quiescent, dividing very infrequently under normal conditions in the adult. Quiescent satellite cells (Figs 2.6.1, 2.6.2) are situated in indentations between the sarcolemma and the basal lamina (Muir et al. 1965), and remain associated with the surface of the developing myofiber during muscle development (Charge and Rudnicki 2004). Following

activation by various mechanisms (Le Grand and Rudnicki 2007), satellite cells move outside the basal lamina, proliferate and their progeny (Fig. 2.6.2) progress down a myogenic lineage (Hawke and Garry 2001, Schultz and McCormick 1994). Therefore, activated satellite cells are essential for postnatal muscle growth and repair (Bischoff 1994, Relaix and Zammit 2012, Seale and Rudnicki 2000). Following activation, a subset of satellite cell progeny returns to the quiescent state to maintain the satellite cell pool (Dhawan and Rando 2005) for future rounds of regeneration (Fig. 2.6.2).

Muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase. In the degenerative phase, a disruption of the myofiber sarcolemma, as well as myofiber necrosis, take place (Charge and Rudnicki 2004). Subsequently, an immune response ensues, in which factors released by the injured muscle provide chemotactic signals for circulating inflammatory cells (Rappolee and Werb 1992, Tidball 1995). Neutrophils invade the injured muscle first (Fielding et al. 1993, Orimo et al. 1991), followed by macrophages, which clear debris (Arnold et al. 2007, Tidball and Villalta 2010); this immune response is critical for proper muscle regeneration (Arnold et al. 2007, Grounds 1987). In the regenerative phase, quiescent satellite cells re-enter the cycle and proliferate, differentiate, migrate, adhere and fuse with one another (Fig. 2.6.2) or with existing myofibers (Papadimitriou et al. 1990, Robertson et al. 1990, Robertson et al. 1993b). Subsequently, small myotubes fuse with one another (Robertson et al. 1990) and with the stumps of the parent myofiber (Papadimitriou et al. 1990, Robertson et al. 1993b), eventually giving rise to the fully regenerated myofiber (Fig. 2.6.2).

### 2.1.2.1 Branched Myofibers During Myogenesis

Branched myofibers, more commonly known as "split myofibers," constitute an abnormal regeneration phenotype, in which new myotubes are formed within the old basal lamina following myofiber necrosis. Ultimately, each new myotube becomes enclosed in its own basal lamina, and the old basal lamina is lost; as a result, the basal lamina of the parent myofiber is contiguous with that of several smaller daughter myofibers (Bourke and Ontell 1984, Ontell 1986, Ontell and Feng 1981). Myofiber branching can be either simple, with one branch, or complex, with many branches forming an "anastomosing syncytial reticulum" (Isaacs et al. 1973, Tamaki and Akatsuka 1994, Tamaki et al. 1997, Tamaki et al. 1993). The patterns of myofiber branching reported in the literature (Fig. 2.6.3) are bifurcations at one end of the myofiber (bifurcated-end), a single narrow branch (process), or an opening in the center (split) (Lovering et al. 2009). Although there are occasional reports of branched myofibers in normal muscles of the chicken (Gollnick et al. 1983) and rat (Gollnick et al. 1981), the occurrence of myofiber branching under these conditions is extremely low (Tamaki et al. 1992) or even absent in some muscles (Gollnick et al. 1983). Myofiber branching was initially described in the literature about 100 years ago as longitudinal fiber division, commonly occurring in muscular dystrophy (Durante 1902, Erb 1891), and was later shown to be elevated in response to age (Bockhold et al. 1998), injury (Hall-Craggs 1972, Tamaki and Akatsuka 1994, Tamaki et al. 1997), hypertrophy (Hall-Craggs 1970, Tamaki et al. 1996), and muscle transplantation (Bourke and Ontell 1984, Ontell et al. 1982). Subsequent reports of myofiber branching in muscular dystrophy also followed (Chan et al. 2007, Head et al. 1992, Isaacs et al. 1973, Lovering et al. 2009, Ontell and

Feng 1981, Schmalbruch 1984, Tamaki et al. 1993), with dystrophic muscle fibers being weaker and more prone to injury than myofibers from non-dystrophic muscles (Chan et al. 2007, Head et al. 1992). More recently, branched myofibers were shown conclusively to be regenerating myofibers, by the presence of centrally located nuclei (Chan et al. 2007, Griffin et al. 2009). In spite of the detrimental effect of branched myofibers on muscle function, the mechanisms involved in the branching process are not well understood. Satellite cells migrate along the growing myofiber to be added to the growing tip (Hurme et al. 1991). In addition, mouse odorant receptor 23 (MOR23) (Griffin et al. 2009, Pavlath 2010a), the first molecule found to regulate myofiber branching during regeneration, controls *in vitro* muscle cell migration and cell-cell adhesion (Griffin et al. 2009). These studies suggest aberrant migration and/or adhesion may be likely mechanisms for myofiber branching. Alternatively, fusion defects have also been proposed to lead to myofiber branching (Schmalbruch 1976), however this hypothesis has not been thoroughly tested. In addition, whether branched myofibers arise due to the longitudinal myofiber splitting into several small myofibers (Schwartz et al. 1976), or alternatively due to incomplete lateral fusion of myotubes within the same basal lamina (Ontell 1986, Schmalbruch 1976, Schmalbruch 1984) is unknown.

### 2.2 Migration and Fusion During Myogenesis

Muscle growth and repair involve a series of ordered steps, including migration and fusion, which are two major points of focus in this dissertation. These two processes, which are critical for proper myogenesis, are also highly dependent on actin remodeling. Therefore, migration and fusion during myogenesis will be described in detail in the

context of muscle growth and repair, with a focus on the molecules controlling actin dynamics.

### 2.2.1 Factors Controlling Muscle Cell Migration

Myogenic cell migration is a pre-requisite for postlesional muscle regeneration (Bischoff 1997). A variety of muscle trauma conditions stimulate migration of myogenic cells including ischemia (Phillips et al. 1987, Schultz et al. 1988), thermal injury (Morgan et al. 1987, Phillips et al. 1990), crushing (Schultz et al. 1985, Watt et al. 1994), and exposure to snake venom toxin (Klein-Ogus and Harris 1983). Localized muscle trauma produces factors that stimulate satellite cell chemotaxis towards the site of injury (Schultz et al. 1985, Watt et al. 1994). Macrophages produce growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor β (TGF-β), potent chemoattractants for myoblasts (Table 2.6.1), leading them to eventually fuse to one another into myofibers (Robertson et al. 1993a). In addition, cytokines and chemokines may also play a role in this process (Robertson et al. 1993a). While satellite cells can migrate within the muscle (Klein-Ogus and Harris 1983, Phillips et al. 1990, Phillips et al. 1987, Schultz et al. 1988, Schultz et al. 1985, Watt et al. 1994) or between adjacent muscles (Watt et al. 1987), very little is known about the molecules involved in regulating migration of muscle cells during regeneration.

Much of the current knowledge about chemotactic factors for muscle cells comes from *in vitro* studies. During *in vitro* myogenesis (Fig. 2.6.4), satellite cells give rise to progeny myoblasts, which migrate and differentiate into myocytes. Myocytes, in turn, also migrate, adhere and fuse, giving rise to myotubes in two distinct stages of fusion

(Abmayr and Pavlath 2012). Muscle cell migration is necessary to achieve cell–cell contact during myogenesis, which is required both to trigger differentiation (Krauss et al. 2005) and to allow myocytes to fuse *in vitro*. Some regulatory factors that influence myoblast migration modulate the velocity or direction of cell migration, whereas others regulate the clearance of extracellular matrix at the leading edge of migrating cells, thus facilitating cell motility (Griffin et al. 2009, Horsley et al. 2003, Jansen and Pavlath 2006, Lafreniere et al. 2006). Myotube formation and growth *in vitro* are enhanced by both positive and negative regulators of cell migration (Simionescu and Pavlath 2011). Potent chemoattractants for proliferating myoblasts *in vitro* include extracellular factors (growth factors, chemokines, cytokines), cell surface proteins (laminins, integrins) and transmembrane proteins (various receptors) (Table 2.6.1). Negative regulators of myoblast migration identified to date, which comprise only cell surface proteins, are much fewer (Table 2.6.1). While positive migratory factors promote cell fusion by increasing the probability of myoblasts being close to one another, negative migratory factors may enhance cell fusion by acting as a "brake" on migrating cells to facilitate cell–cell contact and adhesion. Interestingly, with the exception of the actin cytoskeleton, which is involved in regulating various aspects of myogenesis, no other cytoplasmic proteins have been identified as regulators of muscle cell migration.

While both myoblasts and myocytes migrate during myogenesis, myocytes exhibit less motility than myoblasts (Griffin et al. 2010, Powell 1973). In addition, few migratory factors are known to promote myocyte migration (Table 2.6.1). For example, myocytes do not migrate to hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF), the canonical myoblast migratory factors, but instead migrate more

readily to the extracellular factor stromal cell-derived factor 1 (SDF1) than myoblasts do (Griffin et al. 2010). In addition, two transmembrane proteins, mannose receptor (MR) (Jansen and Pavlath 2006) and mouse odorant receptor 23 (MOR23) (Griffin et al. 2009), are necessary for myocyte migration prior to fusion. Future studies should focus on downstream pathways that might help explain why migratory factors differ between myoblasts and myocytes.

### 2.2.2 Regulation of Myoblast Fusion

Although muscle cell migration is required for fusion to occur, migration is still relatively far removed from the actual fusion process. Fusion of mononucleated myoblasts to form multinucleated myofibers is an essential step in skeletal muscle differentiation and growth (Darr and Schultz 1989, Horsley et al. 2001, Mitchell and Pavlath 2001, Phelan and Gonyea 1997, Rosenblatt and Parry 1992). In addition, myoblast fusion is also important for muscle repair. Some muscle diseases, such as centronuclear myopathy and myotonic dystrophy, may be due partly to defects in myoblast fusion (Farkas-Bargeton et al. 1988, Wockel et al. 1998).

The best-studied model of vertebrate myoblast fusion occurs during adult regenerative myogenesis. No current methods allow direct visualization of the steps of myogenesis *in vivo* in mice; therefore alternative measurements are used as an indirect readout. These measurements include morphological examination of myofiber cross-sectional area (CSA), isolation of single myofibers with attached satellite cells, as well as isolation and purification of primary mouse muscle cells. Indeed, cell culture models of myoblast fusion *in vitro*, utilizing primary muscle cells isolated from mouse, rat or

human muscles, or established mouse muscle cell lines (Abmayr and Pavlath 2012), allow careful dissection and analysis of the ordered steps leading up to the formation of multinucleated myotubes (Fig. 2.6.4), which are equivalent to immature myofibers *in vivo* (Bischoff 1978, Knudsen 1992, Knudsen and Horwitz 1977, Wakelam 1985). Finally, these *in vitro* studies also demonstrate that myoblasts do not generally fuse with other cell types (Okazaki and Holtzer 1965).

Upon mitogen withdrawal, myoblasts differentiate into elongated cells, which migrate towards one other and undergo recognition and adhesion. Following adhesion, alignment occurs through the parallel apposition of the membranes of elongated myoblasts (Wakelam 1985). Finally, membrane union takes place by a mechanism that is incompletely understood, but involves coated vesicles in close proximity to the aligned plasma membranes (Lipton and Konigsberg 1972, Przybylski and Blumberg 1966, Rash and Fambrough 1973), leading to fusion of several differentiated myoblasts with one another to form small, nascent myotubes with few nuclei. Subsequently, differentiated myoblasts fuse with nascent myotubes, to generate mature myotubes containing many nuclei. Therefore, mammalian myoblast fusion is a two-step process, generating nascent and mature myotubes, respectively (Horsley et al. 2001, Horsley et al. 2003, Horsley and Pavlath 2004). Finally, fusion also occurs between myotubes (Horsley et al. 2001, Peckham 2008, Rash and Fambrough 1973, Robertson et al. 1990), although the mechanisms underlying myotube-myotube fusion are not well understood.

The orientation of the fusion process has been studied in mammalian C2C12 myoblasts, where fusion events occur in multiple orientations. Lateral fusion takes place between a myoblast and a myotube, as well as perpendicular fusion of two myoblasts,

followed by perpendicular fusion of the resultant myotube with another myotube (Nowak SJ et al. 2009). These different modes of fusion may be due to spatial and temporal regulation of myoblast fusion. Spatial regulation refers to molecules exhibiting very specific localization during the fusion process (Table 2.6.2): molecules with symmetric localization, found at the contact region between the two cells and present in both cells, include metalloproteases, cell-cell adhesion molecules, and membrane components, but very few cytoplasmic proteins; in contrast, molecules exhibiting asymmetric localization, found at the contact region between two cells, but in only one of the cells, include actin and other cytoplasmic molecules, but very few cell-cell adhesion molecules (Pavlath 2010b). Temporal regulation of myoblast fusion occurs via certain molecules regulating a specific step of the two-step fusion process. Whereas many molecules are involved in regulating the fusion of myoblasts with one another (Horsley and Pavlath, 2004), few molecules regulating the fusion of myoblasts with nascent myotubes are known (Table 2.6.2). In addition, some molecules are only expressed by nascent myotubes, whereas others are expressed by both myoblasts and myotubes during later fusion stages, and these molecules are associated with migration and cell-cell adhesion (Pavlath 2010b). Thus, regulation of myoblast fusion likely occurs by multiple mechanisms.

Many molecules orchestrate the various steps of myoblast fusion, including extracellular factors (prostaglandins, cytokines, growth hormones), cell surface proteins (integrins and other glycoproteins), transmembrane proteins (receptors and others), intracellular proteins (including many actin regulators) and a few nuclear proteins (Table 2.6.2). However, until recently, no molecule deemed essential for myoblast fusion has been found. Interestingly, myomaker, a muscle-specific transmembrane protein, is both

necessary and sufficient to promote myoblast fusion *in vitro* and in *vivo*, suggesting this molecule may be the long-sought myogenic fusion protein controlling mammalian myoblast fusion (Millay et al. 2013).

### 2.2.3 Actin Dynamics During Myogenesis

Extensive cytoskeletal reorganization occurs both before and after fusion (Fulton et al. 1981). Before fusion, cultured myoblasts are highly migratory (Griffin et al. 2010), requiring extensive actin remodeling. In order to move, cells must extend their plasma membrane forward at the front, or leading edge, of the cell (Ridley et al. 2003), resulting in various protrusions (Ridley 2011), among which lamellipodia and filopodia are most extensively studied. However, little is known about actin dynamics during mammalian muscle cell migration in the context of these protrusions.

Prior to fusion, muscle cells exhibit lamellipodia, originally described as thin sheet-like regions (Abercrombie et al. 1970) found at the leading edge of various cell types (Friedl and Gilmour 2009, Weijer 2009), including muscle precursors in chick embryos. Lamellipodia contain branched actin filaments (Abercrombie et al. 1971, Small et al. 2002), driving the forward protrusion of the plasma membrane during extension (Ridley et al. 2003) and directional migration (Suraneni et al. 2012). In lamellipodia, small GTPases Rac1 and Cdc42 activate various actin regulatory proteins (Ridley 2011), leading to actin-related protein-2/3 (Arp2/3)-dependent actin polymerization (Campellone and Welch 2010, Lai et al. 2008, Mullins et al. 1998), but whether these small GTPases regulate lamellipodia formation during muscle cell migration is unknown. Interestingly, lamellipodia are also involved in later stages of myoblast fusion, with

myotube-myotube fusion occurring between the leading edge of lamellipodia of one myotube and the lateral plasma membrane of the other (Mukai and Hashimoto 2008).

Once in differentiation media, both actin filaments and microtubules reorganize in fusing myoblasts (Lu et al. 2001, Musa et al. 2006, Straube and Merdes 2007, Swailes et al. 2006, Swailes et al. 2004). During early myoblast alignment, a cortical actin wall, composed of many parallel and tightly bundled F-actin filaments, extends the length of the plasma membrane of the two aligned cells, possibly providing a temporal barrier for fusion until the myoblasts are completely aligned and ready to fuse (Duan and Gallagher 2009). Fusing myoblasts then exhibit filopodia, which are exploratory extensions from the plasma membrane containing parallel bundles of actin filaments (Ridley 2011). Filopodia are generally utilized by cells to probe their environment (Lewis and Bridgman 1992, Small and Celis 1978, Svitkina et al. 2003), as well as to connect fusing myoblasts along the entire length of the membrane contact for membrane fusion (Stadler et al. 2010). The small GTPase Cdc42 may be required for filopodia formation (Le Clainche and Carlier 2008) during cell spreading (Guillou et al. 2008) and may be involved in Arp2/3-dependent actin regulation in cells (Machesky and Insall 1998, Prehoda et al. 2000, Rohatgi 1999). However, filopodia still form in cells depleted of Cdc42 (Czuchra et al. 2005, Pellegrin and Mellor 2005), and some reports suggest Arp2/3 may be excluded from filopodia (Svitkina and Borisy 1999). However, whether Cdc42 is involved in filopodia formation in fusing myoblasts is unknown.

Many molecules, including cytoplasmic proteins, regulate myoblast fusion and actin polymerization (Table 2.6.2), suggesting various ways of regulating the fusion process via actin regulatory proteins. Importantly, the small GTPases Rac1 and Cdc42 are

required for recruitment of F-actin and other cytoskeletal proteins to contact sites, and are essential for myoblast fusion in the mouse (Vasyutina et al. 2009). In addition, Dedicator of cytokinesis 180/1 (Dock180/Dock1) and Guanine nucleotide exchange protein 100 (GEP100/Brag2) act as GEF proteins for small GTPases, while also regulating myoblast fusion in mice (Pajcini et al. 2008). Moreover, knockdown of Nck-associated protein 1 (Nap1), a molecule part of the WASP-family verprolin-homologous protein (WAVE) actin-remodeling complex, which eventually acts on Arp2/3, results in the accumulation of filamentous actin (F-actin) structures at the plasma membrane; interestingly, these structures correlate with a decrease in myoblast fusion (Nowak SJ et al. 2009).

Cell surface proteins are also involved in actin regulation upstream of small GTPases. Cadherins are linked to and can influence actin organization through α- and β-catenin. Thus, M-cadherin-dependent adhesion activates Rac1 through the RhoGEF Trio, which is required for myoblast fusion, whereas activation of the small GTPase RhoA results in degradation of M-cadherin, and further inhibits fusion (Charrasse et al. 2007, Charrasse et al. 2006). In addition, the small GTPase ADP-ribosylation factor 6 (Arf6) is also part of and required for the M-cadherin/Rac1/Trio complex to form (Donaldson 2008). Integrins are also likely to be important for actin reorganization, as they can bind to extracellular matrix proteins through their extracellular domain, and can also interact with the actin cytoskeleton using their cytoplasmic domain (Arnaout et al. 2005).

### 2.3 Creatine Kinase

ATP is necessary for proper cellular function. The cellular ATP pool is relatively constant, but ATP itself is rather unstable. Creatine kinase (CK) replenishes local cellular

ATP (Ames 2000, Tachikawa et al. 2004, Wyss and Kaddurah-Daouk 2000), and thus plays an important role in energy transduction in tissues with large and fluctuating energy demands, including skeletal or cardiac muscle, brain, retina, and spermatozoa (Wallimann and Hemmer 1994, Wyss and Kaddurah-Daouk 2000). Moreover, dysregulated CK is associated with many diseases, such as heart disease, mental diseases, cancer and inflammatory diseases (Balasubramani et al. 2006, Burklen et al. 2006, Huddleston et al. 2005, Ishikawa et al. 2005, Meffert et al. 2005).

### 2.3.1 Roles of Creatine Kinase in ATP Production and Actin Regulation

Four mammalian creatine kinase (*Ck*) genes encode two cytosolic (brain-type *Ckb* and muscle-type *Ckm*) and two mitochondrial (ubiquitous *Ckmt1* and sarcomeric *Ckmt2*) isoforms (Wyss and Kaddurah-Daouk 2000). The mitochondrial CK isoforms catalyze the reversible transfer of a high-energy phosphate group from ATP to creatine, generating ADP and phosphocreatine. Phosphocreatine then leaves the mitochondria and diffuses through the cytosol to sites of high ATP consumption, where cytosolic CK enzymes replenish the local ATP pool. Finally, creatine diffuses back to the mitochondria, thereby closing the cycle (Wyss and Kaddurah-Daouk 2000).

A major focus of this dissertation is the brain-type creatine kinase (CKB), which has been linked to ATP-dependent processes in various cell types. Thus, CKB is involved in regulating tumor cell motility (Mulvaney et al. 1998), motility patterns of the sea urchin sperm (Tombes and Shapiro 1985), thrombin-mediated shape changes in astrocytes (Mahajan et al. 2000), spreading and migration in astrocytes and fibroblasts (Kuiper et al. 2009), T cell development and activation (Zhang et al. 2009), phagocytosis

(Kuiper et al. 2008), osteoclast-mediated bone resorption (Chang et al. 2008), and high sensitivity hearing in stereocilia of hair cells (Shin et al. 2007). These data suggest a role for CKB in regulating ATP-dependent processes in various cell types.

To elucidate possible cellular functions of CKB, various groups examined CKB interacting proteins using a brain cDNA library. Subsequently, the two CKB interacting proteins found are the cis-Golgi Matrix protein, GM130 (Burklen et al. 2007), and protease-activated receptor-1 (PAR-1) (Mahajan et al. 2000). GM130 belongs to the golgin protein family (Barr 1999) and is involved in the assembly and maintenance of the Golgi apparatus (Rabouille and Jokitalo 2003), whereas PAR-1 is a G-protein coupled receptor with a role in mediating the cellular responses to thrombin during blood coagulation and other processes (Donovan et al. 1997, Even-Ram et al. 1998, Grand et al. 1996). Using bioinformatics tools, CKB and the muscle-type creatine kinase (CKM), another cytosolic CK enzyme, may also interact with each other (Hu et al. 2011), and with myocilin (MYOC), a protein expressed in many ocular tissues including the trabecular meshwork (Joe et al. 2011). The interaction of CKB and CKM with MYOC is predicted based on the fact that MYOC has a cytoskeletal function, and CK enzymes have been previously associated with cytoskeletal roles. However, this interaction remains to be tested more thoroughly. In addition, CKB localized to the distal region of the sea urchin sperm tail (Tombes et al. 1988), where it binds the cytoskeletal protein dynein, linking CKB to microtubules. Indeed, tubulin is a predicted CKB binding partner (Hu et al. 2011). Bioinformatics tools also predicted actin isoforms as binding partners for CK enzymes. Thus, γ-cyto-actin, a cytoplasmic actin isoform (Nowak KJ et al. 2009, Perrin and Ervasti 2010) is a predicted CKB interacting protein (Hu et al. 2011), while β-

cyto-actin, another cytoplasmic actin isoform, has not yet been shown to interact with CKB from these studies. In cultured C2C12 cells, mRNA levels of β-cyto-actin and γ-cyto-actin decline rapidly when differentiation begins (Bains et al. 1984), while mRNA expression of α-cardiac-actin, primarily expressed in heart muscle, is rapidly induced upon muscle differentiation, and decreases as myotube maturation proceeds (Bains et al. 1984). Finally, in mice, α-cardiac-actin may have overlapping functions with α-skeletal-actin (Nowak KJ et al. 2009), the predominant actin isoform in adult mouse skeletal muscle, whose mRNA levels reach a peak during later stages of fusion in the mouse myoblast cell line C2C12 (Bains et al. 1984, Vandekerckhove et al. 1986). Overall, these studies predict CKB could interact with different classes of molecules in multiple cell types, including the actin cytoskeleton; however, the interaction of CKB with these molecules remains to be thoroughly tested. Moreover, whether CKB provides ATP for the actin cytoskeleton, or for any other potential binding partner discussed here, is also unknown.

### 2.3.2 Creatine Kinase Functions During Myotube Formation

Prior work in the Pavlath lab showed treatment of muscle cells with creatine enhanced cell fusion in a CK-dependent manner (O'Connor et al. 2008). In addition, inhibition of actin polymerization prevented myonuclear addition following creatine treatment, suggesting phosphocreatine (PCr) hydrolysis is coupled to actin dynamics during myoblast fusion. Unlike other cell types where CK is necessary for cell migration, creatine treatment did not alter muscle cell motility (O'Connor et al. 2008), suggesting a role for the PCr/CK system in regulating a step beyond cell migration during myoblast

fusion. Interestingly, CKB becomes progressively localized near the ends of myotubes in later stages of myoblast fusion (O'Connor et al. 2008). Although fusion events occur in multiple orientations (Nowak SJ et al. 2009), myoblasts preferentially fuse with myotube ends during secondary myogenesis in rats (Zhang and McLennan 1995), the postnatal period in mice (Kitiyakara and Angevine 1963, Yaffe and Saxel 1977) and during fusion of cultured mouse myoblasts (Peckham 2008). Whereas these data suggest myotube ends may be areas of active fusion, the molecular mechanisms driving fusion at these sites are currently unknown.

**2.4 BAR Domain Proteins**

Bin-Amphiphysin-Rvs (BAR) domain proteins form a rapidly expanding protein family implicated in many diverse cellular processes, including endocytosis, vesicle fusion and trafficking, specialized membrane organization, actin organization, cell polarity, stress signaling, transcription and tumor suppression (Ren et al. 2006). Alterations in BAR domain proteins, such as mutations or deletions, have been associated with various aspects of cancer and blood disorders, including auto-inflammatory disease (Chen et al. 2012, Wise et al. 2002). Other BAR domain proteins have been linked to neurological diseases, including Alzheimer's disease (Kuwano et al. 2006), Huntington's disease (Holbert et al. 2003, Modregger et al. 2002), centronuclear myopathy (Nicot et al. 2007) and mental retardation/cerebellar hypoplasia (Billuart et al. 1998, Endris et al. 2002, Pirozzi et al. 2011, Tentler et al. 1999). Finally, some BAR domain proteins may play a role in other diseases including Tourette syndrome (Paschou et al. 2004), diabetes mellitus (Pietropaolo et al. 1993, Spitzenberger et al. 2003) and paraneoplastic stiff-

person syndrome (Folli et al. 1993). For several of these diseases, the mechanisms by which alterations in BAR domain proteins can lead to disease are poorly understood.

### 2.4.1 BAR Domain Proteins as Membrane Modulating Proteins

The BAR domain protein family is defined by the presence of an evolutionarily conserved region named after its founding members: *B*in1, *A*mphiphysin, and *R*vs167 (BAR) (Sakamuro et al. 1996, Sivadon et al. 1995). The BAR domain was predicted to have a coiled-coil domain structure, and was shown to act as a homo- and heterodimerization domain (Ramjaun et al. 1999, Slepnev et al. 1998, Wigge et al. 1997). Structural studies subsequently showed that the BAR domain is a crescent-shaped dimer, whose surface is covered with positively charged residues, allowing it to directly interact with negatively charged membrane lipids, such as phosphoinositides and phosphatidylserine (Frost et al. 2008, Frost et al. 2009, Peter et al. 2004, Shimada et al. 2007). In addition, biochemical studies indicated the BAR domain itself is capable of sensing and inducing membrane curvature, as it binds preferentially to highly curved membranes and can tubulate membranes *in vitro* (Peter et al. 2004). Generally, BAR domains bend membranes to a curvature corresponding to the angle of the BAR domain dimer, thereby generating specific membrane geometries (Itoh and De Camilli 2006), depending on the type of curvature generated by the BAR domain (Suetsugu 2010). The apparent origin of BAR domain proteins in eukaryotes suggests a function unique to eukaryotic organisms, although the features that confer folding of a polypeptide into a structure that can bind and bend membranes are still not fully understood (Ren et al. 2006).

The BAR domain superfamily has been divided into several families, including the "classical" BAR, the Fes/CIP4 Homology-BAR/FCH-BAR (F-BAR) and the Inverse-BAR/IMD-BAR/IRSp53-MIM Homology (I-BAR) domain-containing proteins (Frost et al. 2007, Frost et al. 2009). For "classical" BAR and F-BAR domain proteins, the concave surface of the BAR domain interacts with the membrane, generating various degrees of positive curvature. As a result, several plasma membrane invaginations arise, such as the well-characterized clathrin-coated pits and caveolae (Chen et al. 2012, Frost et al. 2009, Suetsugu 2010), as well as other less well-characterized invaginations (Doherty and McMahon 2009). In contrast, for I-BAR domain proteins, the convex surface of the BAR domain interacts with the membrane, generating negative curvature, resulting in the formation of outward membrane protrusions, such as filopodia and lamellipodia (Chen et al. 2012, Frost et al. 2009, Saarikangas et al. 2009, Suetsugu 2010).

In addition to generating positive or negative membrane curvature, other features of BAR domain-containing proteins reveal unique adaptations. For example, the presence of well-defined phosphoinositide binding motifs, such as the PhoX (PX) or Pleckstrin homology (PH) domains of PX-BAR and BAR-PH domain protein families, respectively, can direct BAR domain proteins to specific locations in the cell membrane enriched in phosphoinositides, where they can induce specific membrane curvature (Chen et al. 2012, Frost et al. 2009, Itoh and De Camilli 2006). Finally, for the N-terminal amphipathic helix-BAR (N-BAR) domain proteins, containing an amphipathic $\alpha$-helix adjacent to the N-terminal end of the BAR domain, insertion of the $\alpha$-helix into the membrane may facilitate the subsequent insertion of the BAR domain into the lipid bilayer, likely enhancing or further stabilizing membrane curvature (Chen et al. 2012, Frost et al. 2009).

These types of adaptations suggest that BAR domains can be used in a variety of contexts for different functions.

### 2.4.2 BAR Domain Proteins Implicated in Actin Regulation

Besides modulating membrane dynamics, several BAR domain proteins provide a link between the membrane and the membrane-associated cytoskeleton. BAR and F-BAR domain proteins contain interacting motifs for phosphoinositides and small GTPases (GTPases of the Rho, Rab, Arl and Arf subfamilies), two classes of molecules that function as major regulators of cytosol-membrane interactions (Behnia and Munro 2005, De Matteis and Godi 2004, Di Paolo and De Camilli 2006). Both phosphoinositides and small GTPases regulate the recruitment of cytosolic factors implicated in various functions, including actin regulation, to membranes (Itoh and De Camilli 2006); among these factors are two Arp2/3 activators, Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) (Takenawa and Suetsugu 2007). In addition, BAR domain proteins can functionally dimerize the verprolin-cofilin-acidic (VCA) domains of the C termini of WASP/WAVE, a potent means of activating the Arp2/3 complex (Padrick et al. 2008). By interacting with WASP/WAVE proteins, BAR domain proteins can indirectly induce actin-based protrusions, such as lamellipodia or filopodia, or invaginations, such as caveolae, endosomes and clathrin-coated pits (Suetsugu and Gautreau 2012). Finally, in some cases, BAR domain proteins can bind to and “bundle” actin fibers directly (Takenawa and Suetsugu 2007).

Interactions of BAR domain proteins with phosphoinositides or small GTPases can be mediated by either the BAR domain, or by flanking modules (Frost et al. 2009,

Habermann 2004, Itoh and De Camilli 2006). Although there are at least six distinct members of the Rho GTPase family (Foster et al. 1996, Hall 1994), the most commonly studied members are those belonging to the Rho family, namely RhoA, Rac1 and Cdc42. Small GTPases of the Rho family orchestrate cytoskeletal dynamics, and cycle between an inactive GDP-bound state and an active GTP-bound state. This transition is regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP (Rossman et al. 2005), and by GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTPase activity (Bernards and Settleman 2004). BAR domain proteins often contain a RhoGAP/GEF domain, as well as one or more scaffolding domains, allowing actin regulation, as well as other functions, to occur, thereby working at the interface between plasma membrane remodeling and Rho GTPase signaling. A few interesting examples in this category include the well-studied Tuba and GRAF1 proteins, which will be described below in more detail.

Tuba, which belongs to the "classical" BAR domain protein family, is a scaffold protein concentrated at synapses in the brain. Beside the BAR domain, Tuba contains several Src homology 3 (SH3) domains, which enable its binding to dynamin, a GTPase critical for fission of endocytic vesicles, as well as to a variety of actin regulatory proteins, including Neural Wiskott-Aldrich syndrome protein (N-WASP), WASP-family verprolin-homologous protein 1 (WAVE1) and Enabled/Vasodilator-stimulated phosphoprotein (Ena/VASP). In addition, the Dbl homology (DH) domain of Tuba functions as a Cdc42-specific GEF, involving Tuba in actin assembly. Thus, Tuba may link dynamin to GTPase signaling, and to the actin cytoskeleton (Cestra et al. 2005, Salazar et al. 2003).

GRAF1, which is a BAR-PH protein family member, binds to Focal Adhesion Kinase (FAK) (Hildebrand et al. 1996) and to the membrane scission protein dynamin (Lundmark et al. 2008) via the SH3 domain, suggesting a role for GRAF1 in endocytosis. Indeed, the BAR and PH domains of GRAF1 localize it to tubular and vesicular membranes that define the CLathrin-Independent Carriers/GPI-Enriched Endocytic Compartments (CLIC/GEEC) endocytic pathway, which mediates internalization in a manner dependent on Cdc42 activation (Lundmark et al. 2008, Sabharanjak et al. 2002). Whereas the BAR and PH domains of GRAF1 are involved in regulating membrane binding and curvature (Lundmark et al. 2008), GRAF1 also stimulates the GTPase activity of RhoA and Cdc42 via the GAP domain (Hildebrand et al. 1996), linking Rho GTPase signaling with membrane remodeling to facilitate endocytic internalization. Finally, GRAF1 depletion, which leads to impaired CLIC/GEEC function, also reduces cell spreading and migration (Doherty GJ et al. 2011), thus linking membrane trafficking with cell shape and motility.

Other BAR domain proteins lacking a RhoGAP/GEF domain can regulate actin dynamics by interacting with actin regulatory proteins using scaffolding domains. Pacsin proteins, which belong to the F-BAR protein family, are cytoplasmic phosphoproteins regulating vesicle formation and transport. Using SH3 domains, Pacsin proteins interact with the endocytic proteins dynamin and synaptojanin1 (Modregger et al. 2000), the actin regulator N-WASP (Modregger et al. 2000), as well as the small GTPase Rac1, leading to negative regulation of Rac1 activity, along with cell spreading and migration (de Kreuk et al. 2011). Although much less studied than other BAR domain protein family members, some I-BAR domain proteins have also been linked to actin regulation via small GTPases

and N-WASP/WAVE proteins via additional domains besides the BAR domain (de Kreuk and Hordijk 2012).

BAR domain proteins containing only a BAR domain can still regulate actin dynamics by interacting with other proteins. Arfaptin-2, a "classical" BAR domain protein family member, contains only a BAR domain, whose concave surface interacts with the membrane and with the small GTPase Rac (Tarricone et al. 2001), raising the question of whether these interactions are mutually exclusive or sequential for BAR domain proteins (Habermann 2004). Arfaptin-2 is also a putative cytosolic target of, and mediates crosstalk between, small GTPases Arf and Rac1 (Shin and Exton 2001, Tarricone et al. 2001), suggesting that BAR domain proteins could mediate crosstalk between various signaling pathways mediated by small GTPases (Habermann 2004).

### 2.4.3 Mammalian Studies of the Amphiphysin/Bin Family of Proteins

Members of the N-BAR family of proteins, namely Amphiphysin1, Bridging integrator 1 (Bin1), Bin2 and Bin3 (Frost et al. 2009), are a major focus of this dissertation. Therefore, these proteins will be discussed extensively in this section, in terms of their domain structure, as well as their known cellular functions, some of which are either intertwined with, or alternatively independent from, actin regulation.

Amphiphysin1 is a neuronal cytosolic protein concentrated in nerve terminals, which was originally identified as an antigen enriched in chicken synaptic membranes (Lichte et al. 1992), and as the dominant autoantigen in paraneoplastic stiff-person syndrome (David et al. 1994, De Camilli et al. 1993, Folli et al. 1993). Amphiphysin1 contains an N-BAR domain (Dawson et al. 2006, Peter et al. 2004) which regulates

membrane curvature and tubulation (Ren et al. 2006), clathrin/AP-2 (CLAP)-binding sites (Ren et al. 2006, Slepnev et al. 2000), and an SH3 domain which binds the endocytic proteins dynamin1 (David et al. 1996, Grabs et al. 1997, Wigge and McMahon 1998) and synaptojanin1 (Ren et al. 2006). Amphiphysin1 mRNA expression is confined to the brain of adult mice (Prendergast et al. 2009) and humans (Ge K. and Prendergast G. C. 2000). Amphiphysin1 is important for the formation of filopodia and growth cones in neurons (Mundigl et al. 1998, Yoo et al. 2002), but the role of Amphiphysin1 in the brain appears to be independent of endocytosis (Di Paolo et al. 2002). Finally, Amphiphysin1 also regulates Rac1-dependent actin polymerization and phagocytosis in Sertoli cells (Yamada et al. 2007), linking Amphiphysin1 to actin regulation in a manner that is not completely understood.

The second isoform or Amphiphysin, named Bin1, was described by various groups on the basis of its Amphiphysin-like structure, SH3 domain, or binding to c-Myc/c-Abl oncoproteins (Butler et al. 1997, Kadlec and Pendergast 1997, Sakamuro et al. 1996, Sparks et al. 1996, Tsutsui et al. 1997, Wechsler and Dang 1992). Bin1 transcripts are subject to differential splicing, leading to a diversity of Bin1 isoforms, including the ubiquitous and brain isoforms (Prendergast et al. 2009, Ren et al. 2006). All Bin1 isoforms contain an N-terminal BAR domain and a C-terminal SH3 domain, and some isoforms also contain the Myc-binding domain (MBD) and the Clathrin/AP2 (CLAP)-binding region (Prendergast et al. 2009, Ren et al. 2006). The mRNA expression of the ubiquitous Bin1 isoform is highest in adult mouse brain and skeletal muscle (Prendergast et al. 2009), and this isoform inhibits transcription by interacting with the c-Myc transcription factor (Sakamuro et al. 1996, Sakamuro and Prendergast 1999). In contrast,

the brain isoform of Bin1, also known as Amphiphysin2, can heterodimerize with Amphiphysin1 (Wigge et al. 1997) and regulate clathrin-mediated endocytosis (Simpson et al. 1999) by associating with components of clathrin-coated synaptic vesicles (Owen et al. 1998, Ramjaun and McPherson 1998); however, Bin1 may also contribute to postinternalization traffic (Leprince et al. 2003).

The initial observation of a polyclonal Bin1 antiserum cross reacting with non-Bin1 polypeptides in cells that do not express Amphiphysin (Sakamuro et al. 1996) suggested the likely presence of additional BAR domain proteins. Homology searches revealed two transcripts encoded by the *BIN2* gene (Ge K. and Prendergast G. C. 2000), whose function had not been extensively studied. The cytosolic Bin2 protein contains an N-BAR domain (Sánchez-Barrena et al. 2012) by which it can tubulate membranes *in vitro* (Sánchez-Barrena et al. 2012), as well as a C-terminal extension with acidic and serine/proline rich segments; however, unlike other N-BAR protein family members, Bin2 lacks an SH3 domain (Ge K. and Prendergast G. C. 2000), indicating that Bin2 may not interact with other proteins in a similar manner to the other N-BAR family members. Bin2 also forms a complex with Bin1 in COS7 cells via the BAR domain (Ge K. and Prendergast G. C. 2000), but the function of this interaction is unknown. Also, unlike Amphiphysin1 and Bin1, Bin2 mRNA expression is lymphoid cell/tissue specific (Ge K. and Prendergast G. C. 2000), with Bin2 regulating podosome formation, as well as motility and phagocytosis in leucocytes (Sánchez-Barrena et al. 2012). Finally, also unlike other N-BAR domain proteins, Bin2 is not implicated in receptor-mediated endocytosis, and does not have tumor-suppressor features (Ge K. and Prendergast G. C.

2000). Bin2 is the least studied of all the N-BAR domain protein family members, and its cellular functions remain to be defined extensively.

The final member of this family, named Bin3, was identified on based on structural similarity to its budding yeast ortholog (Ren et al. 2006, Routhier et al. 2001). Unlike Bin1, Bin3 is only a single transcript, expressed ubiquitously in adult tissues with the sole exception of the brain (Prendergast et al. 2009, Ren et al. 2006). Bin3 is also a target of miR-184 in human lens epithelial cells (Tian et al. 2010), although the connection between BAR domain proteins and miRNAs has not been extensively investigated. In contrast to the other family members, Bin3 protein contains only an N-BAR domain (Ren et al. 2006). *BIN3* null mice develop juvenile cataracts, characterized by numerous large vacuoles and striking loss of F-actin from lens fiber cells (Ramalingam et al. 2008), in addition to an elevated incidence of lymphoma in the lung, liver, intestine, spleen and lymph nodes in mice beyond one year of age (Ramalingam et al. 2008). *BIN3* null MEFs also exhibit increased proliferation and invasive motility (Ramalingam et al. 2008), indicating that Bin3 may exhibit tumor suppressor functions. Finally, localization of HA-Bin3 to cytosolic vesicular membranes in COS kidney cells (Ramalingam et al. 2008) suggests a possible function for Bin3 in endocytosis, but this likely function for Bin3 has not yet been explored.

### 2.4.4 Yeast Studies of the Amphiphysin/Bin Family of Proteins

Much of the current knowledge on the potential functions of Amphiphysin/Bin proteins in mammalian cells arises from studies in yeast cells. *Bin1* and *Bin3* are evolutionarily conserved classic members of the BAR protein family. *Bin1* and *Bin3*

orthologs have been investigated extensively in the budding yeast *S. cerevisiae*, where they are termed Reduced viability upon starvation 161 and 167 (*RVS167* and *RVS161*), respectively. Both *RVS* genes were identified in screens for mutants that exhibit reduced viability upon nutrient starvation (rvs phenotype) (Bauer et al. 1993, Crouzet et al. 1991, Munn et al. 1995). *RVS* mutants also exhibit endocytosis defects, a depolarized actin cytoskeleton, and sensitivity to high salt and amino acid analog concentrations (Bauer et al. 1993, Munn et al. 1995, Sivadon et al. 1995). Whereas both Rvs161p and Rvs167p contain an N-BAR domain, Rvs167p contains two additional domains, namely a region rich in glycine, proline, and alanine (GPA), as well as an Src homology 3 (SH3) domain (Ren et al. 2006). As Rvs167p and Rvs161p form a heterodimer via their BAR domains (Ren et al. 2006, Youn et al. 2010), Rvs161p may exert its functions via this interaction. Rvs161p and Rvs167p localize to cortical actin patches, which are sites of endocytosis (Balguerie et al. 1999, Brizzio et al. 1998, Kaksonen et al. 2005). Indeed, Rvs161p regulates both fluid-phase and receptor-mediated endocytosis (Ren et al. 2006) independently from its role in cell-cell fusion during mating (Brizzio et al., 1998), which requires an interaction with Fus2, a non-BAR domain protein (Brizzio et al. 1998, Paterson et al. 2008, Ren et al. 2006). Rvs167p may also have a unique role in linking the actin cytoskeleton to the cell cycle (Lee et al. 1998), due to the phosphorylation of Rvs167p by Pho85, a yeast cell-cycle dependent kinase related to the mammalian enzyme Cyclin-dependent kinase 5 (Cdk5) (Huang et al. 1999, Nishizawa et al. 1999). Both genes interact with proteins derived from mutant alleles of *ACT1* and *MYO1*, encoding actin and myosin, respectively (Balguerie et al. 1999, Breton and Aigle 1998), as well as with other cytoskeletal components encoded by the *ABP1*, *SAC6*, *SLA1*, *SLA2*, and *SRV2*

genes (Lila and Drubin 1997). Together, these studies highlight the importance of Bin orthologs in regulating cell-cell fusion, actin dynamics and endocytosis in the budding yeast.

*Bin1* and *Bin3* orthologs have been studied to a lesser extent in the fission yeast *S. pombe*, where they are termed *hob1*$^{+}$ and *hob3*$^{+}$ (homolog of *Bin1* and *Bin3*, respectively) (Routhier et al. 2001, Routhier et al. 2003). Interestingly, Hob3p has a domain structure similar to that of Rvs161p, meaning it contains only an N-BAR domain, while Hob1p has a domain structure similar to Rvs167p, containing an N-BAR domain, a central domain, and an SH3 domain (Ren et al. 2006). In spite of significant homology with the budding yeast counterparts, *hob1*- and *hob3*-deficient yeast do not exhibit reduced viability upon nutrient starvation, increased osmolar sensitivity, or defective endocytosis (Ren et al. 2006). In addition, *hob3*-deficient yeast do not exhibit a defect in cell-cell fusion during mating (Routhier et al. 2001, Routhier et al. 2003), but have defects in F-actin organization and cytokinesis (Routhier et al. 2001), whereas *hob1*-deficient yeast exhibit defects in cell cycle arrest (Routhier et al. 2003). Interestingly, the mammalian *Bin1* and *Bin3* genes can complement their respective defects in yeast (Routhier et al. 2001, Routhier et al. 2003). While human Bin3 can completely rescue actin localization defects in *hob3*-deficient yeast, Rvs161p can only partially rescue this phenotype (Routhier et al., 2001). However, Hob3p can rescue growth in *rvs161*-deficient yeast, whereas human Bin3 or Bin1 cannot (Routhier et al. 2001, Routhier et al. 2003), indicating that functional conservation may exist both within the yeast counterparts as well as with the human orthologs. Hob3p can then recruit and activate the small GTPase Cdc42 to sites of cell division, likely for mediating cytokinesis in a complex that also contains a GEF protein,

Gef-1. In this manner, Hob3p facilitates the Gef-1-Cdc42 interaction and Cdc42 activation in yeast (Coll et al. 2007). These studies highlight the importance of Amphiphysin/Bin proteins in regulating Cdc42-dependent cellular processes.

### 2.4.5 BAR Domain Proteins Involved in Skeletal Muscle Myogenesis

Various steps of myoblast fusion require both membrane and actin dynamics, and BAR domain proteins are capable of regulating both of these processes. Known functions of the mammalian N-BAR domain protein Bin1 in skeletal muscle cells have been discovered mostly from *in vitro* studies using the muscle Bin1 isoform. Bin1 knockdown and overexpression studies in the muscle cell line C2C12 revealed a role for Bin1 in differentiation (Wechsler-Reya et al. 1998). Whereas Bin1 overexpression also caused enhanced myotube formation (Wechsler-Reya et al. 1998), this effect is likely due to the role of Bin1 in regulating differentiation. Bin1 is also expressed in the somites of mouse embryos, and interacts with actin and myosin via the SH3 domain. While transgenic overexpression of the Bin1 SH3 domain did not affect fusion *in vivo* (Fernando et al. 2009), it resulted in increased myofiber size with sarcomeric disorganization (Fernando et al. 2009), suggesting that Bin1 may be crucial in promoting muscle differentiation and sarcomere assembly. Bin1 is also required for the maintenance of excitation-contraction coupling in skeletal muscle (Razzaq et al. 2001, Zelhof et al. 2001), and regulation of the contractile response was attributed to the ability of the Bin1 BAR domain to enhance sarcolemmal membrane curvature, influencing T-tubule assembly and maturation (Lee et al. 2002). Mutations in Bin1 cause centronuclear myopathy, characterized by muscle weakness and abnormal centralization of nuclei in muscle fibers, possibly due in part to

disrupting the remodeling of T-tubules and/or endocytic membranes (Nicot et al. 2007). Finally, the Bin1 SH3 domain is also necessary for interaction with Dynamin2, driving normal muscle function and nuclear positioning (Nicot et al. 2007). As dynamin2 mutations also cause centronuclear myopathy (Bitoun 2005), and dynamin is implicated in syncytium formation (Richard et al. 2011), this work implicates Bin1 in regulating muscle formation and function in the context of muscle disease. Finally, Bin1 may also regulate cardiac muscle development in mouse embryos (Muller et al. 2003).

Besides Bin1, the other well characterized BAR domain protein in myogenesis is the GTPase regulator associated with focal adhesion kinase-1 (GRAF1) protein, a RhoGAP-containing BAR-PH domain protein necessary for, and can promote, skeletal muscle differentiation by limiting RhoA/ROCK signaling (Doherty JT et al. 2011). GRAF1 promotes skeletal muscle differentiation in a GAP- and SH3-domain-dependent manner, and promotes myoblast fusion in a BAR- and GAP-domain-dependent manner (Doherty JT et al. 2011). The BAR domain of GRAF1 is required for the induction of multinucleated myotubes beyond the step of differentiation (Doherty JT et al. 2011, Richard et al. 2011), presumably by regulating membrane bending/sculpting. In myoblasts, GRAF1 localizes to leading edge lamellipodia and dorsal ruffles, as well as to a cytoplasmic perinuclear compartment; however, upon differentiation, GRAF1 localization shifts to the bi-polar tips of elongating myoblasts, which are completely devoid of actin-based structures (Doherty JT et al. 2011), suggesting that GRAF1 limits the extent of Rho GTPase-dependent F-actin polymerization at these sites. Finally, GRAF1 is also expressed in the somites of *Xenopus laevis* embryos, where GRAF1 depletion leads to somite degeneration, swimming defects and embryonic lethality

(Doherty JT et al. 2011). In developing tadpoles, GRAF1, a bona-fide RhoGAP, is necessary for skeletal muscle differentiation/maturation (Doherty JT et al. 2011), making GRAF1 the first RhoGAP protein identified to regulate muscle maturation *in vivo*.

**2.5 Summary**

Skeletal muscle myogenesis is fundamental to muscle growth and regeneration. Proper myogenesis depends on the ability of satellite cells, the adult muscle stem cells, to proliferate, differentiate, migrate, adhere and fuse, giving rise to myotubes *in vitro* or myofibers *in vivo.* Various steps of myoblast fusion, including migration and fusion, two major points of focus in this dissertation, require extensive membrane and actin dynamics. Although many molecules are known to regulate migration and fusion during myogenesis, very few have been studied in the context of membrane and/or actin dynamics. This dissertation is focused on two particular classes of molecules, creatine kinase enzymes and BAR domain proteins. Creatine kinase B (CKB) has been linked to various actin-dependent processes in cells, likely replenishing ATP for actin dynamics. In addition, CKB may also be important for myotube formation. However, the molecular pathways by which CKB regulates myotube formation in an ATP-dependent manner are unknown. In contrast, BAR domain proteins regulate both membrane and actin dynamics in cells via small GTPases, but have been little studied in skeletal muscle. Overall, CK enzymes and BAR domain proteins are novel regulators of myogenesis, and elucidating their mechanism of action can provide valuable insights into the regulation of both membrane and actin dynamics during muscle growth and repair.

## 2.6 Figures and Tables

**Figure 2.6.1: Skeletal muscle structure**

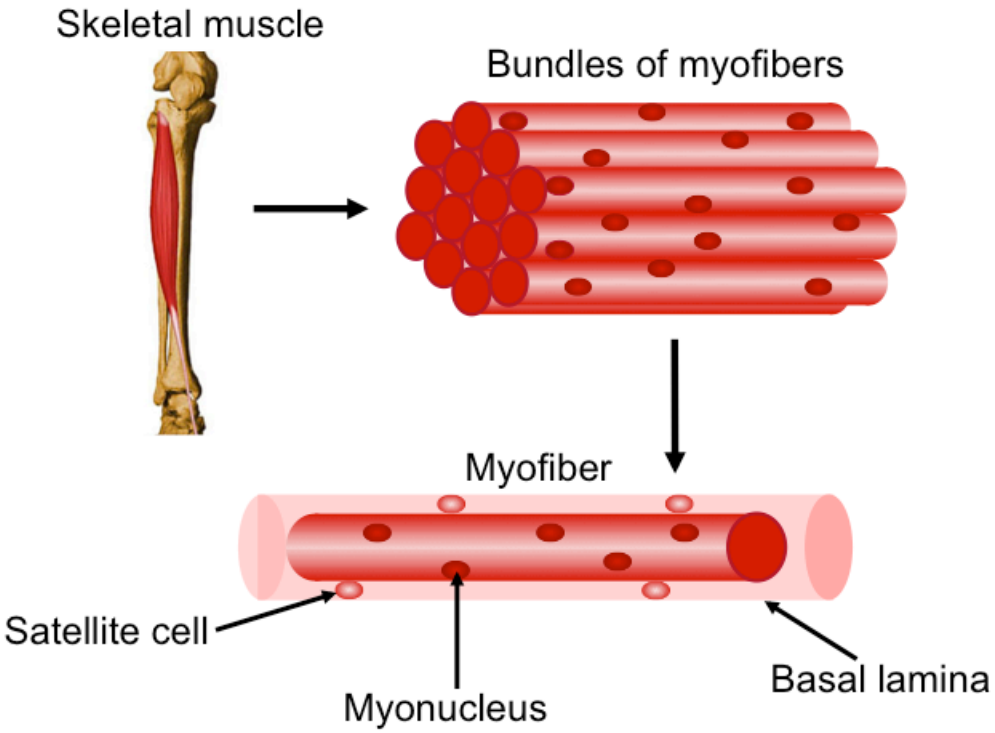

**Figure 2.6.1: Skeletal muscle structure**

Skeletal muscle is tethered to the bone, and is composed of bundles of multinucleated muscle cells, the myofibers. Each myofiber is surrounded by a basal lamina, a connective tissue layer, and contains multiple postmitotic myonuclei with a continuous cytoplasm. The myogenic stem cells (satellite cells) are situated between the basal lamina and the myofiber plasma membrane.

**Figure 2.6.2: Myogenesis *in vivo***

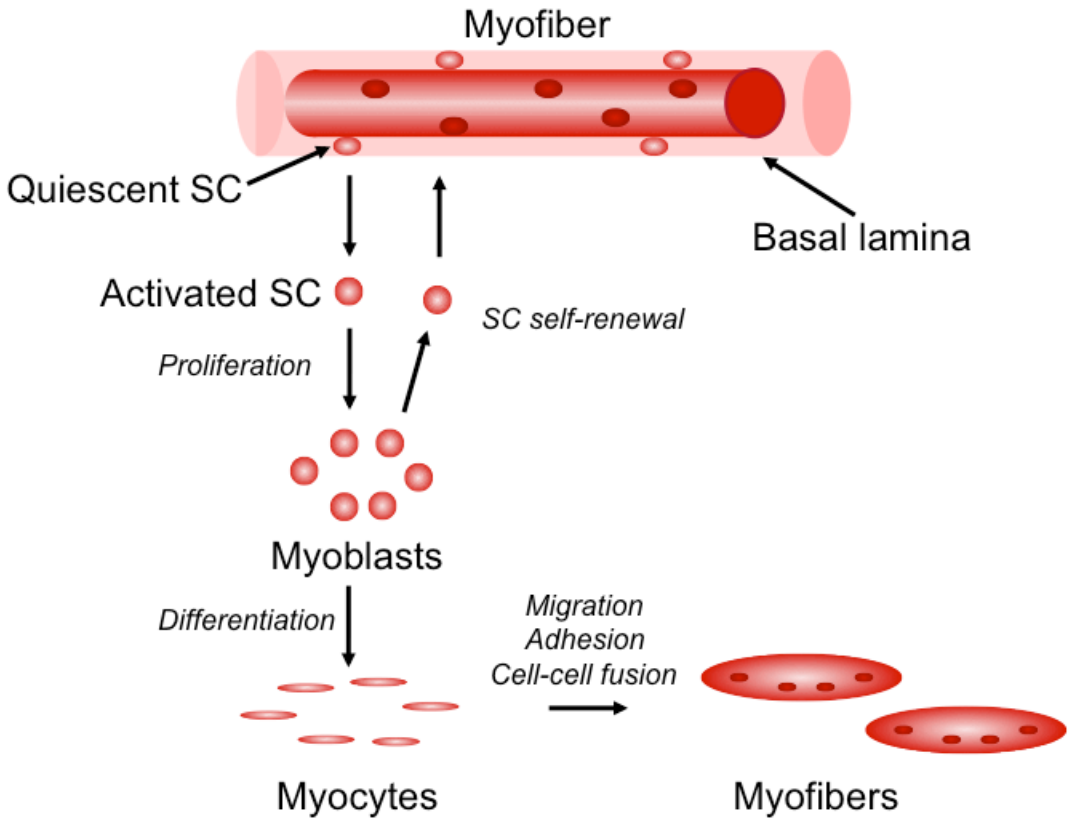

**Figure 2.6.2: Myogenesis *in vivo***

During the regenerative phase of muscle repair, quiescent satellite cells (SCs) become activated, meaning they re-enter the cell cycle, and proliferate, giving rise to progeny myoblasts. Myoblasts then differentiate into myocytes, which migrate, adhere and fuse, giving rise to myofibers in two distinct fusion stages (not shown).

**Figure 2.6.3: Patterns of myofiber branching *in vivo***

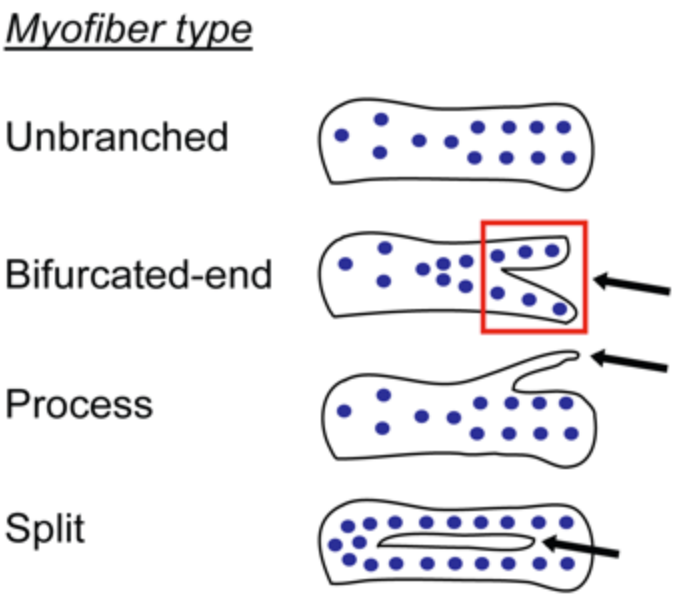

**Figure 2.6.3: Patterns of myofiber branching *in vivo***

Schematic of various types of branching observed in regenerated myofibers, as indicated by rows of centrally located nuclei: bifurcated-end, process, and split. An unbranched regenerated myofiber is shown for comparison. The arrows and red box indicate myofiber branching.

**Figure 2.6.4: Myogenesis *in vitro***

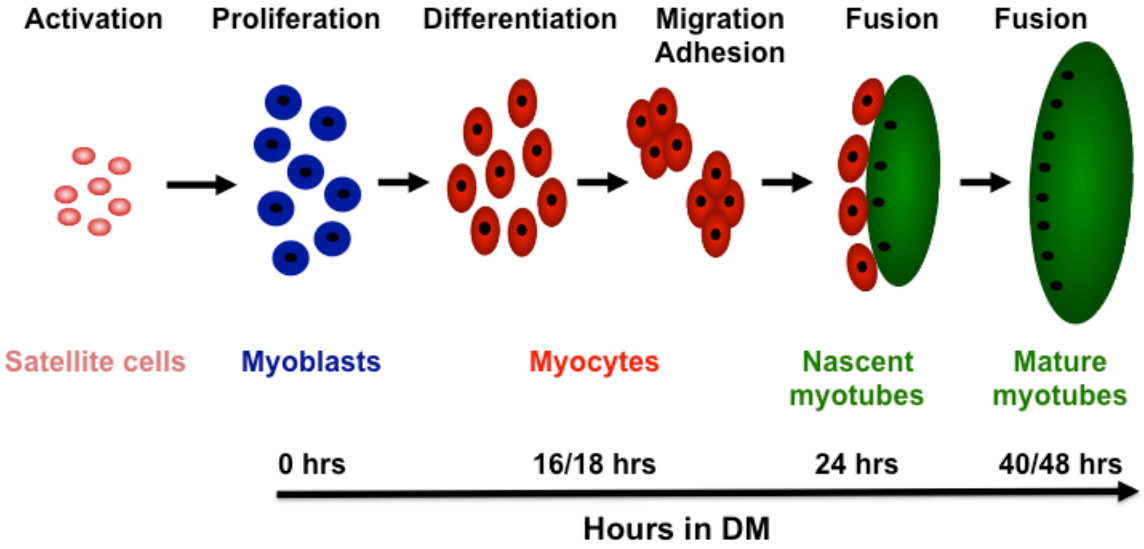

**Figure 2.6.4: Myogenesis *in vitro***

Satellite cells become activated, giving rise to myoblasts, which proliferate in media containing serum and growth factors. In serum-free differentiation media (DM), myoblasts (0 h in DM) withdraw from the cell cycle and differentiate into myocytes (16/18 h in DM). Myocytes then migrate, adhere and fuse to one another, initially giving rise to nascent myotubes (24 h in DM). Subsequently, myocytes fuse with nascent myotubes, giving rise to mature myotubes (40/48 h in DM).

**Table 2.6.1: Regulators of muscle cell migration *in vitro***

| Location | Type | Molecule | References |
|---|---|---|---|
| **Extracellular** | Prostaglandin | Prostacyclin ($PGI_2$)** | (Bondesen et al. 2007) |
| | Growth factors | Hepatocyte growth factor (HGF) | (Allen et al. 2003, Bischoff 1997, Corti et al. 2001, Griffin et al. 2010, Kawamura et al. 2004, Lee et al. 1999) |
| | | Platelet-derived growth factor (PDGF)# | (Allen et al. 2003, Bischoff 1997, Griffin et al. 2010, Robertson et al. 1993a) |
| | | Epidermal growth factor (EGF) | (Bischoff 1997, Corti et al. 2001) |
| | | Fibroblast growth factor (FGF)# | (Allen et al. 2003, Corti et al. 2001, Robertson et al. 1993a) |
| | | Insulin-like growth factor (IGF) | (Allen et al. 2003, Becciolini et al. 2006, Lafreniere et al. 2004, Neuhaus et al. 2003, Suzuki et al. 2000) |
| | | Vascular endothelial growth factor (VEGF) | Germani et al., 2003 |
| | | Transforming growth factor-β (TGF-β)# | (Bischoff 1997, Robertson et al. 1993a) |
| | Chemokines | Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) | (Corti et al. 2001) |
| | Cytokines | Stromal cell-derived factor 1 (SDF1)* | (Griffin et al. 2010, Odemis et al. 2007) |
| | | Interleukin-4 (IL4) | (Horsley et al. 2003, Lafreniere et al. 2006) |
| | | Tumor necrosis factor α (TNFα) | (Torrente et al. 2003) |

| | | |
|---|---|---|
| **Cell surface** | CD44 | (Mylona et al. 2006) |
| | CD164 | (Bae et al. 2008) |
| | Laminin | (Goodman et al. 1989, Ocalan et al. 1988, Yao CC et al. 1996) |
| | Integrin | (Boettiger et al. 1995, Crawley et al. 1997, Echtermeyer et al. 1996) |
| | Decorin** | (Olguin et al. 2003) |
| | Sphingosine 1-phosphate (S1P)** | (Becciolini et al. 2006) |
| **Membrane** | Mannose receptor (MR)* | (Jansen and Pavlath 2006) |
| | Mouse odorant receptor 23 (MOR23)* | (Griffin et al. 2009) |
| **Nucleus** | The nuclear factor of activated T-cells 5 (NFAT5) | (O'Connor et al. 2007) |

*Molecules important for myocyte migration

**Negative regulators of muscle cell migration

[#]Chemotactic agents for myoblasts *in vivo*

**Table 2.6.2: Regulators of mammalian myoblast fusion *in vitro***

| Location | Type | Molecule | References |
|---|---|---|---|
| **Extracellular** | Peptide hormone | Growth Hormone (GH)** | (Sotiropoulos et al. 2006) |
| | Cytokine | Interleukin-4 (IL4)** | (Horsley et al. 2003, Lafreniere et al. 2006) |
| | Prostaglandin | Prostaglandin F2$\alpha$** | (Horsley and Pavlath 2003) |
| | | Prostaglandin $E_1$ ($PGE_1$) [#] | (David and Higginbotham 1981, Entwistle et al. 1988, Rossi et al. 1989, Zalin 1977) |
| | Protease | Cathepsin B[#] | (Jane et al. 2002) |
| | Glycoprotein | Follistatin** | (Iezzi et al. 2004, Pisconti et al. 2006) |
| | Soluble gas | Nitric oxide[&] | (Lee et al. 1994, Long et al. 2006, Pisconti et al. 2006) |
| | Earth metal, intracellular signaling and extracellular protein activity | $Ca^{2+}$ | (Constantin et al. 1996, Knudsen and Horwitz 1977, Knudsen and Horwitz 1978, Shainberg et al. 1969, Wakelam 1985) |
| | Metallo-protease | A disintegrin and metalloprotease 12 (ADAM12) [#a] | (Yagami-Hiromasa et al. 1995) |
| | Extracellular matrix protease | Membrane type 1 metalloprotease (MT1-MMP) | (Ohtake et al. 2006) |
| | | Very late antigen-4 (VLA-4) [#] | (Rosen et al. 1992) |

| | | | |
|---|---|---|---|
| **Cell surface** | Integrins* | α3-integrin[a] | (Brzoska et al. 2006) |
| | | α9-integrin[a] | (Lafuste et al. 2005) |
| | | β1 integrin[#a] | (Schwander et al. 2003, Tachibana and Hemler 1999) |
| | Glycoprotein | Neural cell adhesion molecule (NCAM) | (Suzuki et al. 2003) |
| | | Vascular cell adhesion molecule 1 (VCAM-1)[#] | (Rosen et al. 1992) |
| | | Nephrin** | (Sohn et al. 2009) |
| | | M-cadherin*[a] | (Bach et al. 2010, Charrasse et al. 2007, Charrasse et al. 2006) |
| | | N-cadherin[b] | (Knudsen et al. 1990) |
| | Sialomucin | CD164 | (Bae et al. 2008) |
| **Membrane** | Transmembrane 4 superfamily protein | CD9[&] | (Tachibana and Hemler 1999) |
| | | CD81[&] | |
| | Transmembrane receptor | Mannose receptor (MR)** | (Jansen and Pavlath 2006), |
| | G-protein coupled receptor | Mouse odorant receptor 23 (MOR23) | (Griffin et al. 2009) |
| | Phospholipid binding protein | Myoferlin**[a] | (Doherty et al. 2005) |
| | Structural component of | Caveolin-3[&] | (Galbiati et al. 1999) |

| | | | |
|---|---|---|---|
| **Membrane** | caveolae | | |
| | Phospholipid component | Phosphatidylserine (PS) [a] | (van den Eijnde et al. 2001) |
| | Ion channels | Inward rectifying K+ channels[#] | (Fischer-Lougheed et al. 2001) |
| | | T-type Ca2+ channels[#] | (Bijlenga et al. 2000) |
| | Chaperone glycoprotein | Glucose-related protein 94 (GRP94)[#] | (Gorza and Vitadello 2000) (Wanderling et al. 2007) |
| | Sterol, membrane fluidity | Cholesterol[a] | (Mukai et al. 2009) |
| **Cytoplasm** | Microtubule binding protein | End-binding protein 3 (EB3) [a] | (Straube and Merdes 2007) |
| | Globular protein, cytoskeleton | Actin[ab] | (Nowak SJ et al. 2009) |
| | Integrin-associated adaptor protein | Kindlin-2[a] | (Dowling et al. 2008) |
| | Structural protein | Non-muscle myosin 2A[b] | (Swailes et al. 2006) |
| | RhoA inhibitor | RhoE | (Fortier et al. 2008) |
| | Enzyme for production of phosphatidic acid | Phospholipase D* | (Bach et al. 2010) |
| | Scaffold | Syntrophin[b] | (Abramovici and Gee |

| | | | |
|---|---|---|---|
| **Cytoplasm** | protein | | 2007) |
| | Kinases | Focal adhesion kinase (FAK) | (Quach et al. 2009) |
| | | Diacylglycerol (DAG) kinase ζ[b] | (Abramovici and Gee 2007) |
| | | The mammalian target of rapamycin (mTOR)** | (O'Connor et al. 2008) (Park and Chen 2005, Sun et al. 2010) |
| | | c-Src[&] | (Fornaro et al. 2006) |
| | | Rho/Rho-associated protein kinase (Rock)[&] | (Nishiyama et al. 2004) |
| | | Protein kinase A (PKA)* | (Mukai and Hashimoto 2008) |
| | Phosphatase for c-src | Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2)** | (Fornaro et al. 2006) |
| | Integrin-associated adaptor protein | Talin 1, 2 | (Conti et al. 2009) |
| | Molecule regulating cell-cell adhesion and gene transcription | β-catenin[a] | (Zeschnigk et al. 1995) |
| | Myoferlin-associated protein | EH-domain protein 2 (EHD2)** | (Doherty et al. 2008) |

| | | | |
|---|---|---|---|
| **Cytoplasm** | $Ca^{2+}$-dependent cysteine protease | Calpain[#] | (Schollmeyer 1986) |
| | Calpain inhibitor protein | Calpastain[#] | (Balcerzak et al. 1995, Temm-Grove et al. 1999) |
| | $Ca^{2+}$-binding protein | Calmodulin[#] | (Bar-Sagi and Prives 1983) |
| | Cyclic nucleotide | cGMP[&] | (Lee et al. 1994, Pisconti et al. 2006) |
| | Second messenger | cAMP* | (Mukai and Hashimoto 2008) |
| | Small GTPases | Cell division cycle 42 (Cdc42)* | (Vasyutina et al. 2009) |
| | | Ras-related C3 botulinum toxin substrate 1 (Rac1)*[b] | (Charrasse et al. 2007, Vasyutina et al. 2009) |
| | | ADP-ribosylation factor 6 (Arf6)* | (Bach et al. 2010, Chen et al. 2003) |
| | GEFs | Brag2/Guanine nucleotide exchange protein 100 (Arf-$GEP_{100}$)* | (Pajcini et al. 2008) |
| | | Dedicator of cytokinesis 1/180,5 (Dock1/180,5)* | (Laurin et al. 2008, Pajcini et al. 2008) |
| | | Triple functional domain protein (Trio)* | (Charrasse et al. 2007) |
| | Actin cross-linking protein | Filamin C* | (Dalkilic et al. 2006) |

| | | | |
|---|---|---|---|
| **Cytoplasm** | Member of WAVE-activating remodeling complex | Nck-associated protein 1 (Nap 1)* | (Nowak SJ et al. 2009) |
| | Actin nucleation promoting factor | Neural Wiskott-Aldrich syndrome protein (N-WASP)* | (Kim et al. 2007) |
| | Actin binding protein | WASP-interacting protein (WIP)* | (Kim et al. 2007) |
| **Nucleus** | Transcription factor | The nuclear factor of activated T-cells, cytoplasmic 2 (NFATC2)** | (Horsley et al. 2001) |
| | | Forkhead box protein O1a (FOXO1a) [#] | (Bois et al. 2005, Bois and Grosveld 2003, Nishiyama et al. 2004) |
| | Component of spliceosome | Survival of motor neurons (Smn)[&] | (Shafey et al. 2005) |

*Regulators of actin dynamics

[#]Involved in the first step of fusion

**Involved in the second step of fusion

[&]Fusion step regulated unknown

[a]Molecules with symmetric localization

[b]Molecules with asymmetric localization

# Chapter 3: Materials and Methods

# Chapter 3: Materials and Methods

## Animals

Wild-type (WT) and Bin3 null (KO) mice were maintained on a mixed C57BL/6J-129/SvJ genetic background (Ramalingam et al. 2008). In addition, WT C57BL/6 or BALB/c mice were also used for some experiments. Mice were age- and sex- matched in experiments and used between 4 and 23 weeks of age for *in vitro* experiments and between 10 and 34 weeks of age for *in vivo* experiments in accordance with the IACUC guidelines of Emory University.

## Muscle injuries

For analyses of myofiber cross-sectional area, muscle injury was induced by a single injection of 50 μL of 1.2% $BaCl_2$ in PBS into the tibialis anterior muscles of mice as described previously (O'Connor et al. 2007). Muscles were collected at various timepoints after injury and histological sections were prepared and imaged as described previously (Jansen and Pavlath 2006). Myofiber cross-sectional area was quantified using ImageJ. A total of 480-1200 myofibers from four to seven mice were analyzed per genotype for each timepoint.

For analyses of myofiber branching, severe muscle injury was induced by two consecutive injections of 50 μL of 1.2% $BaCl_2$ in PBS two days apart into the gastrocnemius muscles of mice. Muscles were collected 21 days after the second injury. Subsequently, muscles were enzymatically digested for 1 h 20 min with gentle agitation and single myofibers were isolated as described previously (Kafadar et al. 2009), fixed with 3.7% formaldehyde in PBS and stained with 4’,6-diamidino-2-phenylindole (DAPI)

to identify nuclei. Regenerated myofibers, identified by the presence of central nuclei, were analyzed for the number and type of branches. A total of 300-450 myofibers from four mice were analyzed per genotype.

**Primary muscle cell culture, differentiation and fusion assays**

Primary myoblasts were isolated from the hindlimb muscles of WT and Bin3 KO mice of C57BL/6J-129/SvJ background (Ramalingam et al. 2008), WT C57BL/6 or WT BALB/c mice as previously described with the exception of a Percoll gradient (Bondesen et al. 2004). Cells were cultured in growth media (GM; Ham's F10, 20% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 μg/mL streptomycin, 5 ng/mL fibroblast growth factor (FGF)) on collagen-coated plates. Cultures were >95% myogenic as defined by MyoD immunostaining.

To induce differentiation, primary mouse myoblasts were plated on entactin-collagen IV-laminin (ECL; Millipore) in GM and after 1 h switched to differentiation media (DM; DMEM, 100 U/mL penicillin G, 100 μg/mL streptomycin, 1% Insulin-Transferrin-Selenium-A supplement (ITS; Gibco)) for 24 or 40-48 h. Cells were then fixed with 3.7% formaldehyde and immunostained with an antibody (F1.652; Developmental Studies Hybridoma Bank) against embryonic myosin heavy chain as previously described (Horsley et al. 2001).

The differentiation index was determined by dividing the total number of nuclei in embryonic myosin heavy chain (eMyHC) positive cells by the total number of nuclei counted (Jansen and Pavlath 2006). The fusion index was determined by dividing the total number of nuclei in myotubes by the total number of nuclei counted (Jansen and

Pavlath 2006). The average myonuclear number was determined by dividing the total number of nuclei in myotubes (≥ 2 nuclei) by the total number of myotubes counted (Jansen and Pavlath 2006). A total of 2500-5400 nuclei were analyzed per genotype or condition for each timepoint. To quantify myotube diameter using ImageJ, a range of 3-30 measurements were taken perpendicular to the axis of the myotube at equal distances along the axis of the myotube, depending on the length of the myotube. To quantify myotube length using ImageJ, a line was drawn along the entire myotube and only myotubes visible in their entirety were analyzed. Three to four independent isolates were analyzed per genotype or condition.

**Cell mixing experiments**

Myoblasts were differentiated into myocytes or nascent myotubes using different initial plating densities for 24 h in DM. Myocytes were then incubated with 2.5 μM CellTracker orange CMTMR dye (Invitrogen), and nascent myotubes with 0.5 μM CellTracker green CMFDA dye (Invitrogen) in DM for 10 min at 37°C. Subsequently, the dyes were removed, and the cells were incubated in DM for another 30 min at 37°C. Cells were trypsinized, mixed and plated in DM for an additional 24 h (Jansen and Pavlath 2006), at which time the cells were fixed in 3.7% formaldehyde and stained with DAPI. A total of 60-200 myotubes per experiment were analyzed for the presence of dual label. Three independent isolates were analyzed per genotype.

**Endocytosis assays**

Myoblasts were differentiated into myocytes (18 h in DM) or myotubes (24 h in DM) and switched to serum-free media lacking transferrin (DMEM, 10 mM Hepes pH 7.4, 0.2% bovine serum albumin (BSA)) at 37°C for 3 h prior to the assay. Cells were cooled to 4°C to inhibit endocytic internalization, followed by incubation with Alexa-594 conjugated transferrin (Invitrogen) in the presence or absence of Dynasore (Abcam), to allow both transferrin and Dynasore to equilibrate at the cell surface. Subsequently, in the continuous presence or absence of Dynasore, cells were either incubated at 37°C for 35 min to allow transferrin internalization, or kept at 4°C to prevent it. All cells were then returned to 4°C in the presence or absence of an acidic buffer (0.5 M NaCl, 15 mM MES (2-(N-Morpholino) ethanesulfonic acid hydrate (Sigma-Aldrich) pH 4.5 in PBS), to remove cell surface transferrin. Finally, while still at 4°C, all cells were fixed with 4% paraformaldehyde, and stained with DAPI. Intensities were measured with MetaMorph software version 6.1 (Molecular Devices, Sunnyvale, CA) as integrated pixel intensity, and the ratio (internalized/total) was plotted for some experiments. A total of 20-90 cells were analyzed per genotype or condition. One to three independent isolates were analyzed per genotype or condition.

**Cell migration assays**

Cell migration experiments were performed as previously described (Jansen and Pavlath 2006). Myoblasts (0 h in DM) or myocytes (18 h in DM) were visualized using an Axiovert 200M microscope with a 0.3 NA 10X Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.), and images were recorded using a camera (Qimaging) and OpenLab

5.5.2 (Improvision) software every 5 min for 3 h using time-lapse microscopy. Using ImageJ, the migratory paths of 60 individual cells were analyzed per genotype and mean cell velocities were calculated. Three to four independent isolates were analyzed per genotype.

**Phalloidin staining**

Differentiated muscle cells at 18, 24 or 40 h in DM were fixed with 3.7% formaldehyde and incubated in blocking buffer (0.1% Triton-X 100, 1% BSA in PBS) for 20 min, followed by Fluorescein isothiocyanate (FITC)-phalloidin (Enzo Life Sciences) in PBS with 1% BSA for 20 min. Nuclei were counterstained with DAPI. The percentage of cells with lamellipodia was quantified. A total of 130-160 cells were analyzed per genotype for each timepoint. Three independent isolates were analyzed per genotype.

**Immunostaining**

To detect endogenous or Human influenza hemagglutinin (HA)-tagged recombinant Bin3, myoblasts were differentiated for 18 or 24 h in DM, fixed with 3.7% formaldehyde and incubated in blocking buffer (5% donkey serum, 0.1% or 0.25% Triton X-100, 0.5% or 1% BSA in PBS), followed by an overnight incubation at 4°C with Bin3 hybridoma 3A4 (Ramalingam et al. 2008) or HA (Covance) primary antibodies. Primary antibodies were detected using biotin-conjugated donkey-anti mouse IgG (Jackson ImmunoResearch), HRP-conjugated streptavidin (PerkinElmer) and the tyramide signal amplification red reagent (Tyramide Signal Amplifcation (TSA) kit, Perkin Elmer). Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole).

To detect CKB, myoblasts were differentiated for 0, 18 or 40 h in DM, fixed and immunostained as previously described (O'Connor et al. 2008), using the TSA red reagent (Perkin Elmer). To detect CKM, myoblasts were differentiated for 0, 18 or 48 h in DM, fixed and immunostained with rabbit anti-CKM (a gift from Dr. Theo Wallimann, Institute of Cell Biology, Swiss Federal Institute of Technology Zurich) and the appropriate secondary antibody. Nuclei were counterstained with DAPI.

For CKB and α-sarcomeric actin colocalization studies, myoblasts were differentiated for 40 h in DM and immunostained for CKB as previously described (O'Connor et al. 2008). Immunostaining for α-sarcomeric actin was performed using anti-α-sarcomeric actin (Sigma) antibody, and the appropriate secondary antibody. Nuclei were counterstained with DAPI.

**Cyclocreatine experiments**

Myoblasts were incubated in the presence or absence of 5 mM 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine) from 18 to 42 h in DM. Cultures were then fixed and stained for various experiments. In all assays, three independent isolates were analyzed.

For phalloidin staining, cultures were fixed and stained as previously described (Simionescu-Bankston et al. 2013). A total of 100-150 myotubes were analyzed per condition. Intensities from 20 myotubes per condition were measured using ImageJ software as integrated pixel intensity. For CKB immunostaining, cultures were fixed and immunostained as previously described (O'Connor et al. 2008). A total of 80-200

myotubes were analyzed per condition. For all experiments, nuclei were counterstained with DAPI.

For fusion assays, cultures were fixed and immunostained with an antibody against eMyHC as previously described (Horsley et al. 2001). The differentiation and fusion indices, as well as the percentage of myotubes with > 5 nuclei were determined as previously described (Horsley and Pavlath 2003). A total of 6000-6800 nuclei were analyzed per condition.

**Retroviral infection**

Mouse Bin3 cDNA (Open Biosystems) with 3xHA tags at the C-terminus was cloned into the pBABE retroviral vector (Morgenstern and Land 1990) together with an IRES-EGFP marker. A control vector with an IRES-EGFP marker was also generated. WT and Bin3 KO myoblasts were subjected to 2-4 rounds of retroviral infection (Abbott et al. 1998) using either the HA-Bin3 vector or the control vector. Infected cells were subsequently grown in GM under puromycin selection at 0.75 μg/mL for a minimum of 48 h. The infection efficiency was >95% based on cell survival in the presence of puromycin.

**Real-time PCR**

Total RNA was isolated from WT myocytes using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, followed by treatment with DnaseI (Life Technologies). DnaseI-treated RNA (2 μg) was reverse transcribed using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). mRNA

levels were determined by real-time PCR using the SYBR Select Master Mix (Invitrogen), the StepOnePlus Real-Time PCR System and StepOne Software version 2.2.2 (Applied Biosystems, Life Technologies). All reactions were run in duplicate. Primers for mouse *AMPH* (PPM30542A), *BIN1* (PPM25097A), *BIN2* (PPM66366A) and *BIN3* (PPM26566A) were obtained from SABiosciences. Three independent isolates were analyzed. Mouse gastrocnemius muscle or brain was used as a positive control.

**RT-PCR**

Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, followed by treatment with DnaseI (Life Technologies). Subsequently, total RNA (2.5 μg) was reverse transcribed and PCR was performed as previously described (O'Connor et al. 2007), with the exception of utilizing *Taq* DNA polymerase (Qiagen) and the primers listed in Table 4.4.2. To control for genomic contamination, all primers spanned an intron-exon boundary for the genes that contained one. As a control, 18S cDNA was amplified using QuantumRNA 18S rRNA primers (Ambion), and PCR products were resolved and visualized as previously described (O'Connor et al. 2007). Reactions were performed on a minimum of two independent isolates.

**Plasmid construction**

To generate an N-terminal LexA-CKB bait construct for yeast two-hybrid assays, a truncated cDNA fragment encoding the human *CKB* gene (accession no.

NM_001823.4) (Burklen et al. 2007) was generated using PCR and cloned into the pBTM116 vector (Bartel et al. 1993).

To generate an N-terminal GFP-tagged CKB construct for co-immunoprecipitation experiments, a cDNA fragment encoding the full-length human *CKB* gene was generated using PCR and cloned into the pEGFP/C2 vector (Clontech). To generate N-terminal Myc-tagged constructs of the mouse genes of interest for co-immunoprecipitation experiments, cDNA fragments encoding the full-length mouse genes together with *Myc* were generated using PCR and cloned into a pCDNA3 plasmid (Clontech), or directly cloned into an N-terminal Myc tag-containing pCDNA3 plasmid.

To generate an N-terminal FLAG-copGFP-tagged CKB construct for actin binding assays, a cDNA fragment encoding the full-length human *CKB* gene together with *copGFP* and *FLAG* were cloned into pENTR/D-TOPO (Invitrogen), recombined in pDEST8 (Invitrogen), and expressed in Sf9 insect cells using the Bac-to-Bac system (Invitrogen). To generate an N-terminal FLAG-tagged Fascin-2 construct, a cDNA fragment encoding the mouse full-length *Fscn2* together with *FLAG* were cloned into pENTR/D-TOPO (Invitrogen), recombined into pDEST8 and expressed in Sf9 cells as previously described (Perrin et al. 2013). For all experiments, fusion protein expression was verified by immunoblotting.

**Yeast two-hybrid assays**

The yeast two-hybrid screen was performed using *S. cerevisiae* strain L40 (*MAT***a** *his3 trp1 leu2 ade2 LYS*::(*lexA op*)$_4$-*HIS3 URA3*::(*lexA op*)$_8$-*lacZ*) that harbors the *HIS3* and *lacZ* reporter genes (Hollenberg et al. 1995). The strain was initially transformed

with the LexA-CKB bait construct in the pBTM116 plasmid (Bartel et al. 1993), containing the *TRP1* marker. Once expression of the bait protein in yeast was verified using an anti-LexA antibody (Santa Cruz), L40 cells containing LexA-CKB were transformed with a Matchmaker 17-day-old mouse embryo cDNA library (Clontech) fused to the GAL4 activation domain in the pACT2 prey plasmid (Clontech), containing the *LEU2* marker. Subsequently, transformants were plated onto selective medium (lacking Leu, Trp, and His) and incubated for 5 days at 30°C. $His^+$ transformants were further screened for the formation of blue colonies in the β-galactosidase filter assay with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The library plasmids from positive clones were isolated, electroporated into *Escherichia coli* HB101 cells and retransformed into L40 cells containing the LexA-CKB construct, and blue colony formation assays were then repeated for plasmid linkage. Plasmid DNA isolated from positive clones was sequenced to identify the genes encoding the interacting proteins.

**Immunoprecipitation and immunoblotting**

To perform Rac1 and Cdc42 activity assays, myocytes were lysed in supplied magnesium lysis buffer (MLB; Millipore) containing protease/phosphatase inhibitors (Sigma), and centrifuged at 14,000 x *g* for 10 min. The pellet was then discarded and the supernatant was incubated for 30 min with 10 mM EDTA. The reaction was stopped by the addition of 60 mM $MgCl_2$. An equal amount of protein from each sample was incubated with 20 μL PAK1-PBD beads (Millipore) for 1.5 h at 4°C. The beads were then washed, spun quickly and the supernatants were subjected to SDS-PAGE and immunoblotting. The relative amounts of active Rac1 and Cdc42 were determined by

densitometric analysis. Two to three independent isolates were analyzed per genotype or condition.

For co-immunoprecipitation experiments, HEK 293 cells were co-transfected with 10 μg pEGFP-CKB/C2 plasmid and 10 μg pCDNA3 plasmid containing the Myc-tagged gene of interest using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post transfection, cells were harvested in RIPA-2 buffer containing protease inhibitors. Cell lysates were centrifuged and the supernatant was subjected to immunoprecipitation.

To immunoprecipitate GFP-CKB, Dynabeads Protein-A Kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, rabbit anti-GFP antibody (Invitrogen) or control rabbit IgG antibody (Santa Cruz) was cross-linked to the beads prior to the addition of protein extract (300-400 μg). After washing the beads, the immunoprecipitate was eluted, followed by SDS-PAGE and immunoblotting.

To immunoprecipitate Myc-tagged proteins, Protein A/G PLUS-Agarose beads (Santa Cruz) were used according to the manufacturer's instructions. Briefly, protein extract (500 μg) was incubated with mouse anti-Myc antibody (Santa Cruz) or control mouse IgG antibody (Santa Cruz) and then, Protein A/G PLUS-Agarose bead slurry was added. After washing the beads, the supernatants were resuspended in SDS loading buffer and subjected to SDS-PAGE and immunoblotting.

Samples were lysed in RIPA-2 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (Mini Complete; Roche) and centrifuged at 21,000 x *g* for 15 min. The pellet was then discarded and the supernatant was subjected to SDS-PAGE and immunoblotting. An

equal amount of protein from each sample was loaded onto 10% or 12% SDS-polyacrylamide gels and transferred to either a 0.2 μm or a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories). After blocking non-specific binding, the membranes were incubated with the appropriate primary antibodies. Primary antibodies used were as follows: Bin3 (3A4; Ramalingam et. al, 2008), Cdc42 (Santa Cruz), EF1α (Millipore), eMyHC (F1.652; Developmental Studies Hybridoma Bank), GFP (Invitrogen), HA (Covance), Hsp90 (Santa Cruz), Myc (Covance), Myogenin (F5D; Developmental Studies Hybridoma Bank), Rac1 (BD Biosciences) and Tubulin (Sigma-Aldrich). Primary antibodies were detected using appropriate horseradish peroxidase (HRP)-conjugated IgG (Jackson ImmunoResearch) secondary antibodies, followed by enhanced chemiluminescence.

**Protein expression and purification for actin binding assays**

Recombinant baculoviral DNA was transfected into small cultures of Sf9 insect cells using CellFectin II (Invitrogen). Four days later, recombinant baculovirus was harvested and the transfected cells were analyzed for FLAG-copGFP-CKB or FLAG-Fascin-2 protein expression by immunoblotting using the M2 anti-FLAG antibody (Sigma). After verification of fusion protein expression, large cultures were incubated for three days with amplified baculovirus before being harvested for protein purification. Cells were lysed and protein purified using anti-FLAG M2 affinity beads (Sigma) as previously described (Rybakova et al. 2002). Purified protein was dialyzed against PBS, concentrated and used for *in vitro* actin binding assays.

**F-actin binding assay**

Purified α-skeletal-actin (Cytoskeleton) was utilized for *in vitro* co-sedimentation assays as previously described (Henderson et al. 2010). Briefly, 0.5 μM FLAG-copGFP-CKB or 0.5 μM FLAG-Fascin-2 (Perrin et al. 2013) was incubated with 0-15 μM F-actin and subjected to high-speed centrifugation. Equal volumes of the supernatant (*S*) and the pelleted (*P*) F-actin filaments together with the interacting protein were resolved by SDS-PAGE, stained using Coomassie blue, and imaged with the Odyssey infrared scanner (Li-cor). The relative amount of F-actin-bound protein was determined by densitometric analysis, and quantified as the $P/(S+P)$ ratio. Data were plotted and fitted using nonlinear regression analysis (Graph-Pad Prism). Two independent experiments were performed with different FLAG-tagged protein and F-actin preparations.

**G-actin binding assay**

Purified non-filamentous globular actin (G-actin) (1 μM, Cytoskeleton) was incubated in the presence or absence of 1 μM purified FLAG-copGFP-CKB or anti-FLAG M2 affinity beads (Sigma) for 1 h at 4°C in G-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT). The beads were then pelleted and the supernatant collected. Subsequently, the beads were washed, and FLAG-copGFP-CKB was eluted with 0.1 mg/mL FLAG peptide (University of Minnesota Genomics Center). Equal volumes of starting material, FLAG-depleted supernatant and FLAG elution were electrophoresed using SDS-PAGE, and stained and imaged as described above.

**Image acquisition**

For the analysis of myofiber branching *in vivo*, the differentiation and fusion assays *in vitro*, as well as the Bin3 immunostaining in differentiated cells, images were obtained using an Axiovert 200M microscope (Carl Zeiss MicroImaging) with a 0.3 NA 10X Plan-Neofluar objective (Carl Zeiss MicroImaging) and were recorded using a camera (Qimaging) and OpenLab 5.5.2 (Improvision) software. For all other experiments, images were obtained using an Axioplan microscope (Carl Zeiss MicroImaging) with either a 0.3 NA 10X Plan-Neofluar objective (Carl Zeiss MicroImaging) or with a 0.8 NA 25X Plan-Neofluar objective (Carl Zeiss MicroImaging) and were recorded with a camera (Carl Zeiss MicroImaging) and Scion Image 1.63 (Scion Corporation) software. All images were assembled using Adobe Photoshop CS5.1 for Macintosh (Adobe) and equally processed for size, color levels, brightness, and contrast.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.0 for Macintosh (GraphPad Software). Statistical analysis to determine significance between two groups was performed using a Student's *t* test. One-way or two-way ANOVA with Bonferroni's posttest was used for comparisons between multiple groups as appropriate. Differences were considered to be statistically significant at $P<0.05$.

**Acknowledgements**

We thank the Emory University Custom Cloning Core Facility for generating the HA-Bin3 and control constructs. This work was supported by grants from the National Institutes of Health (AR-04731408 to G.K.P.; AR049899 to J.M.E.; AR007612 to J.T.O. and J.J.B; DK59888 to A.N.; GM077569 and NS42599 to V.F.) and from the Emory University Research Committee to V.F.

# Chapter 4: Creatine Kinase B Interacts with and Modulates the Actin Cytoskeleton During Myoblast Fusion

**A portion of this chapter is submitted as:**

**Simionescu-Bankston, A.**, Apponi, L.H., Wang, Y., Steeds, C., Olthoff, J.T., Belanto, J.J., Ervasti, J.M. and Pavlath, G.K. (2013) Creatine Kinase B interacts with and modulates the actin cytoskeleton during myoblast fusion. *Manuscript under review.*

**Contributions:**

Yanru Wang (Figures 4.4.3, 4.4.4, 4.4.5), James M. Ervasti lab (Figure 4.4.8).

# Chapter 4: Creatine Kinase B Interacts with and Modulates the Actin Cytoskeleton During Myoblast Fusion

## 4.1 Introduction

Formation, growth and repair of multinucleated skeletal muscle cells are dependent on fusion of progenitor myoblasts (Abmayr and Pavlath 2012). In spite of the importance of myoblast fusion, the molecular mechanisms underlying this process are incompletely understood. Cell culture models have been valuable for defining the various stages of myogenesis. During myogenesis, myoblasts differentiate into elongated myocytes, which migrate and adhere to one another. At cell-cell contact sites, a high density of adhesion molecules is found (Vasyutina et al. 2009). Following adhesion, downstream signaling pathways are activated, leading to localized actin changes at the cell-cell contact site (Peckham 2008). Next, membrane union takes place, leading to fusion of several differentiated myoblasts with one another to form nascent myotubes with few nuclei. Finally, differentiated myoblasts preferentially fuse with the ends of nascent myotubes (Peckham 2008) to generate mature myotubes containing many nuclei.

During myotube formation, some molecules become spatially restricted to specific cellular domains. A number of molecules regulating mammalian myoblast fusion localize to cell-cell contact sites in opposing muscle cells, including adhesion molecules and molecules associating with their intracellular domains. However, other molecules localize to cell-cell contact sites between two muscle cells in only one of the cells, and these are mainly intracellular molecules associated with regulating the actin cytoskeleton (Pavlath 2010b). Although the exact reasons for such localization during myogenesis are unknown, understanding why some molecules localize in this manner provides valuable

insights into the process of myoblast fusion.

We previously reported that Creatine Kinase B (CKB), the brain isoform of cytosolic CK enzymes, is prominently localized at myotube ends *in vitro* (O'Connor et al. 2008). CKB catalyzes the transfer of a phosphate group from phosphocreatine to ADP, thereby replenishing local ATP at sites of high ATP turnover (Wyss and Kaddurah-Daouk 2000). These findings suggested that the ends of myotubes are sites of high ATP demand. However, the molecules that require ATP generated by CKB for their cellular function are unknown. Such molecules may be functionally important for myogenesis. Interestingly, CKB can also localize to specific regions in non-muscle cells. CKB transiently accumulates in membrane ruffles of astrocytes and fibroblasts during cell spreading and migration, and ablation of CKB activity negatively affects these two processes (Kuiper et al. 2009). In addition, CKB is transiently recruited to the phagocytic cup of macrophages during phagocytosis, where inhibition of CKB activity diminishes actin accumulation (Kuiper et al. 2008). Finally, CKB localizes to hair bundles in the ear and CKB-deficient mice exhibit hearing loss (Shin et al. 2007). These studies indicate CKB activity is required at specific cellular locations for various functions. Further examination of CKB localization in muscle cells would enhance our understanding of the mechanisms underlying myotube formation.

To better understand the function of CKB at myotube ends, we sought to identify CKB interacting proteins using a yeast two-hybrid screen. We identified molecules with a broad diversity of roles, including actin polymerization, intracellular protein trafficking and alternative splicing, as well as sarcomeric components. In-depth studies of α-skeletal-actin and α-cardiac-actin, two predominant skeletal muscle actin isoforms, demonstrated

their biochemical interaction and partial colocalization with CKB near the ends of mouse myotubes *in vitro*. Importantly, inhibition of cytosolic CK activity resulted in both depolymerized filamentous actin (F-actin) in myotubes, as well as reduced myotube number and size. Together, our results suggest cytosolic CK enzymes play a key role in myotube formation by modulating actin dynamics during myogenesis.

## 4.2 Results

### CKB is localized in both myocytes and myotubes

To study CKB localization during *in vitro* myogenesis (Fig. 4.4.1A), we performed immunostaining in primary mouse muscle cells. We previously showed CKB is localized in nascent and mature myotubes (O'Connor et al. 2008), suggesting CKB activity is likely critical at the ends of myotubes. However, we show here that CKB also localizes at the ends of elongated myocytes (Fig. 4.4.1B), extending our previous results by suggesting that CKB activity is also important in myocytes prior to fusion. Thus, CKB may provide ATP for various molecules at the ends of differentiated muscle cells throughout myotube formation. In contrast, the muscle isoform of cytosolic CK enzymes (CKM), which localizes to the contractile apparatus (Schafer and Perriard 1988) and exhibits functional redundancy with CKB in replenishing ATP (Renema et al. 2007), was absent from the ends of differentiated muscle cells (Fig. 4.4.1C). The reciprocal localization of CKB and CKM suggests that these two enzymes may exhibit differential functions during myogenesis. Given the localization of CKB to the ends of differentiated muscle cells, proposed sites of fusion *in vitro* (Peckham 2008), we focused our subsequent studies on CKB.

### Screen for CKB interacting proteins by yeast two-hybrid assay

To gain insights into the function of CKB at the ends of differentiated muscle cells, we performed a yeast two-hybrid screen using the LexA-CKB fusion protein as bait to identify novel CKB interacting proteins. Since we were unable to express the full-length CKB protein in yeast, we instead utilized a previously described (Burklen et al.

2007) N-terminally truncated CKB construct (Fig. 4.4.2A), which includes the isoform-specific box B 260 that distinguishes CKB from CKM (Stolz and Wallimann 1998), and the highly conserved cysteine 283 (Cys 283) near the catalytic site. We confirmed expression of LexA-CKB in yeast by immunoblotting (Fig. 4.4.2B). Using this bait, we screened $1.2x10^6$ clones from an embryonic day 17 mouse cDNA library for CKB interacting proteins. Eight positive clones were isolated based on their ability to grow in SC-Histidine media and confirmed by activation of the *lacZ* reporter (Fig. 4.4.2C). No autoactivation of the *lacZ* reporter was observed for these clones (data not shown).

The positive clones obtained in the yeast two-hybrid screen encode proteins with a diversity of functions, including regulation of the actin cytoskeleton (Table 4.4.1). These results are significant given the importance of actin regulation for several steps in myotube formation, including elongation, migration and fusion (Abmayr and Pavlath 2012). We identified α-skeletal-actin, a skeletal muscle-specific actin isoform (Perrin and Ervasti 2010), as a putative CKB interacting protein. Additional putative CKB interacting proteins with roles in actin dynamics include Bridging integrator 3 (Bin3), Heat shock 27 kD protein 3 (Hsbp3), Septin-8 and Rho GTPase-activating protein 23 (Arhgap23). N-BAR domain proteins, such as Bin3, link membrane dynamics to the actin cytoskeleton (Frost et al. 2009, Ren et al. 2006). In addition, we recently showed that Bin3 is important for lamellipodia formation and muscle cell motility, as well as myotube formation and growth (Simionescu-Bankston et al. 2013). Small heat shock proteins, such as Hsbp3, inhibit actin polymerization by acting as capping proteins, or may protect the actin cytoskeleton against disruption induced by various stressful conditions (Mounier and Arrigo 2002). Septin family proteins, such as Septin-8, are small GTP-binding

proteins that can self-assemble into filaments and rings (Mostowy and Cossart 2012), help organize actin bundles (Kinoshita et al. 2002), and coordinate changes in cytoskeletal and membrane organization (Weirich et al. 2008). RhoGAP proteins, such as Arhgap23, negatively regulate the function of Rho GTPases (Moon and Zheng 2003) with critical roles in modulating actin dynamics (Hall 1998). Finally, several proteins identified in our screen are involved in regulating cellular processes other than the actin cytoskeleton. Transmembrane emp24-like trafficking protein 10 (Tmed10) and Gametogenetin binding protein 2 (Ggnbp2), are involved in intracellular protein trafficking (Strating and Martens 2009, Zhang et al. 2005), whereas Muscleblind-like RNA-binding proteins, such as Muscleblind-like 3 (Mbnl3), inhibit muscle differentiation and regulate alternative splicing of pre-mRNA (He et al. 2009, Lee et al. 2008, Squillace et al. 2002, Teplova and Patel 2008). Overall, the proteins identified in our screen regulate a number of highly ATP-dependent processes, which could require a localized source of ATP provided by CKB in muscle cells.

Since our goal was to gain insights into CKB function in muscle cells, we next examined whether the candidate proteins identified in our screen using a non-muscle library were expressed in muscle cells. We analyzed the expression of these candidates in pure cultures of primary mouse myoblasts differentiated into nascent myotubes (24 h) or mature myotubes (48 h) by RT-PCR. The genes identified in our screen were expressed at all stages of myogenesis, except *Acta*1, coding for α-skeletal-actin, which was present exclusively in differentiated muscle cells (Fig. 4.4.3).

**CKB interacts with α-actin and actin regulatory proteins**

Given the importance of actin regulation for myoblast fusion (Gruenbaum-Cohen et al. 2012, Kim et al. 2007, Nowak SJ et al. 2009, Vasyutina et al. 2009), we subsequently focused on studying the interaction of CKB with α-skeletal-actin and with actin regulatory proteins. The mRNA levels of α-skeletal-actin, the predominant actin isoform in adult mouse skeletal muscle, reach a peak during later stages of fusion in the mouse myoblast cell line C2C12 (Bains et al. 1984, Vandekerckhove et al. 1986). To verify the interaction between CKB and α-skeletal-actin biochemically, we expressed full-length GFP-CKB and Myc-α-skeletal-actin constructs in HEK 293 cells and performed co-immunoprecipitation experiments. Immunoprecipitation using either GFP or Myc antibodies, followed by immunoblotting, verified that GFP-CKB and Myc-α-skeletal-actin interacted in mammalian cells (Fig. 4.4.4A).

To determine whether the interaction between CKB and α-skeletal-actin is direct, we performed *in vitro* binding assays with purified proteins. As actin can exist in either a filamentous (F-actin) or globular (G-actin) state, we tested the interaction of CKB with α-skeletal-actin in each of these forms. Incubation of a fixed amount of purified FLAG-copGFP-CKB with increasing amounts of F-actin, followed by high-speed sedimentation, indicated CKB did not measurably cosediment with F-actin (Fig. 4.4.8A,B), whereas the positive control FLAG-Fascin-2 protein did (Perrin et al. 2013). Similarly, purified FLAG-copGFP-CKB did not co-immunoprecipitate with purified G-actin using an anti-FLAG antibody (Fig. 4.4.8C). These results indicate that CKB likely interacts with α-skeletal-actin indirectly via intermediary proteins.

Since α-skeletal-actin is only one of six mammalian actin isoforms, which include the other muscle isoforms (α-cardiac-actin, α-smooth-actin, γ-smooth-actin) and the cytoplasmic isoforms (β-cyto-actin, γ-cyto-actin) (Perrin and Ervasti 2010), we next determined whether CKB displays specificity in binding to any of these isoforms by co-immunoprecipitation. We focused on the isoforms expressed to a significant degree in cultured skeletal muscle cells during myogenesis: α-cardiac-actin, β-cyto-actin and γ-cyto-actin. α-cardiac-actin mRNA is rapidly induced upon differentiation, and decreases as myotube maturation proceeds (Bains et al. 1984); in contrast, β-cyto-actin and γ-cyto-actin mRNA levels decline rapidly when differentiation begins (Bains et al. 1984). To determine whether CKB interacted with these actin isoforms biochemically, we expressed full-length GFP-CKB and Myc-tagged-actin constructs in HEK 293 cells and performed co-immunoprecipitation experiments. Immunoprecipitation using either GFP or Myc antibodies, followed by immunoblotting, indicated that GFP-CKB interacted with Myc-α-cardiac-actin (Fig. 4.4.4B), but not with Myc-β-cyto-actin (Fig. 4.4.4C) or Myc-γ-cyto-actin (Fig. 4.4.4D), in HEK 293 cells. Together, these data demonstrate that CKB interacts specifically with α-actin isoforms in mammalian cells, but not with β- or γ-actin isoforms.

Finally, we also performed co-immunoprecipitation experiments to confirm the interaction between CKB and actin regulatory proteins identified in our screen (Fig. 4.4.3C, Table 4.4.1). We found Myc-Bin3 (Fig. 4.4.5A), Myc-Septin-8 (Fig. 4.4.5B) and Myc-Hsbp3 (Fig. 4.4.5C) also co-immunoprecipitated with GFP-CKB in HEK 293 cells. These data indicate CKB can interact with multiple molecules important for actin dynamics, a highly ATP-dependent process (Lodish et al. 2000).

**CKB activity is necessary for actin polymerization in myotubes, as well as myotube formation**

We next analyzed the functional relevance of CKB activity for the actin cytoskeleton in muscle cells. We initially examined the co-localization of CKB with α-skeletal-actin and α-cardiac-actin, the two predominant muscle actin isoforms. Primary mouse myoblasts were differentiated into myotubes and co-stained for CKB and α-sarcomeric actin using an antibody that recognizes both α-skeletal-actin and α-cardiac-actin, which are 99% identical (Vandekerckhove et al. 1986). These immunofluorescence analyses revealed that CKB and α-sarcomeric actin partially colocalized near myotube ends (Fig. 4.4.6), where α-sarcomeric actin extended all the way to the tips of myotubes, whereas CKB did not. In contrast to non-muscle cells, studying the role of CKB in differentiated muscle cells is complicated by both dual expression (Fig. 4.4.1B,C) of and functional redundancy between of CKB and CKM in replenishing ATP (Renema et al. 2007). In the presence of cyclocreatine, cytosolic CK isoforms are unable to generate ATP (Wyss and Kaddurah-Daouk 2000). To inhibit the activity of both cytosolic CK enzymes during myotube formation, we treated muscle cells with cyclocreatine at the onset of cell-cell fusion occurring at 18 h of differentiation. Subsequently, myotube cultures were fixed and F-actin was visualized using FITC-phalloidin 24 h later. Cyclocreatine treatment caused prominent changes in F-actin localization, as well as actin depolymerization in myotubes, manifested by lack of actin filaments (Fig. 4.4.7A). Quantification of the FITC-phalloidin fluorescent signal revealed a 54% loss of F-actin signal in myotubes following cyclocreatine treatment (Fig. 4.4.7B), indicating that CK activity is important for actin organization in myotubes. Interestingly, CKB localization

in myotubes remained unchanged following cyclocreatine treatment (Fig. 4.4.9), suggesting that cytosolic F-actin is not responsible for CKB localization at myotube ends.

Given the importance of actin dynamics for myoblast fusion (Gruenbaum-Cohen et al. 2012, Kim et al. 2007, Nowak SJ et al. 2009, Vasyutina et al. 2009), we also examined whether cyclocreatine treatment affected myotube formation. Immunostaining of muscle cells at 42 h of differentiation for embryonic myosin heavy chain (eMyHC), a marker of terminal differentiation, revealed decreased myotube formation (Fig. 4.4.7C) following cyclocreatine treatment. Subsequently, quantitative analyses showed a 49% decrease in the number of myotubes (Fig. 4.4.7D), a 56% decrease in the fusion index (Fig. 4.4.7E), and a 76% decrease in the percentage of myotubes with >5 nuclei (Fig. 4.4.7F) following cyclocreatine treatment. Since cyclocreatine was added after differentiation was mostly complete, the differentiation index was not significantly altered (Fig. 4.4.10A). Furthermore, cyclocreatine treatment did not cause significant cell death, as no change in the number of nuclei per field was noted (Fig. 4.4.10B). Overall, these data indicate cytosolic CK activity plays a key role in regulating actin polymerization in myotubes, as well as myotube formation.

**4.3 Discussion**

The dramatic relocalization of CKB to the ends of differentiated muscle cells during myotube formation suggested CKB activity is necessary for the function of molecules that turn over ATP rapidly at these sites. Using a yeast two-hybrid screen, we identified a number of novel CKB interacting proteins, many of which are involved in actin polymerization, an ATP-intensive process. We showed two muscle-specific actin isoforms, α-skeletal-actin and α-cardiac-actin, interacted and partially colocalized with CKB near myotube ends. Importantly, our studies identified a critical role for CK activity in both actin dynamics and myotube formation.

The finding that CKB interacts only with muscle-specific isoforms of actin, but not with the ubiquitously expressed β-cyto-actin or γ-cyto-actin isoforms, is novel. As skeletal-actin and α-cardiac-actin are 99% identical (Perrin and Ervasti 2010), whereas β-cyto-actin and γ-cyto-actin are 98% identical and 93% identical to the sarcomeric actin isoforms (Kashina 2006). Many actin-binding proteins, including ezrin (Yao X et al. 1996), βCAP73 (Shuster et al. 1996) and l-plastin (Namba et al. 1992), interact preferentially with non-muscle actin isoforms, such as β-cyto-actin, but not with α-actin, by co-sedimentation assays. Thus, very small differences among actin isoforms are enough to confer differential binding affinities to various proteins. However, how the specificity of the interaction between actin-binding proteins and various actin isoforms occurs is unknown.

Pharmacologic inhibition of CKB and CKM activity in differentiated muscle cells led to depolymerized F-actin, as well as to decreased myotube formation, suggesting CKB activity contributes in part to the regulation of actin dynamics necessary for

myoblast fusion. Additionally, other molecules necessary for myoblast fusion, whose function depends on the production of local ATP, may have also been affected by inhibition of cytosolic CK activity. Genetic or pharmacologic inhibition of cytosolic CK enzymes results in changes of the actin cytoskeleton in various cell types. For example, expression of a dominant-negative CKB enzyme (Hornemann et al. 2000) decreased actin accumulation in phagocytic cups of macrophages (Kuiper et al. 2008). Furthermore, CKB knockdown or treatment of osteoclasts with cyclocreatine reduced the formation of actin sealing-ring structures (Chang et al. 2008). Similarly, treatment of neonatal rat cardiomyocytes with the creatine analog β-guanidinopropionic acid (Wyss and Kaddurah-Daouk 2000), in the presence of the hypertrophic agonist phenylephrine (Yue et al. 2000), also led to actin depolymerization (Diguet et al. 2011). All these cellular processes are dependent on actin remodeling, which requires ATP at various steps. Therefore, cytosolic CK enzymes play an important role in rapid replenishment of local ATP at sites with high ATP turnover in multiple cell types.

A number of dynamic actin-based cellular structures play important roles at various steps during myotube formation, including migration and fusion. Prior to fusion, muscle cells exhibit lamellipodia, actin-based protrusions containing branched actin filaments, which are associated with motility (Small et al. 2002). Later in myogenesis, myoblasts align in preparation for fusion (Wakelam 1985). During early alignment of elongated myoblasts (Wakelam 1985), a cortical actin wall extends the length of the plasma membrane of the two aligned cells, possibly providing a temporal barrier for fusion until the myoblasts are completely aligned and ready to fuse (Duan and Gallagher 2009). Fusing myoblasts then exhibit filopodia, exploratory extensions from the plasma

membrane containing parallel bundles of actin filaments (Ridley 2011), which are used to connect fusing myoblasts along the entire length of the membrane contact (Stadler et al. 2010). Finally, during late myogenesis, myotube-myotube fusion occurs between the leading edge of lamellipodia of one myotube and the lateral plasma membrane of the other (Mukai and Hashimoto 2008). Given the involvement of lamellipodia and filopodia in various stages of myogenesis, disruption of actin polymerization in these protrusions by inhibition of cytosolic CK activity could impair the formation of multinucleated muscle cells. Furthermore, inhibition of cytosolic CK activity may also impair critical actin polymerization occurring at contact sites between two fusing muscle cells.

The finding that CKB localizes near myotube ends, where it partially colocalizes with sarcomeric actins, is highly significant given that myoblasts preferentially fuse with myotube ends (Peckham 2008), which are sites of extensive actin remodeling (Mukai and Hashimoto 2008). The dynamic nature of actin-based protrusions found at myotube ends may facilitate myoblast recognition, adhesion and fusion in this region. Actin remodeling near myotube ends may consume ATP too rapidly for replenishment by diffusion due to the large size of myotubes; therefore, these regions may require localized ATP production. Interestingly, CKB also localizes to specific cellular regions in conjunction with non-sarcomeric actins in non-muscle cells. In spreading astrocytes, a fraction of CKB and F-actin jointly accumulate in membrane ruffles, where actin-based structures are most dynamic (Kuiper et al. 2009). In macrophages and microglia not undergoing phagocytosis, CKB localizes to the cytoplasm; however, in cells undergoing phagocytosis, a portion of CKB is cytoplasmic and another is found at nascent phagosomes, suggesting a partial shift in localization (Kuiper et al. 2008). Interestingly,

F-actin is also found at nascent phagosomes in an almost complete overlap with CKB (Kuiper et al. 2008). These studies suggest CKB is localized to specific cellular regions of actin remodeling in multiple cell types.

How CKB becomes localized at myotube ends is unknown. Actin regulatory proteins identified in our screen, such as Bin3, could be responsible for CKB localization at these sites. However, CKB localization in myotubes was not altered in cells lacking Bin3 (data not shown). Furthermore, cyclocreatine treatment did not affect CKB localization in muscle cells, suggesting F-actin is not structurally required for maintaining CKB at myotube ends. In contrast, CKB localization in astrocytes was F-actin-dependent, as treatment with cytochalasin D, an inhibitor of actin polymerization (Casella et al. 1981), reduced the accumulation of both F-actin and CKB around fibronectin beads (Kuiper et al. 2009). These results suggest CKB localization is likely controlled by distinct mechanisms in different cell types. Further studies are required to identify the mechanisms regulating CKB localization in differentiated muscle cells.

In summary, our data suggest local ATP produced by CKB near the ends of differentiated muscle cells plays an important role in regulating actin polymerization and myotube formation. Future studies should be directed towards elucidating how CKB and its interacting partners work together to regulate myotube formation. Such studies will provide valuable insights into the regulation of myoblast fusion, and could further allow the development of therapies to enhance muscle growth.

## 4.4 Figures and Tables

**Figure 4.4.1: CKB and CKM localization during *in vitro* myogenesis**

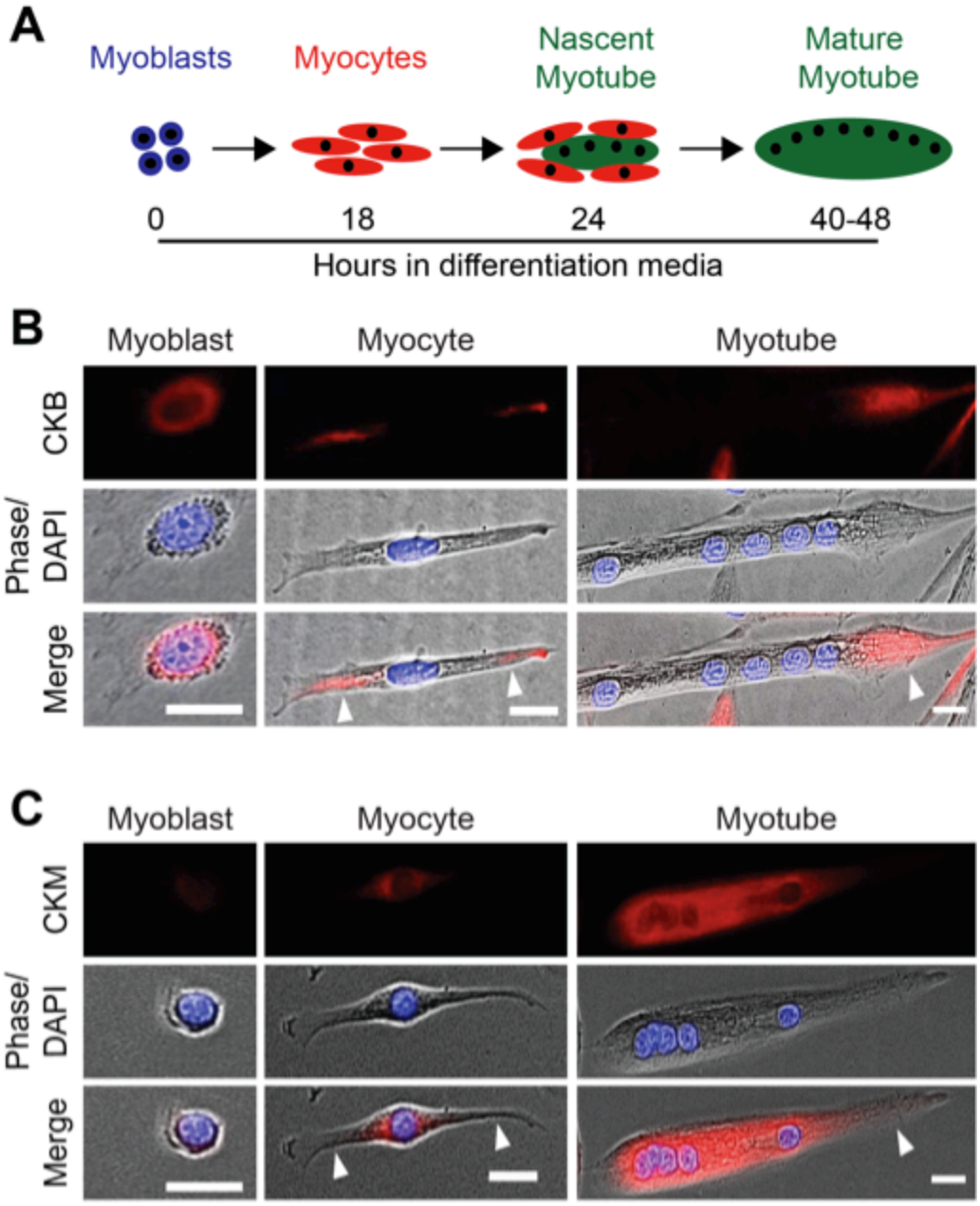

**Figure 4.4.1: CKB and CKM localization during *in vitro* myogenesis**

**(A)** During *in vitro* myogenesis, myoblasts differentiate into myocytes, which fuse to one another to form nascent myotubes. Subsequently, myocytes fuse with nascent myotubes, giving rise to mature myotubes. **(B, C)** Pure cultures of mouse muscle cells were immunostained for CKB or CKM at various stages of myogenesis. CKB localized throughout the cytoplasm in myoblasts, but near the ends of myocytes and myotubes (arrowheads). In contrast, CKM was not expressed in myoblasts, and was absent from the ends of both myocytes and myotubes (arrowheads). Nuclei were counterstained with DAPI. Bar, 50 μm.

**Figure 4.4.2: Identification of CKB interacting proteins by yeast two-hybrid assay**

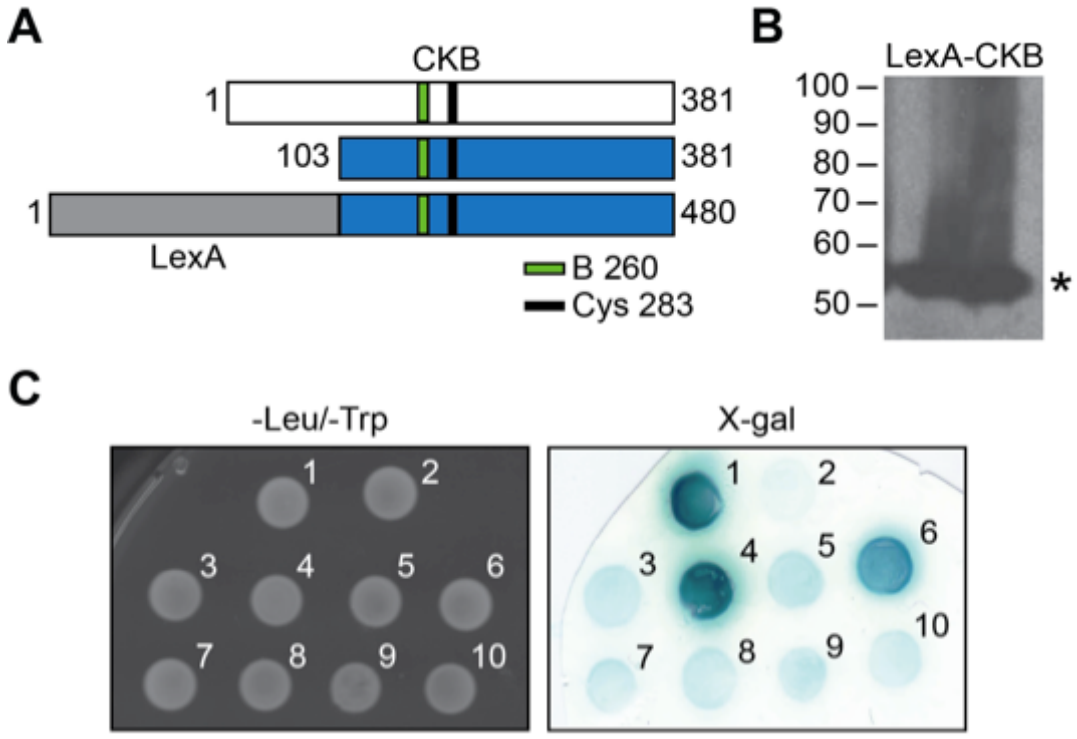

**Figure 4.4.2: Identification of CKB interacting proteins by yeast two-hybrid assay** **(A)** Full-length human CKB (white box) is composed of 381 amino acids. A truncated human CKB construct (amino acids 103-381, blue box) was fused to the LexA DNA binding domain (grey box) and used as bait in the yeast two-hybrid screen. This bait protein contains the CKB-specific box B 260 (green line) and the highly conserved cysteine 283 (Cys 283) (black line) near the catalytic site. **(B)** Expression of LexA-CKB (*) in yeast was verified by immunoblot. **(C)** Yeast expressing LexA-CKB were transformed with various constructs, including Creatine kinase, Muscle (*CKM*) (positive control, 1), vector alone (negative control, 2), as well as the positive clones obtained in the yeast two-hybrid screen: *Bin*3 (3), *Sept*8 (4), *Tmed*10 (5), *Ggnbp*2 (6), *Acta*1 (7), *Arhgap*23 (8), *Hsbp*3 (9) and *Mbnl*3 (10). Transformants were plated on selective medium (lacking Leu, Trp), and assayed for β-galactosidase activity (X-gal). Positive interactions are indicated by blue color on X-gal plates.

**Figure 4.4.3: Candidates isolated in the yeast two-hybrid screen are expressed in muscle cells**

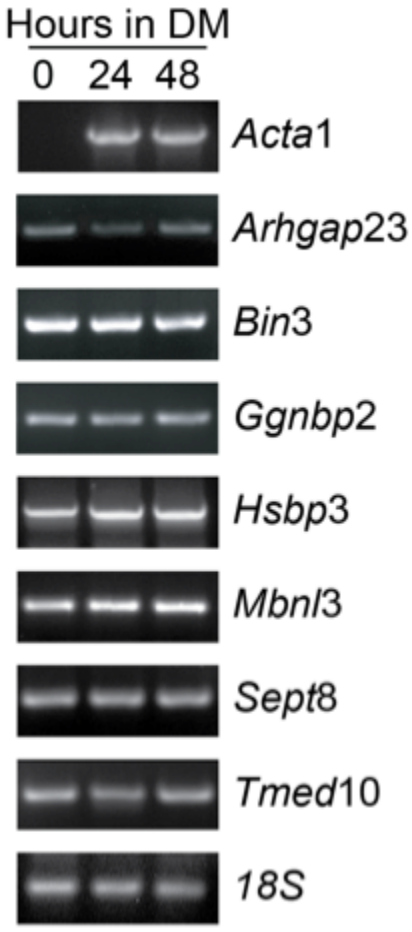

**Figure 4.4.3: Candidates isolated in the yeast two-hybrid screen are expressed in muscle cells**

mRNAs for the genes identified in the yeast two-hybrid screen were present in muscle cells using RT-PCR. All mRNAs except for *Acta*1 were expressed throughout differentiation, whereas *Acta*1 was only expressed at 24 and 48 h in differentiation media (DM). 18S rRNA was used as an internal control. *n*=2-3 independent isolates.

**Figure 4.4.4: CKB interacts with α-actin, but not with β-cyto-actin or γ-cyto-actin isoforms**

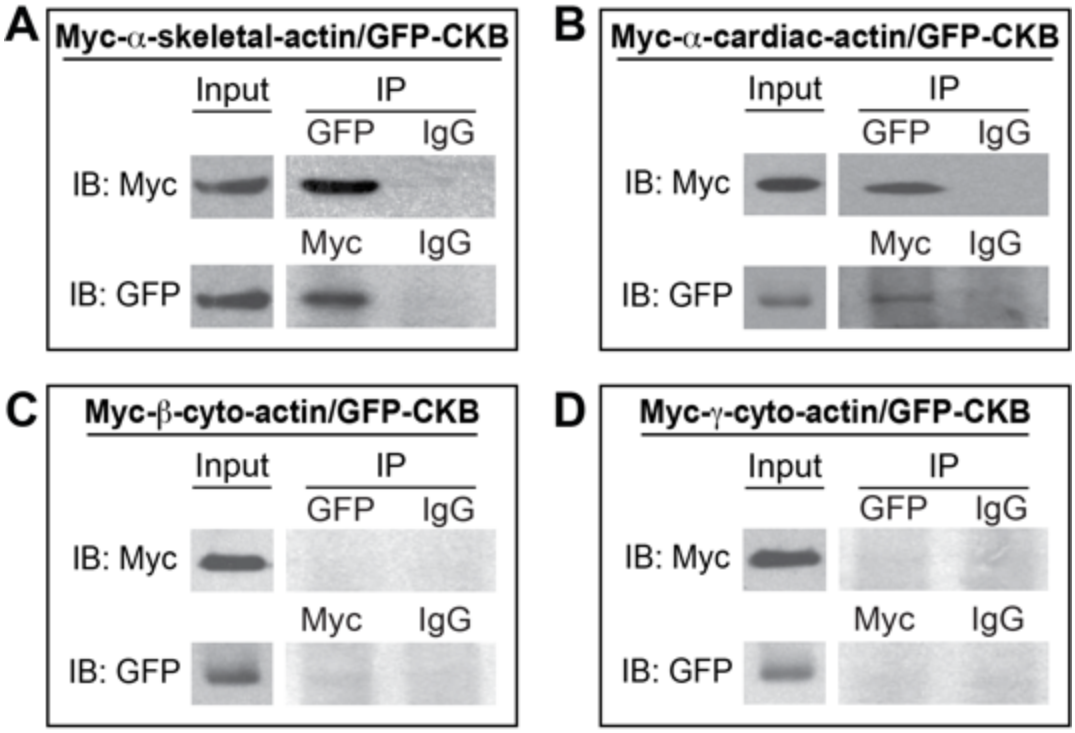

**Figure 4.4.4: CKB interacts with α-actin, but not with β-cyto-actin or γ-cyto-actin isoforms**

Co-immunoprecipitation experiments were performed using GFP-CKB and various Myc-tagged actin fusion proteins. GFP-CKB co-immunoprecipitated with **(A)** Myc-α-skeletal-actin and **(B)** Myc-α-cardiac-actin, but not **(C)** Myc-β-cyto-actin or **(D)** Myc-γ-cyto-actin as shown by immunoblot (IB). Immunoprecipitations (IP) were performed using either GFP or Myc antibodies. As a negative control, IgG was substituted for the primary antibody.

**Figure 4.4.5: CKB interacts with actin regulatory proteins**

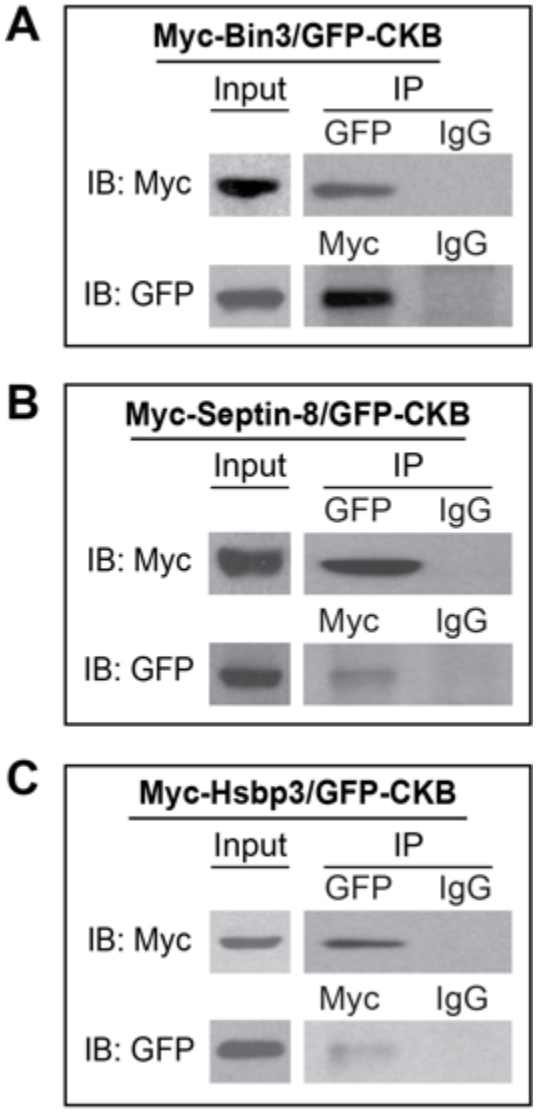

**Figure 4.4.5: CKB interacts with actin regulatory proteins**

GFP-CKB co-immunoprecipitated with **(A)** Myc-Bin3, **(B)** Myc-Septin-8 and **(C)** Myc-Hsbp3 as shown by immunoblot (IB). Immunoprecipitations (IP) were performed using either GFP or Myc antibodies. As a negative control, IgG was substituted for the primary antibody.

**Figure 4.4.6: CKB and α-sarcomeric actin colocalize near the ends of myotubes**

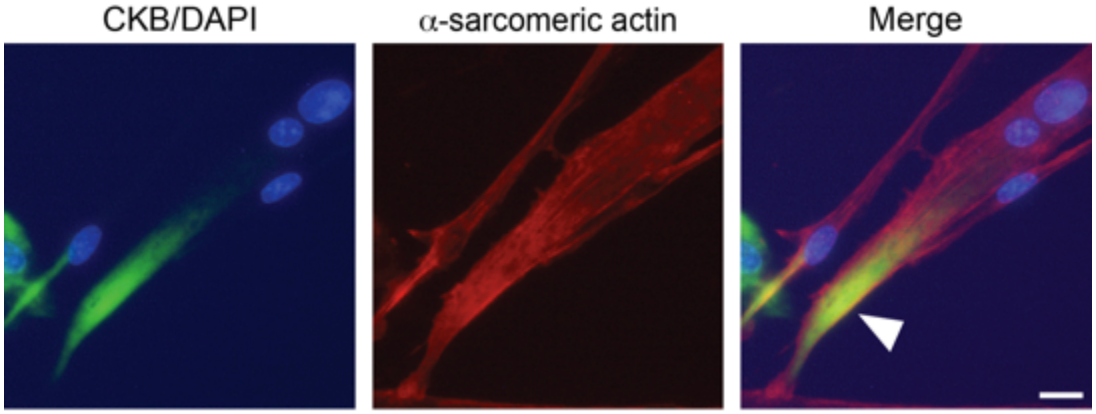

**Figure 4.4.6: CKB and α-sarcomeric actin colocalize near the ends of myotubes**

CKB did not extended all the way to the tips of myotubes (left panel), whereas α-sarcomeric actin did (middle panel). The merge shows colocalization of the two proteins near the ends of myotubes (right panel, arrowhead). Nuclei were counterstained with DAPI. Bar, 50 μm.

**Figure 4.4.7: Cyclocreatine inhibits actin polymerization in myotubes, as well as myotube formation**

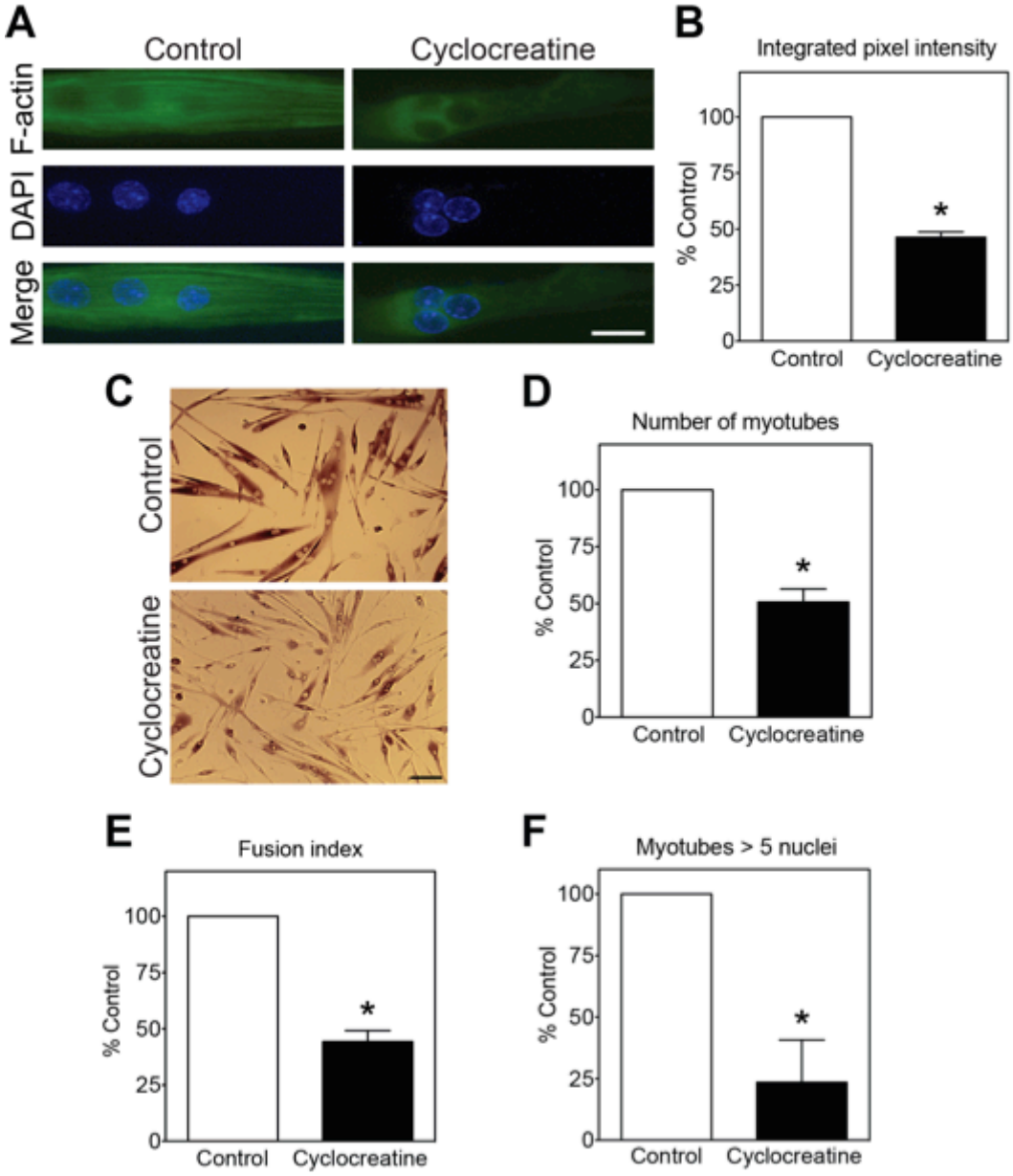

**Figure 4.4.7: Cyclocreatine inhibits actin polymerization in myotubes, as well as myotube formation**

Muscle cells were treated with cyclocreatine from 18 to 42 h of differentiation. **(A)** Cyclocreatine altered F-actin (FITC-phalloidin) localization in myotubes. Nuclei were counterstained with DAPI. Bar, 50 μm. **(B)** The FITC-phalloidin fluorescent signal (integrated pixel density) (**P*<0.05) in myotubes was decreased by cyclocreatine treatment. **(C)** Cultures immunostained for eMyHC are shown at 42 h of differentiation. Bar, 150 μm. **(D)** The number of myotubes (**P*<0.05), **(E)** fusion index (**P*<0.05) and **(F)** the percentage of myotubes with >5 nuclei (**P*<0.05) were decreased by cyclocreatine treatment. Data are mean ± s.e.m., *n*=3 independent isolates.

**Figure 4.4.8: CKB does not directly interact with α-skeletal actin**

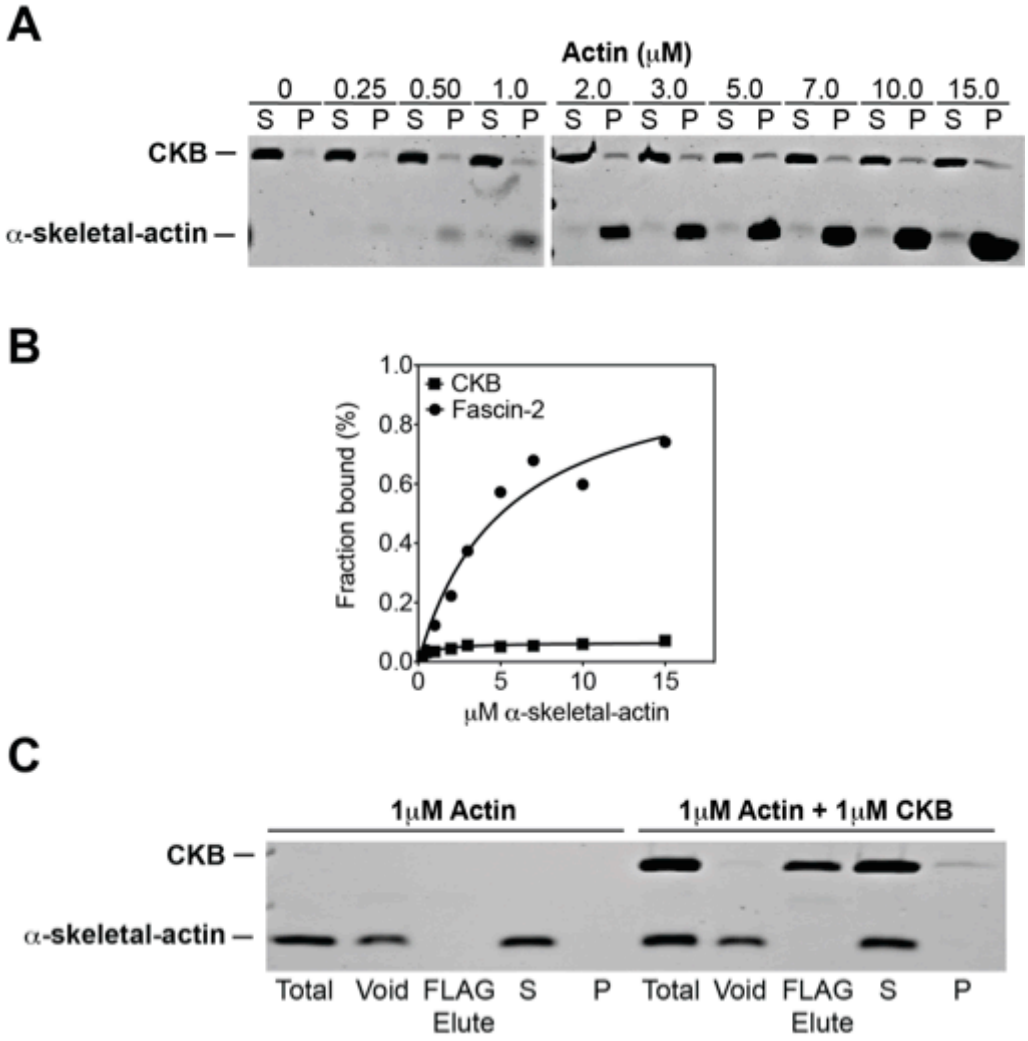

**Figure 4.4.8: CKB does not directly interact with α-skeletal actin**

**(A)** Increasing amounts of F-actin were incubated with FLAG-copGFP-CKB or FLAG-Fascin 2 with subsequent high-speed centrifugation. Coomassie blue-stained SDS-PAGE gel of supernatants (*S*) and pellets (*P*) is shown. **(B)** The fraction of F-actin-bound FLAG-copGFP-CKB (square symbols) or FLAG-Fascin-2 (round symbols) was plotted against the concentration of F-actin, and the binding data were fitted using nonlinear regression analysis. FLAG-Fascin-2 data were previously published (Perrin et al. 2013) and shown here for comparison. Data are mean, $n$=2 independent experiments performed with different FLAG-tagged protein or F-actin preparations. **(C)** Shown is a Coomassie blue-stained SDS-PAGE gel of non-filamentous G-actin incubated in the presence (right) or absence (left) of purified FLAG-copGFP-CKB with anti-FLAG M2 affinity beads followed by elution with a FLAG peptide. Equivalent samples were also subjected to high-speed centrifugation and the resulting supernatants (S) and pellets (P) analyzed to assess the polymerization state of actin.

**Figure 4.4.9: Cyclocreatine does not modify CKB localization in myotubes**

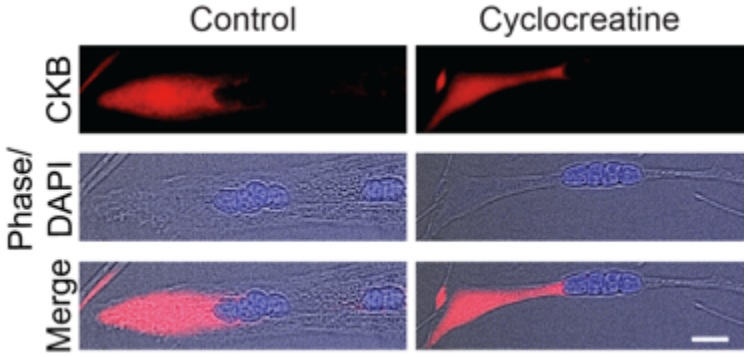

**Figure 4.4.9: Cyclocreatine does not modify CKB localization in myotubes**

Muscle cells were treated with cyclocreatine from 18 to 42 h of differentiation. No difference in CKB localization in myotubes was noted. Nuclei were counterstained with DAPI. Bar, 50 μm. *n*=3 independent isolates.

**Figure 4.4.10: Cyclocreatine does not alter differentiation or the number of nuclei per field**

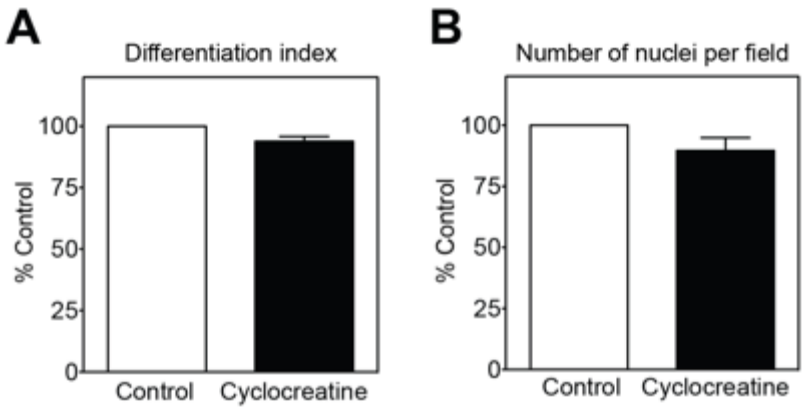

**Figure 4.4.10: Cyclocreatine does not alter differentiation or the number of nuclei per field**

Muscle cells were treated with cyclocreatine from 18 to 42 h of differentiation, followed by eMyHC immunostaining. No significant differences were noted in **(A)** the differentiation index or **(B)** the number of nuclei analyzed per field. Data are mean ± s.e.m., *n*=3 independent isolates.

**Table 4.4.1: CKB-interacting proteins identified in the yeast two-hybrid screen**

| Gene Symbol | Protein Name | Type of Molecule | Function* |
|---|---|---|---|
| *Acta*1 | α-skeletal-actin | Skeletal muscle-specific cytoskeletal protein | *Sarcomere component* |
| *Bin*3 | Bridging integrator 3 | BAR domain protein | *Actin polymerization* |
| *Hsbp*3 | Heat shock 27 kD protein 3 | Small heat shock protein | |
| *Sept*8 | Septin-8 | GTP-binding protein | |
| *Arhgap*23 | Rho GTPase-activating protein 23 | GTPase-activating protein towards Rho/Rac/Cdc42 | |
| *Tmed*10 | Transmembrane emp24-like trafficking protein 10 (yeast) | Type I membrane protein | *Intracellular protein trafficking* |
| *Ggnbp*2 | Gametogenetin binding protein 2 | Zinc finger protein | |
| *Mbnl*3 | Muscleblind-like 3 (Drosophila) | RNA-binding protein | *Alternative splicing* |

*As many of these proteins have several functions, only the function most relevant for myogenesis is listed. In addition, some of these protein functions are only predicted, or have only been shown for some family members.

**Table 4.4.2: RT-PCR primers used to study mRNA expression**

| Gene | Accession no. | Primer Sequence (5' ->3') |
|---|---|---|
| *Acta*1 | NM_001272041.1 | Fwd: ATGTGCGACGAAGACGAGACC<br>Rev: CGGAATTCCTAGAAGCATTTGCGGTGC |
| *Arhgap*23 | NM_021493.2 | Fwd: AAGGCAGATGAACCTTGGAT<br>Rev: GAGGGCAAAAGTGAAGGTTG |
| *Bin*3 | NM_021328.3 | Fwd: GGGAGTATGGAAAACTGCAG<br>Rev: GTTGAGGCTTGGGAATATGC |
| *Ggnbp*2 | NM_153144.2 | Fwd: GTGATGGAATTTCCTGACAA<br>Rev: TTTGCATCAGTCATGCAGCT |
| *Hsbp3* | NM_019960.2 | Fwd: AGTGCGTTATCAGGAGGAGT<br>Rev: GTGTTCGTCCATTCTGGTTC |
| *Mbnl*3 | NM_134163.4 | Fwd: CTAATCCTGCCATGGCTTTC<br>Rev: CGGCCTTTAATGTAATCCAT |
| *Sept*8 | NM_033144.2 | Fwd: TCTTCAACACGACCTTTGAG<br>Rev: CGTGATGAAGTAGAGGCAAA |
| *Tmed*10 | NM_026775.4 | Fwd: ACAGATTCTGCTGGCCATAT<br>Rev: TTAACAATGGACTCGGAAAG |

# Chapter 5: The N-BAR Domain Protein, Bridging Integrator 3, Regulates Rac1- and Cdc42-Dependent Processes in Myogenesis

**A portion of this chapter is published as:**

**Simionescu-Bankston**, **A.**, Leoni, G., Wang, Y., Pham, P.P., Ramalingam, A., DuHadaway, J.B., Faundez, V., Nusrat, A., Prendergast, G.C. and Pavlath, G.K. (2013) The N-BAR domain protein, Bin3, regulates Rac1- and Cdc42-dependent processes in myogenesis. *Dev. Biol.* 382:160-171.

**Contributions:**

Peter P. Pham (Figure 5.4.2 data), Giovanna Leoni (Figure 5.4.7 data).

# Chapter 5: The N-BAR Domain Protein, Bridging Integrator 3, Regulates Rac1- and Cdc42-Dependent Processes in Myogenesis

## 5.1 Introduction

Skeletal muscle growth and repair occur by the process of myogenesis, in which myogenic progenitor cells differentiate, migrate and fuse with one another to form multinucleated myofibers (Abmayr and Pavlath 2012). The plasma membranes of myogenic cells undergo dynamic changes to facilitate various stages of myogenesis (Abramovici and Gee 2007, Kim et al. 2008, Mukai et al. 2009, Stadler et al. 2010, Yoon et al. 2007), including inward membrane invaginations occurring with endocytosis, and outward membrane protrusions during cell migration (Suetsugu et al. 2010). Coordinated changes in actin polymerization at the plasma membrane provide the force for these dynamic membrane alterations. A key question is how changes in the plasma membrane and rearrangements of the actin cytoskeleton are regulated and coordinated at different stages of myogenesis.

The Bin-Amphiphysin-Rvs (BAR) domain superfamily of proteins regulates both membrane and actin dynamics via BAR domains, crescent shaped dimers that bind to membranes and can either sense or induce membrane curvature (Habermann 2004). The inward or outward direction of membrane bending is generally dependent on the particular class of BAR domain, such as classical BAR, N-terminal amphipathic helix-BAR (N-BAR), Fes/CIP4 Homology-BAR/FCH-BAR (F-BAR), BAR-Pleckstrin Homology (BAR-PH), PhoX-BAR (PX-BAR) or Inverse-BAR/IMD-BAR/IRSp53-MIM Homology domain (I-BAR) (Frost et al. 2009, Habermann 2004, Quinones and Oro 2010,

Suetsugu 2010, Suetsugu et al. 2010). Many BAR domain proteins also regulate Rho GTPases and/or other actin regulatory proteins (de Kreuk and Hordijk 2012), and therefore may link membrane dynamics to rearrangements of the actin cytoskeleton. Based on these functions, BAR domain proteins would be predicted to be key regulators of myogenesis; however, these proteins have been little studied in skeletal muscle.

The best characterized BAR domain protein in mammalian skeletal muscle is GTPase regulator associated with focal adhesion kinase-1 (GRAF1), a BAR-PH domain protein containing a Rho GTPase-activating protein (RhoGAP) domain and an SH3 domain (Doherty JT et al. 2011, Hildebrand et al. 1996). GRAF1 regulates differentiation and fusion in the mouse muscle cell line C2C12, and is critical for muscle development in *Xenopus laevis* embryos (Doherty JT et al. 2011). During myogenesis, GRAF1 is localized to the tips of elongating myoblasts, where it is proposed to locally limit the polymerization of filamentous actin (F-actin) (Doherty JT et al. 2011). Additional functions ascribed to GRAF1 include cell spreading and migration in HeLa cells (Doherty JT et al. 2011) and fluid-phase endocytosis in fibroblasts (Lundmark et al. 2008). The only other BAR domain protein studied in mammalian skeletal muscle is Bridging integrator 1 (Bin1), an N-BAR domain protein with an SH3 domain, which regulates differentiation and fusion in C2C12 cells (Wechsler-Reya et al. 1998) and in primary myoblasts *in vitro* (Fernando et al. 2009), and also facilitates sarcomere organization in muscles of mice *in vivo* (Fernando et al. 2009). These studies highlight the importance of BAR domain proteins in muscle differentiation and fusion, but raise questions about the interplay between BAR domain proteins of various classes in regulating myogenesis.

We studied the role of Bridging integrator 3 (Bin3), a ubiquitously expressed (Prendergast et al. 2009) and evolutionarily conserved (Ren et al. 2006) N-BAR domain protein in skeletal muscle. In contrast to the previously studied BAR domain proteins in myogenesis, Bin3 contains only the N-BAR domain (Ren et al. 2006). Both the budding and fission yeast orthologs of Bin3, Rvs161p and Hob3p, respectively, have critical roles in F-actin localization in yeast (Ren et al. 2006). The ability of Hob3p to modulate actin dynamics has been proposed to result from its interaction with the Rho GTPase Cdc42 (Coll et al. 2007, Routhier et al. 2001). Interestingly, Rvs161p also regulates endocytosis and cell-cell fusion (Ren et al. 2006), two cellular processes intimately associated with myotube formation (Abmayr and Pavlath 2012, Doherty et al. 2008, Posey et al. 2011). Loss of Bin3 in mice leads to juvenile cataracts with a near total loss of F-actin in lens fiber cells (Ramalingam et al. 2008). However, the role of Bin3 in regulating endocytosis, cell-cell fusion and actin dynamics during myogenesis is unknown.

Using Bin3 null mice, we show Bin3 is required for proper formation of multinucleated muscles both *in vivo* and *in vitro*. Defects in lamellipodia formation and cell migration were noted in the absence of Bin3 in differentiated muscle cells prior to myotube formation. Importantly, the levels of active Rac1 and Cdc42 were greatly decreased in the absence of Bin3. As Rac1 and Cc42 are important for actin dynamics in muscle cells *in vitro* (Vasyutina et al. 2009), and are essential for muscle cell fusion both *in vitro* and *in vivo* (Vasyutina et al. 2009), these studies identify a major role for a Bin3-dependent signaling pathway in regulating Rac1 and Cdc42- dependent processes during myotube formation.

## 5.2 Results

### Muscle regeneration defects occur in Bin3 KO mice

We observed that the steady-state levels of Bin3 were transiently increased at early stages of muscle regeneration when myogenic progenitor cells are differentiating, migrating and fusing to form small myofibers (Fig. 5.4.1A). These results suggested a potential role for Bin3 in regulating muscle regeneration. To determine the functional role of Bin3 during muscle regeneration, the growth of regenerating myofibers in tibialis anterior muscles of wild-type (WT) and Bin3 null (KO) mice was analyzed at various timepoints after injury (Fig. 5.4.1B). No difference in myofiber cross-sectional area (CSA) was observed between WT and Bin3 KO muscles prior to injury (Fig. 5.4.1C). In contrast, myofiber CSA was transiently decreased by 28% in Bin3 KO muscles at 10 days post injury (Fig. 5.4.1D), indicating a delay in regeneration in the absence of Bin3.

Further analyses of regenerating muscles revealed a pattern suggestive of myofiber branching, an abnormal regenerative outcome associated with severe injury and muscular dystrophy (Pavlath 2010a). In branched myofibers, the plasma membrane of the parent myofiber is contiguous with several smaller myofibers (Pavlath 2010a). To analyze the function of Bin3 in regulating myofiber branching during severe injury, individual myofibers were isolated from the gastrocnemius muscles of WT and Bin3 KO mice 21 days following the second of two injuries. While myofiber branching was increased in both WT and Bin3 KO muscles after injury, Bin3 KO muscles exhibited an 18% increase in the percentage of branched myofibers (Fig. 5.4.2A). However, the percentage of regenerated myofibers, which could affect the overall percentage of branched myofibers, did not differ between WT and Bin3 KO muscles (Fig. 5.4.2B). To

gain a deeper understanding of the myofiber branching observed, we examined both the number and type of branches in WT and Bin3 KO muscles. We found that Bin3 KO muscles exhibited a 27% increase in the percentage of myofibers with two or more branches after injury (Fig. 5.4.2C). Furthermore, we noted three distinct patterns of branched myofibers: bifurcated, split and process (Fig. 5.4.8); however, the percentage of regenerated myofibers with these branching patterns did not differ between WT and Bin3 KO muscles (Fig. 5.4.2D). In contrast, we observed a 2.7 fold increase in the percentage of regenerated Bin3 KO myofibers exhibiting a mix of these different patterns (Fig. 5.4.2D,E), suggesting more complex myofiber branching. Together, these data highlight a function for Bin3 in muscle growth and myofiber branching during muscle regeneration.

**Bin3 is necessary for myotube formation *in vitro***

The regeneration defects observed in Bin3 KO muscles *in vivo* could result from impairments in multiple cell types contributing to the repair process. To distinguish between cell-autonomous and non-autonomous effects of Bin3 on myogenesis, satellite cells were isolated from hindlimb muscles of WT and Bin3 KO mice and analyzed *in vitro* in the absence of other cell types. During *in vitro* myogenesis, satellite cell-derived myoblasts differentiate into myocytes, which then migrate and fuse to one another to form nascent myotubes, small myotubes with few nuclei; subsequently, more myocytes fuse in with nascent myotubes, giving rise to mature myotubes, large myotubes containing many nuclei (Abmayr and Pavlath 2012). Immunoblotting analyses revealed that muscle cells expressed Bin3 at all stages of differentiation (Fig. 5.4.3A). Similarly, by immunostaining, Bin3 was present in all muscle cells in the culture (Fig. 5.4.3B). To

test whether Bin3 regulates myotube formation, WT and Bin3 KO muscle cells were differentiated into nascent myotubes for 24 h, or into mature myotubes for 40 h, and immunostained for embryonic myosin heavy chain (eMyHC), a marker of differentiation. eMyHC staining revealed that Bin3 KO myotubes were smaller at both stages (Fig. 5.4.3C). Quantitative analyses subsequently showed that Bin3 KO myotubes exhibited a 33% decrease in the fusion index at 24 h (Fig. 5.4.3D), and contained 20% fewer nuclei at both 24 and 40 h (Fig. 5.4.3E). In addition, Bin3 KO myotubes were 12% thinner at both 24 and 40 h (Fig. 5.4.3F) and 19% shorter at 24 h (Fig. 5.4.3G). However, the number of nuclei analyzed per field (Fig. 5.4.3H), which could affect the extent of myotube formation, did not differ between WT and Bin3 KO cultures. Moreover, the steady-state levels of myogenin (Fig. 5.4.9A,B) and eMyHC (Fig. 5.4.9C,D), early and late markers of differentiation, respectively, did not differ between WT and Bin3 KO muscle cells. Overall, these data indicate that Bin3-dependent processes within muscle cells are necessary for proper myotube formation.

Functional redundancy by other N-BAR domain proteins during myogenesis may exist and could diminish the effects of Bin3 loss on myotube formation. Therefore, we examined mRNA levels of Bin3 and the most closely related N-BAR domain proteins of the Amphiphysin/Bin family (Table 5.4.1) by real-time PCR. Besides Bin3, only Bin1 was expressed in primary muscle cells (Table 5.4.2). However, since loss of Bin1 results in differentiation defects early in myogenesis (Wechsler-Reya et al. 1998), we could not test whether Bin1 can partially compensate for Bin3 at the later stages of myogenesis analyzed in our studies.

We also assessed whether retroviral-mediated overexpression of recombinant HA-Bin3 in WT cells (Fig. 5.4.10A) was sufficient to enhance myotube size. By eMyHC staining, myotubes did not appear larger at either 24 or 40 h of differentiation (Fig. 5.4.10B). Subsequent quantification revealed slight but statistically insignificant increases in the fusion index (Fig. 5.4.10C) and the number of nuclei per myotube (Fig. 5.4.10D), likely due to a small increase in the number of nuclei per field (Fig. 5.4.10F) following Bin3 overexpression. The differentiation index was not altered following Bin3 overexpression (Fig. 5.4.10E). The inability of Bin3 overexpression to enhance myotube formation is likely due to downstream mediators of Bin3 action being rate-limiting.

**Endocytosis defects are not observed in Bin3 KO myocytes**

One of the yeast orthologs of Bin3 regulates endocytosis (Ren et al. 2006), a process likely to be important for myogenesis. Indeed, molecules regulating endocytosis have recently been implicated in myotube formation (Doherty et al. 2008, Leikina et al. 2013, Posey et al. 2011). Thus, we hypothesized that an endocytic defect could contribute to impaired myotube formation in the absence of Bin3. Since many BAR domain proteins regulate receptor-mediated endocytosis, the most common and well-studied pathway utilized for internalization (Qualmann et al. 2011), we tested whether Bin3 regulates this process in muscle cells using fluorescently labeled transferrin. We labeled WT and Bin3 KO myocytes (18 h in DM) with Alexa-594 conjugated transferrin to allow (37°C) or prevent (4°C) transferrin internalization, in the presence or absence of an acid wash to remove the cell surface transferrin and permit analysis of only the internalized fraction (Fig. 5.4.11A). No difference in the internalized transferrin fraction was observed

between WT and Bin3 KO myocytes (Fig. 5.4.11B). To ensure that we could detect a difference in transferrin internalization, WT myocytes were treated with Dynasore, an inhibitor of dynamin-dependent endocytosis (Macia et al. 2006), prior to performing the internalization assay. Dynasore treatment caused a reduction in transferrin internalization in WT myocytes at 37°C (Fig. 5.4.11C,D). Together, these data suggest Bin3 is not necessary for receptor-mediated endocytosis in myocytes.

**Bin3 plays a role in myocyte migration**

Cell migration is another process critical for myotube formation (Bae et al. 2008, Bondesen et al. 2007, Jansen and Pavlath 2006, Mylona et al. 2006, O'Connor et al. 2007). BAR domain proteins of different classes, including the N-BAR domain protein Bridging integrator 2 (Bin2) (Sánchez-Barrena et al. 2012), have been implicated in regulating cell migration (de Kreuk et al. 2011, Doherty GJ et al. 2011, Guerrier et al. 2009, Pichot et al. 2010, Quinones et al. 2010, Tsuboi et al. 2009). As migration of both myoblasts and myocytes is important for myotube formation, we analyzed the role of Bin3 in regulating the migration of both cell types using time-lapse microscopy. The migratory cell paths of WT and Bin3 KO myoblasts were only slightly different (Fig. 5.4.12A), and their average velocity did not differ significantly (Fig. 5.4.12B,C). In contrast, Bin3 KO myocytes migrated shorter distances than WT cells (Fig. 5.4.4A), and their average velocity was decreased by 30% (Fig. 5.4.4B,C). Retroviral-mediated expression of recombinant HA-Bin3 in Bin3 KO cells (Fig. 5.4.4D) rescued their migration defect and restored the average velocity to WT levels (Fig. 5.4.4E). These results show Bin3 is required specifically for migration of myocytes during myogenesis.

**Bin3 is involved in lamellipodia formation in myocytes**

Actin polymerization is harnessed for cell motility and drives the forward extension of lamellipodia, broad actin-based protrusions associated with motility (Le Clainche and Carlier 2008, Ridley 2011, Small et al. 2002). Interestingly, the two yeast orthologs of Bin3 both regulate F-actin localization (Ren et al. 2006), and Bin3 KO mice exhibit loss of F-actin in lens fiber cells (Ramalingam et al. 2008). Therefore, we reasoned that Bin3 may also be important for actin-dependent processes in muscle cells. We visualized F-actin in WT and Bin3 KO myocytes at 18 and 24 h of differentiation using FITC-phalloidin. We observed fewer Bin3 KO myocytes with lamellipodia at these timepoints (Fig. 5.4.5A). Following quantification, depending on the timepoint, 33-57% fewer Bin3 KO myocytes exhibited lamellipodia (Fig. 5.4.5B). Subsequently, we found that Bin3 and F-actin colocalized in lamellipodia of myocytes (Fig. 5.4.6A). Due to low levels of endogenous Bin3 in muscle cells, we retrovirally expressed recombinant HA-Bin3 and performed HA immunostaining to better examine the localization of Bin3. HA-Bin3 also colocalized with F-actin in lamellipodia of myocytes (Fig. 5.4.6B). Together, these data demonstrate that Bin3 regulates lamellipodia formation in myocytes.

**Decreased levels of active Rac1 and Cdc42 in Bin3 KO myocytes**

Rho GTPases play critical roles in regulating actin dynamics during cell migration (Ridley 2011). In particular, Rac1 and Cdc42 are associated with actin regulation in lamellipodia (Ridley 2011). Based on the function of Bin3 in regulating lamellipodia formation in myocytes, we hypothesized that Bin3 may regulate the activity of Rac1 and Cdc42. Pull-down of active Rac1 and Cdc42 using beads coated with the p21-binding

domain (PBD) of p21-activated protein kinase 1 (PAK1), termed PAK1-PBD, followed by immunoblotting, showed a major decrease in the active levels of these Rho GTPases (Fig. 5.4.7A) in Bin3 KO myocytes. Quantification of immunoblots revealed decreases of approximately 70% in the active levels of both Rho GTPases (Fig. 5.4.7B) in Bin3 KO myocytes. Retroviral-mediated expression of recombinant HA-Bin3 in Bin3 KO cells (Fig. 5.4.7C) led to a 2.4 fold increase in the levels of active Rac1 and a 3.3 fold increase in the levels of active Cdc42 (Fig. 5.4.7D). In addition, HA-Bin3 was detected in a complex with active Rac1 and Cdc42 (Fig. 5.4.7C) in Bin3 KO myocytes. These data indicate that a Bin3-dependent pathway is a key regulator of Rac1 and Cdc42 activity in myocytes.

**5.3 Discussion**

Myofiber formation during myogenesis is key to muscle regeneration. Our data provide insights into the mechanisms by which dynamic rearrangements of the actin cytoskeleton are regulated during myogenesis. We show the N-BAR domain protein, Bin3, is important for myogenesis both *in vitro* and *in vivo*.

Muscle regeneration is a complex process requiring interplay between myogenic and non-myogenic cells (Chazaud et al. 2003, Saclier et al. 2012, Sonnet et al. 2006). Absence of Bin3, a ubiquitously expressed protein (Prendergast et al. 2009), resulted in a transient delay in the growth of regenerating myofibers after injury. Muscle-intrinsic functions of Bin3 likely contributed in part to the growth phenotype, as myocyte migration and myotube formation *in vitro* were impaired in the absence of Bin3. Potentially Bin3 function may also be required in non-myogenic cells during regeneration, and the absence of Bin3 in these cells may have further contributed to the observed growth phenotype. The transient delay in myofiber growth during regeneration may be due to compensation by other molecules that regulate this process. Functional compensation may also explain in part the fact that myofiber size did not differ between WT and Bin3 KO muscles in the absence of injury.

In the absence of Bin3 we also observed abnormal branched myofibers. Although myofiber branching has been studied for many years, Bin3 is the only molecule found to regulate this process besides the G protein coupled receptor, mouse odorant receptor 23 (MOR23) (Griffin et al. 2009). While the mechanisms underlying myofiber branching during muscle regeneration are unknown, interestingly both MOR23 and Bin3 regulate

myocyte migration and myotube formation *in vitro* (Griffin et al. 2009), suggesting aberrations in these processes may contribute to the formation of branched myofibers.

Since muscle cell migration plays a crucial role in myotube formation (Bae et al. 2008, Bondesen et al. 2007, Jansen and Pavlath 2006, Mylona et al. 2006, O'Connor et al. 2007), we initially investigated the role of Bin3 in migration. We found Bin3 was required only for myocyte migration, but not for myoblast migration. Bin3 likely interacts with different downstream molecules in these two cell types, leading to the specificity in regulating stage-specific cellular migration. With the exception of actin, only secreted molecules and transmembrane proteins have been shown to regulate muscle cell migration (Simionescu and Pavlath 2011). Thus, Bin3 is a novel cytoplasmic protein controlling muscle cell migration. Interestingly, Bin2, an N-BAR domain protein with a C-terminal tail containing acidic and serine/proline-rich segments (Ge K. and Prendergast G. C. 2000, Sánchez-Barrena et al. 2012), has recently been implicated in monocyte migration (Sánchez-Barrena et al. 2012). Together, these data associate N-BAR domain proteins with cell migration, a process requiring the formation of outward membrane protrusions, which N-BAR domains are not classically linked with (Suetsugu 2010).

Dynamic changes in the actin cytoskeleton are critical for cells to extend protrusions to sense their environment and subsequently migrate towards a target. Migrating cells extend filopodia, thin exploratory extensions from the plasma membrane containing parallel bundles of actin filaments (Ridley 2011) or lamellipodia, broad protrusions containing branched actin filaments (Le Clainche and Carlier 2008, Ridley 2011, Small et al. 2002). Many BAR domain proteins positively regulate actin polymerization and the formation of outward membrane protrusions (de Kreuk and

Hordijk 2012). Some BAR domain proteins are enriched, and can colocalize with F-actin, in these protrusions (de Kreuk and Hordijk 2012). Indeed, the N-BAR domain protein Bin3 colocalized with F-actin in lamellipodia and was important for lamellipodia formation in myocytes. In contrast, the BAR-PH domain protein GRAF1 localizes to sites devoid of F-actin in muscle cells (Doherty JT et al. 2011). These data suggest that BAR domain proteins of different classes may have complementary roles in regulating actin dynamics in myogenesis.

Myotube formation requires Rac1 and Cdc42-dependent actin polymerization (Vasyutina et al. 2009), but the upstream signals controlling the activity of these Rho GTPases in differentiated muscle cells are unknown. The levels of active Rac1 and Cdc42 were greatly decreased in myocytes in the absence of Bin3, suggesting Bin3 is a major positive regulator of Rac1 and Cdc42 in muscle cells. The fusion defect observed in Bin3 KO myotubes *in vitro* was less severe than seen in Rac1 or Cdc42 KO cells (Vasyutina et al. 2009), possibly due to the residual low levels of these GTPases in Bin3 KO myocytes. In contrast, the BAR-PH domain protein, GRAF1, is a negative regulator of RhoA activity in C2C12 muscle cells but does not affect Cdc42 activity in L6 myoblasts (Doherty JT et al. 2011), suggesting that BAR domain proteins of different classes show specificity in controlling GTPase activity in myogenesis. As we did not observe a visible difference in actin stress fibers between WT and Bin3 KO myocytes, we suggest that levels of active RhoA, a major regulator of stress fiber formation (Pellegrin and Mellor 2007), are unlikely to be affected in Bin3 KO myocytes.

We detected recombinant Bin3 in a complex with active Rac1 or Cdc42 in myocytes. How could Bin3, a protein with only an N-BAR domain, regulate the activity

of Rac1 and Cdc42 in muscle cells? Some BAR domain proteins modulate the activity of Rho GTPases via a RhoGAP or RhoGEF domain (de Kreuk and Hordijk 2012). In contrast, BAR domain proteins that lack a RhoGAP/GEF domain regulate Rho GTPases by recruiting other proteins with RhoGAP/GEF activity. For example, one of the Bin3 yeast orthologs, Hob3p, facilitates the interaction between Gef-1, a Cdc42GEF, and Cdc42 (Coll et al. 2007). Bin3 may similarly interact with an unknown RhoGAP/GEF protein to modulate the activity of Rac1 and Cdc42 in muscle cells. Moreover, Bin3 could heterodimerize with another BAR domain protein (Ren et al. 2006), which ultimately serves as the link to modulating RhoGAP/GEF activity. Additional studies are necessary to define Bin3 interacting partners to better understand the mechanisms by which Bin3 may regulate Rac1 and Cdc42 activity in myocytes. Understanding these mechanisms may help explain the myofiber branching phenotype observed in the absence of Bin3 *in vivo*, and whether Rac1 and Cdc42 are also implicated in this process. In addition, Bin3 may also regulate myogenesis through Rho GTPase-independent mechanisms.

The study of BAR domain proteins is an emerging area in skeletal muscle. Our studies extend previous work in this field (Doherty JT et al. 2011, Wechsler-Reya et al. 1998) and suggest that multiple classes of BAR domain proteins will prove critical for regulating important cellular processes during myogenesis. Understanding the role of BAR domain proteins in muscle growth and repair will likely impact treatments for muscle diseases in which these processes are impaired.

## 5.4 Figures and Tables

**Figure 5.4.1: Bin3 is required for muscle regeneration**

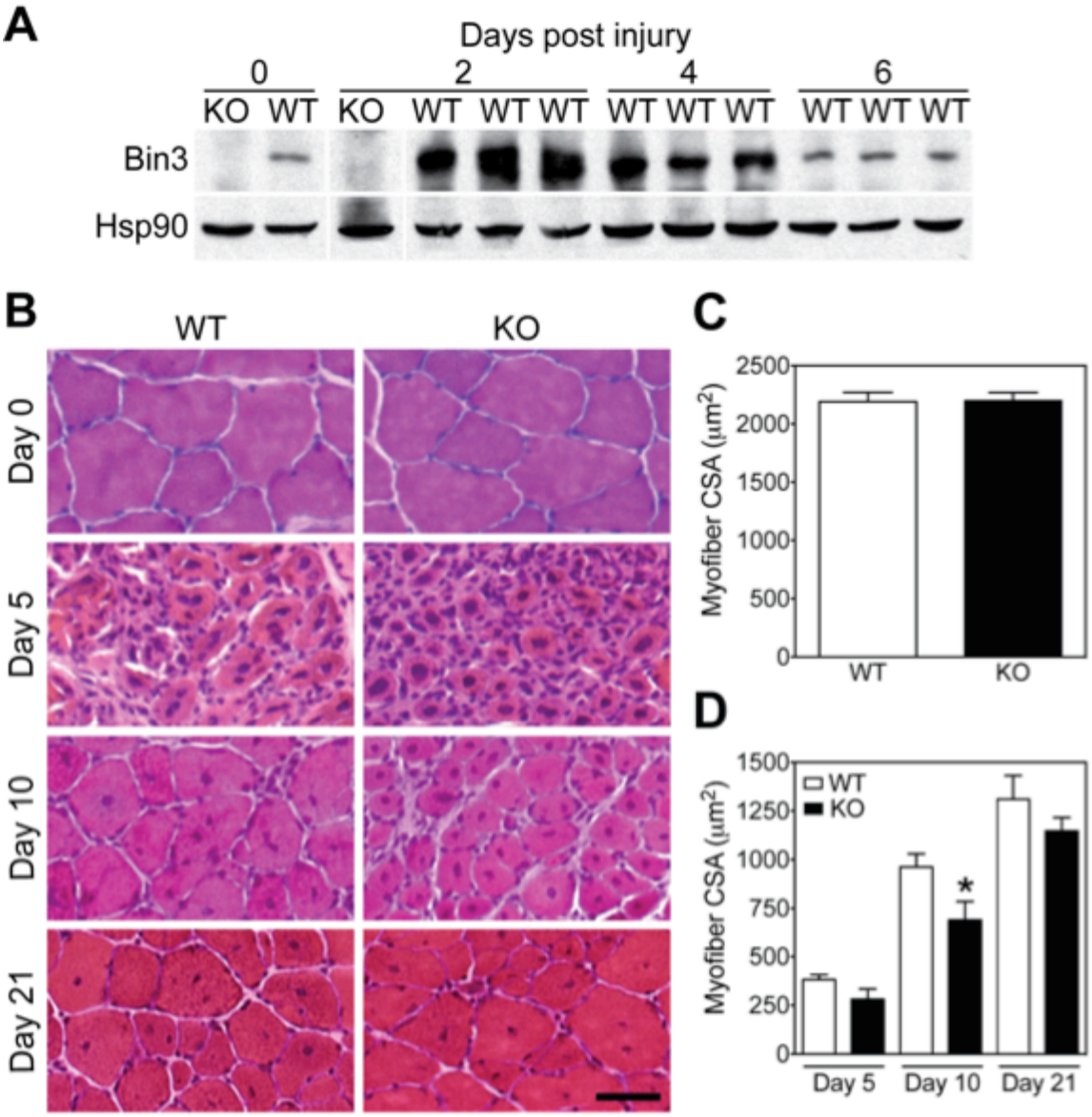

**Figure 5.4.1: Bin3 is required for muscle regeneration**

**(A)** Bin3 protein levels were transiently increased in gastrocnemius muscles of WT mice at 2 and 4 days post injury. Specificity of the Bin3 antibody was demonstrated by lack of antibody reaction in Bin3 KO muscles. Hsp90 was used as a loading control. **(B)** Hematoxylin and eosin stained sections are shown from tibialis anterior (TA) muscles of WT and Bin3 KO mice at 0, 5, 10 and 21 days after injury. Bar, 50 μm. **(C)** No difference in the cross-sectional area (CSA) of uninjured TA myofibers was observed between genotypes. **(D)** The CSA of regenerating TA myofibers was transiently decreased in Bin3 KO mice at 10 days post injury (**P*<0.05). Data are mean ± s.e.m., *n*=4-7 mice for each genotype per timepoint.

**Figure 5.4.2: Bin3 is involved in myofiber branching**

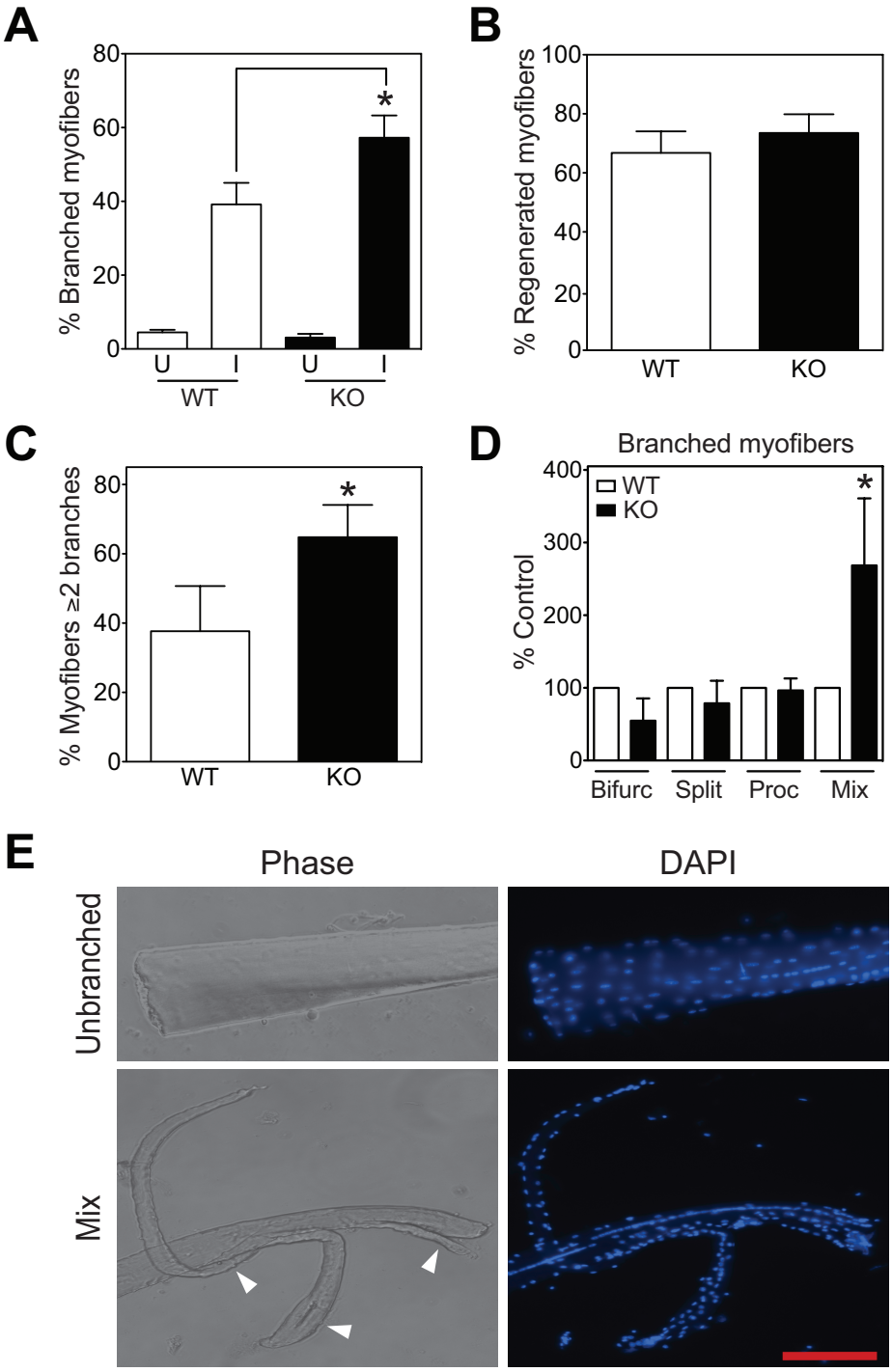

**Figure 5.4.2: Bin3 is involved in myofiber branching**

**(A)** Bin3 KO muscles contained a greater percentage of branched myofibers after injury (I) than WT muscles. Minimal branching was observed in uninjured muscles (U) regardless of genotype (**P*<0.05). **(B)** No difference in the percentage of regenerated myofibers was observed between genotypes. **(C)** Bin3 KO muscles exhibited a greater percentage of myofibers with 2 or more branches after injury (**P*<0.05). **(D)** Bin3 KO muscles contained a greater percentage of regenerated myofibers with a mix of branching patterns (**P*<0.05). Bifurc = Bifurcated; Proc = Process. **(E)** Myofibers after injury visualized with phase contrast microscopy and DAPI are shown. Myofiber with a mix of branching patterns (arrowheads) is shown in comparison to an unbranched myofiber. Bar, 150 μm. Data are mean ± s.e.m., *n*=4 mice for each genotype.

**Figure 5.4.3: Myotube formation *in vitro* is altered in the absence of Bin3**

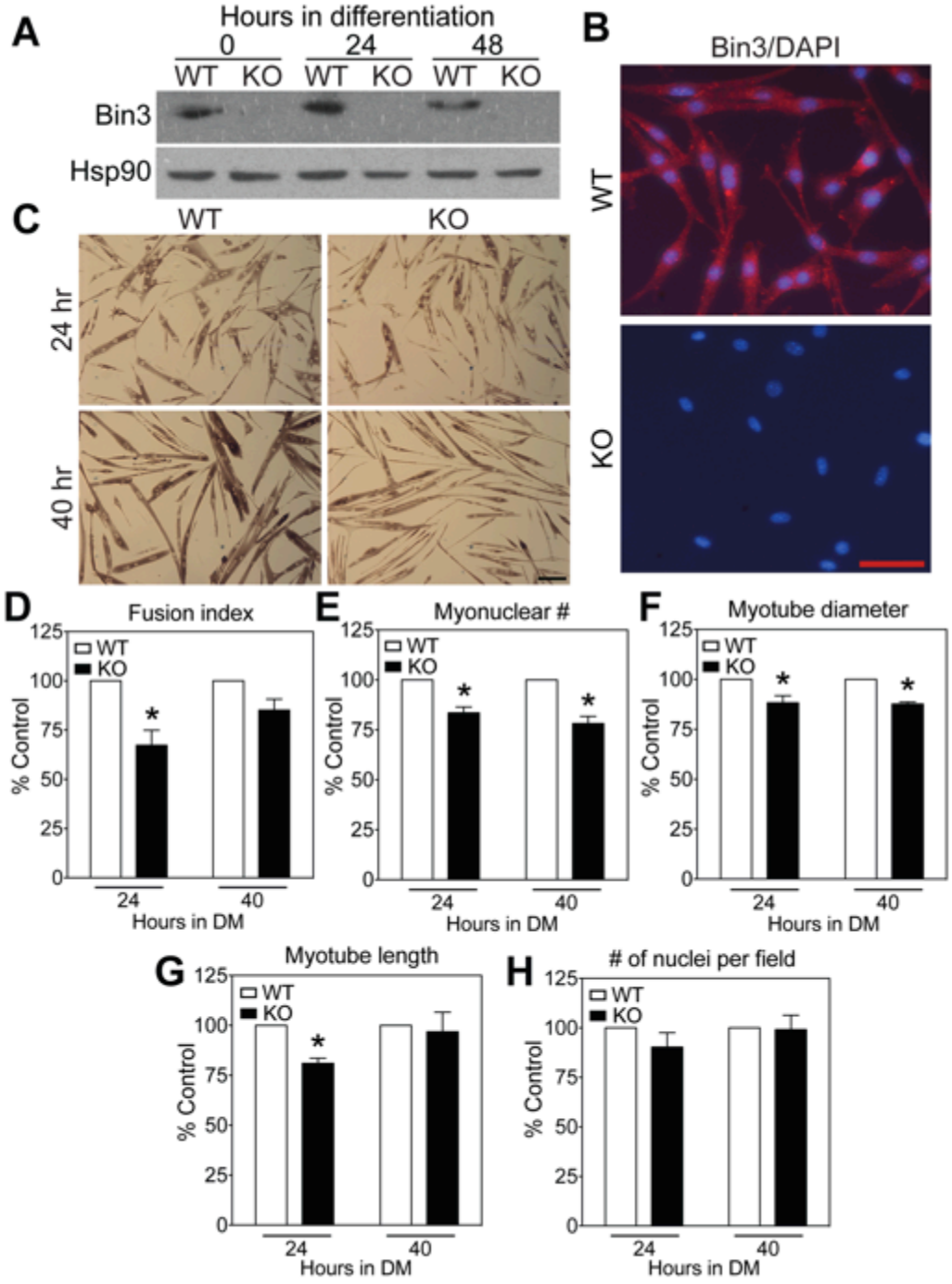

**Figure 5.4.3: Myotube formation *in vitro* is altered in the absence of Bin3**

**(A)** Bin3 protein was expressed in WT muscle cells throughout differentiation. Specificity of the Bin3 antibody was demonstrated by lack of antibody reaction in Bin3 KO muscle cells. Hsp90 was used as a loading control. **(B)** Immunostaining of differentiated WT muscle cells with an anti-Bin3 antibody revealed Bin3 was expressed in all muscle cells in the culture. Nuclei were counterstained with DAPI. Bar, 150 μm. **(C)** WT and Bin3 KO muscle cells immunostained for eMyHC are shown at 24 and 40 h in DM. Bar, 150 μm. **(D)** Fusion index (**P*<0.001), **(E)** myonuclear number (**P*<0.001), **(F)** myotube diameter (**P*<0.01) and **(G)** myotube length (**P*<0.05) were decreased in Bin3 KO muscle cells in DM. No differences were noted in the number of nuclei per field **(H)** between WT and Bin3 KO muscle cells. Data are mean ± s.e.m., *n*=4 independent isolates for each genotype.

**Figure 5.4.4: Bin3 plays a role in myocyte migration**

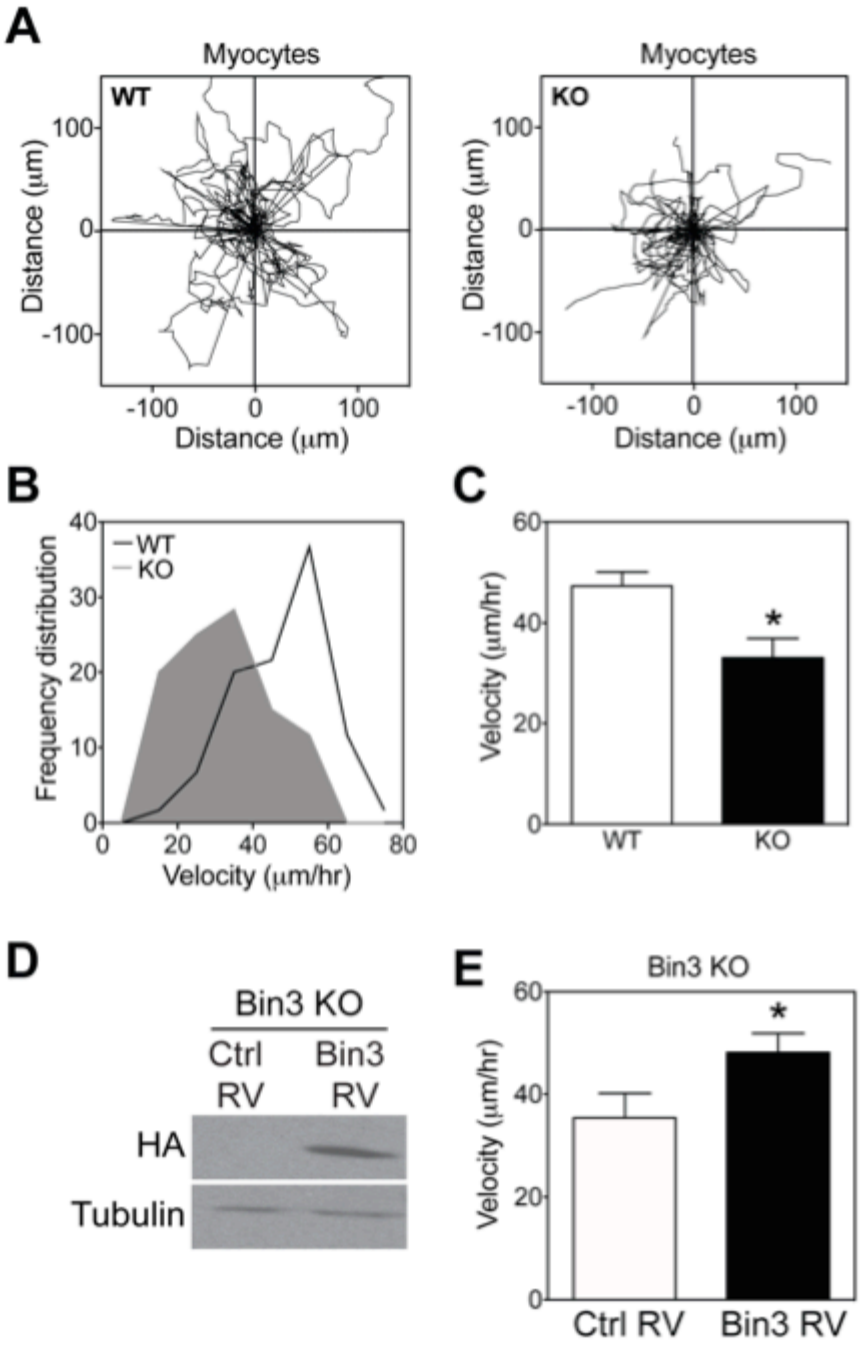

**Figure 5.4.4: Bin3 plays a role in myocyte migration**

**(A)** Time-lapse microscopy revealed that Bin3 KO myocytes migrated shorter distances than WT myocytes. The migratory paths of 30 individual cells per genotype are shown. **(B)** Histogram illustrating the absence of a large population of rapidly moving cells in Bin3 KO myocytes. **(C)** Cell velocity was decreased in Bin3 KO myocytes (**P*<0.05). **(D)** Immunoblot demonstrating expression of recombinant HA-Bin3 (Bin3 RV) in Bin3 KO myocytes after retroviral infection. Ctrl RV = empty vector. Tubulin was used as a loading control. **(E)** Retroviral-mediated expression of HA-Bin3 (Bin3 RV) in Bin3 KO myocytes resulted in increased cell velocity compared to empty vector control (Ctrl RV) (**P*<0.05). In panels B, C and E, 60 individual cells were analyzed per genotype with *n*=3 independent isolates for each genotype. Data in panels C and E are mean ± s.e.m.

**Figure 5.4.5: Bin3 is involved in lamellipodia formation in myocytes**

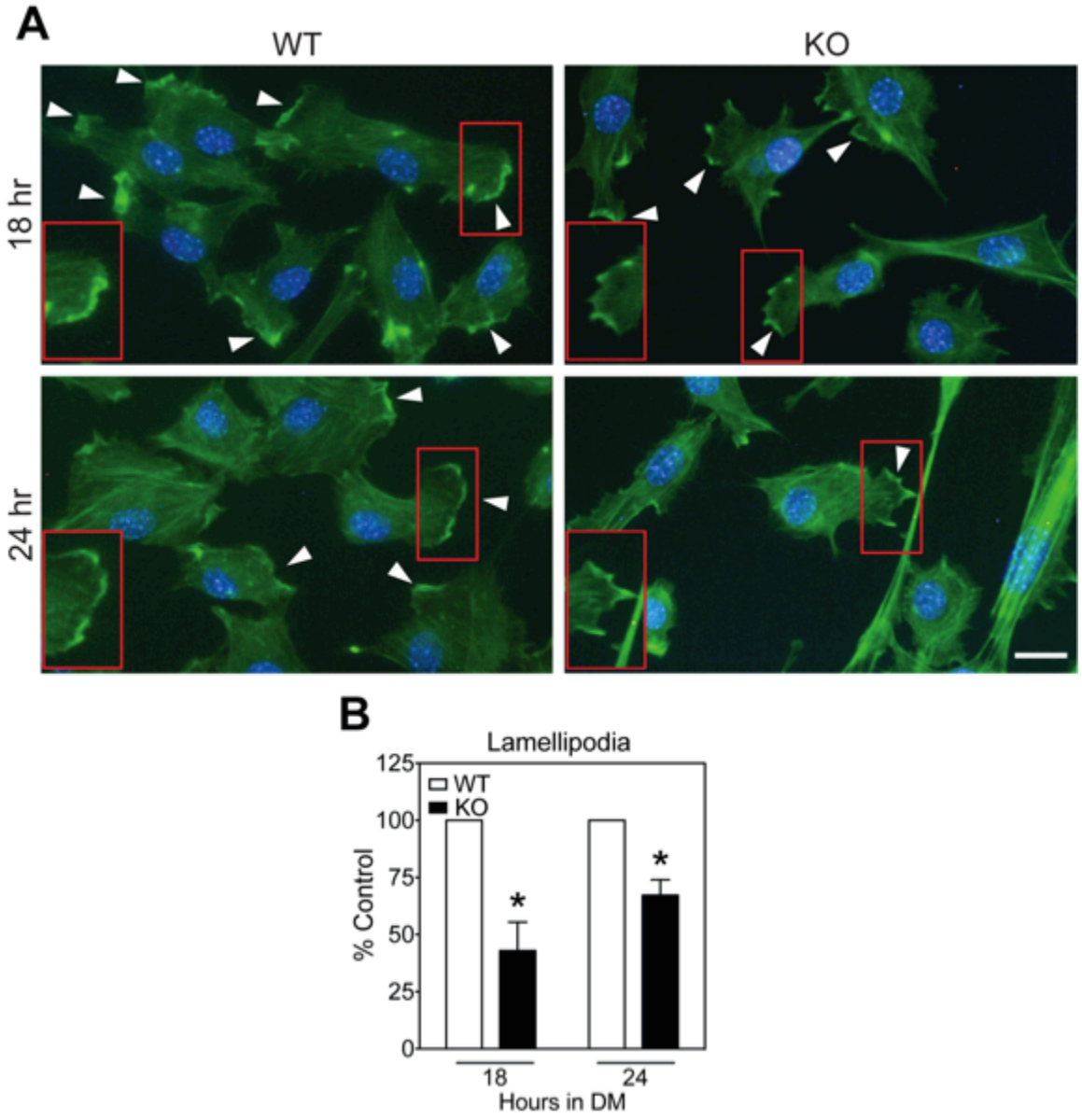

**Figure 5.4.5: Bin3 is involved in lamellipodia formation in myocytes**

**(A)** Lamellipodia were visualized by examining F-actin localization (FITC-phalloidin) in WT and Bin3 KO myocytes at 18 and 24 h in DM (arrowheads). Nuclei were counterstained with DAPI. Insets indicate lamellipodia at higher magnification (red box). Bar, 50 μm. **(B)** Bin3 KO myocytes exhibited a lower percentage of cells with lamellipodia (**P*<0.01) in DM. Data are mean ± s.e.m., *n*=3 independent isolates for each genotype.

**Figure 5.4.6: Bin3 and F-actin colocalize in lamellipodia of myocytes**

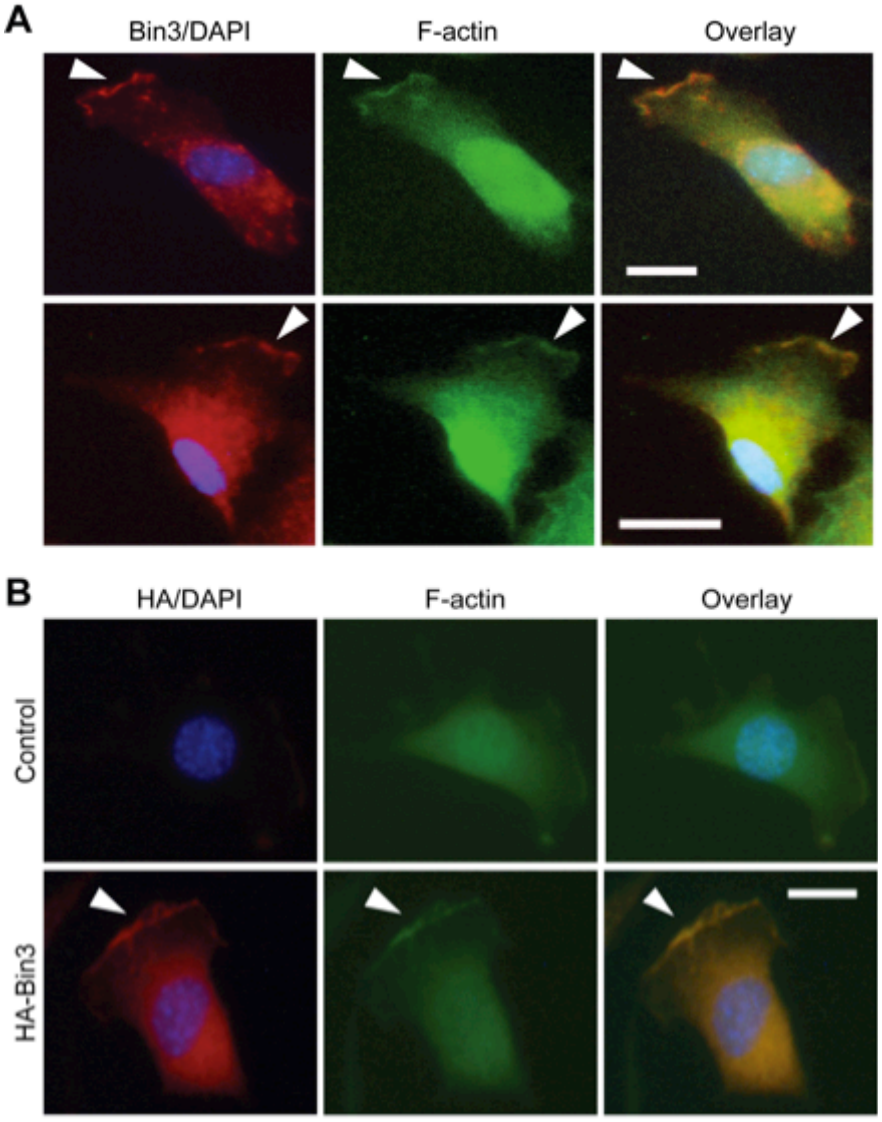

**Figure 5.4.6: Bin3 and F-actin colocalize in lamellipodia of myocytes**

**(A)** Bin3 and F-actin colocalized in lamellipodia of WT myocytes at 18 h in DM (arrowheads). Bar, 50 μm. **(B)** Retrovirally-expressed recombinant HA-Bin3 also colocalized with F-actin in lamellipodia of myocytes (arrowheads, bottom row). The specificity of the HA antibody was demonstrated by lack of antibody reaction in cells with the empty vector control (top row). Bar, 50 μm. Nuclei were counterstained with DAPI.

**Figure 5.4.7: Decreased levels of active Rac1 and Cdc42 in Bin3 KO myocytes**

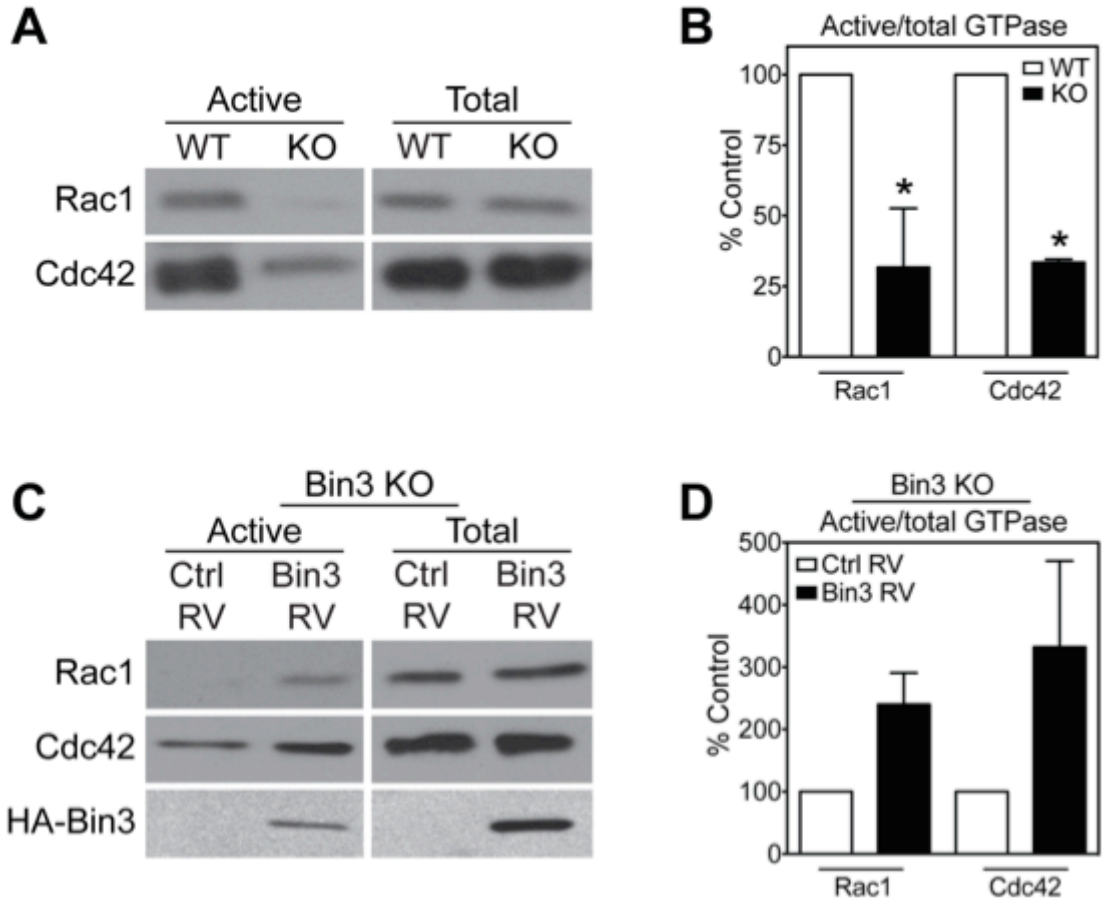

**Figure 5.4.7: Decreased levels of active Rac1 and Cdc42 in Bin3 KO myocytes**

**(A)** Active Rac1 and Cdc42 were pulled down from WT and Bin3 KO myocytes using beads coated with the p21-binding domain of PAK1 (PAK1-PBD). Decreased levels of active Rac1 and Cdc42 were detected in Bin3 KO myocytes. **(B)** The active/total ratios for Rac1 and Cdc42 were decreased in Bin3 KO myocytes (**P*<0.05). **(C)** Active Rac1 and Cdc42 were pulled down from Bin3 KO myocytes retrovirally-expressing recombinant HA-Bin3 (Bin3 RV) or empty vector (Ctrl RV) using PAK1-PBD beads. Increased amounts of active Rac1 and Cdc42 were observed in Bin3 KO myocytes following Bin3 overexpression. Moreover, HA-Bin3 was detected in a complex with active Rac1 and Cdc42. **(D)** The active/total ratios for Rac1 and Cdc42 were increased following Bin3 overexpression. HA immunostaining showed an average of 89% $HA^+$ cells. Data in panel B are mean ± s.e.m., *n*=3 independent isolates for each genotype. Data in panel D are mean ± s.e.m., *n*=2 independent isolates for each condition.

**Figure 5.4.8: Patterns of myofiber branching observed in WT and Bin3 KO muscles**

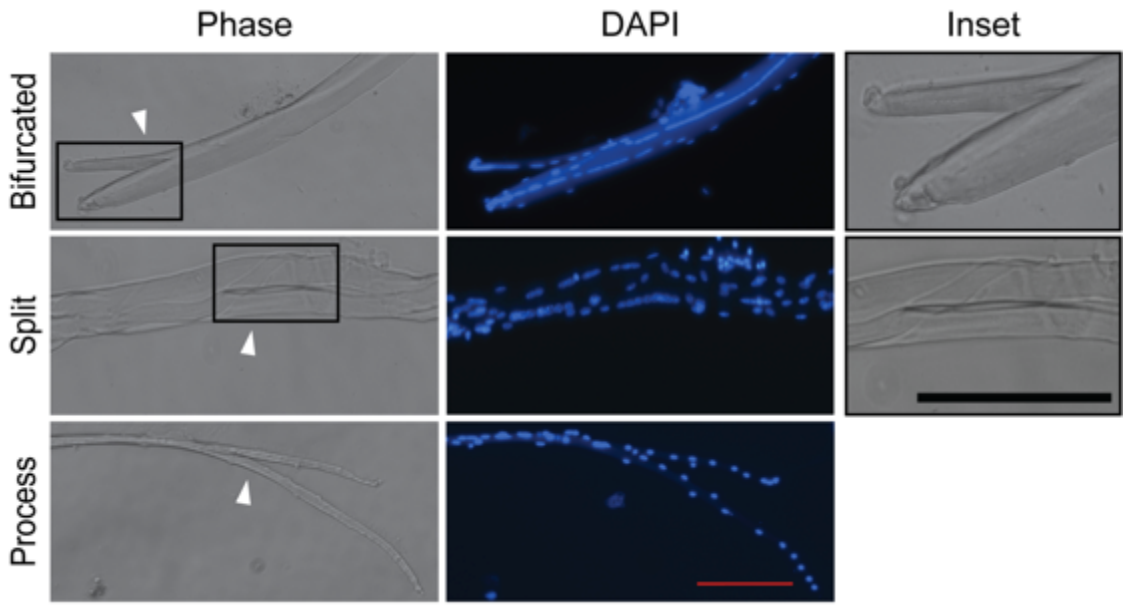

**Figure 5.4.8: Patterns of myofiber branching observed in WT and Bin3 KO muscles**

Myofibers after injury visualized with phase contrast microscopy and DAPI are shown. Various branching patterns (bifurcated, split, process) were observed (arrowheads). Insets indicate two of these patterns (bifurcated, split) at a higher magnification (black box). Bar, 150 μm.

**Figure 5.4.9: Differentiation does not differ between WT and Bin3 KO muscle cells**

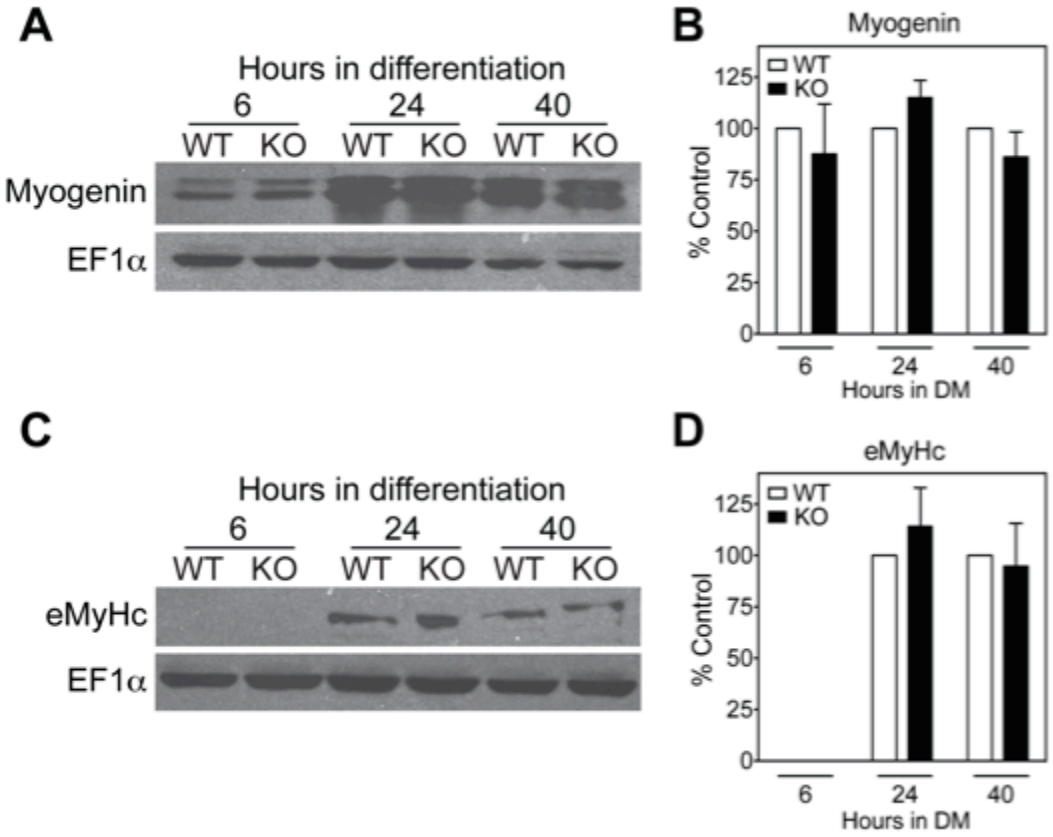

**Figure 5.4.9: Differentiation does not differ between WT and Bin3 KO muscle cells**

Immunoblot demonstrating expression of **(A)** myogenin or **(C)** eMyHC in WT and Bin3 KO cells during differentiation. EF1α was used as a loading control. Densitometric analysis indicated similar levels of **(B)** myogenin or **(D)** eMyHC protein relative to EF1α in WT and KO cells. Data are mean ± s.e.m., *n*=3 independent isolates for each genotype.

**Figure 5.4.10: Bin3 overexpression is not sufficient to enhance myotube size**

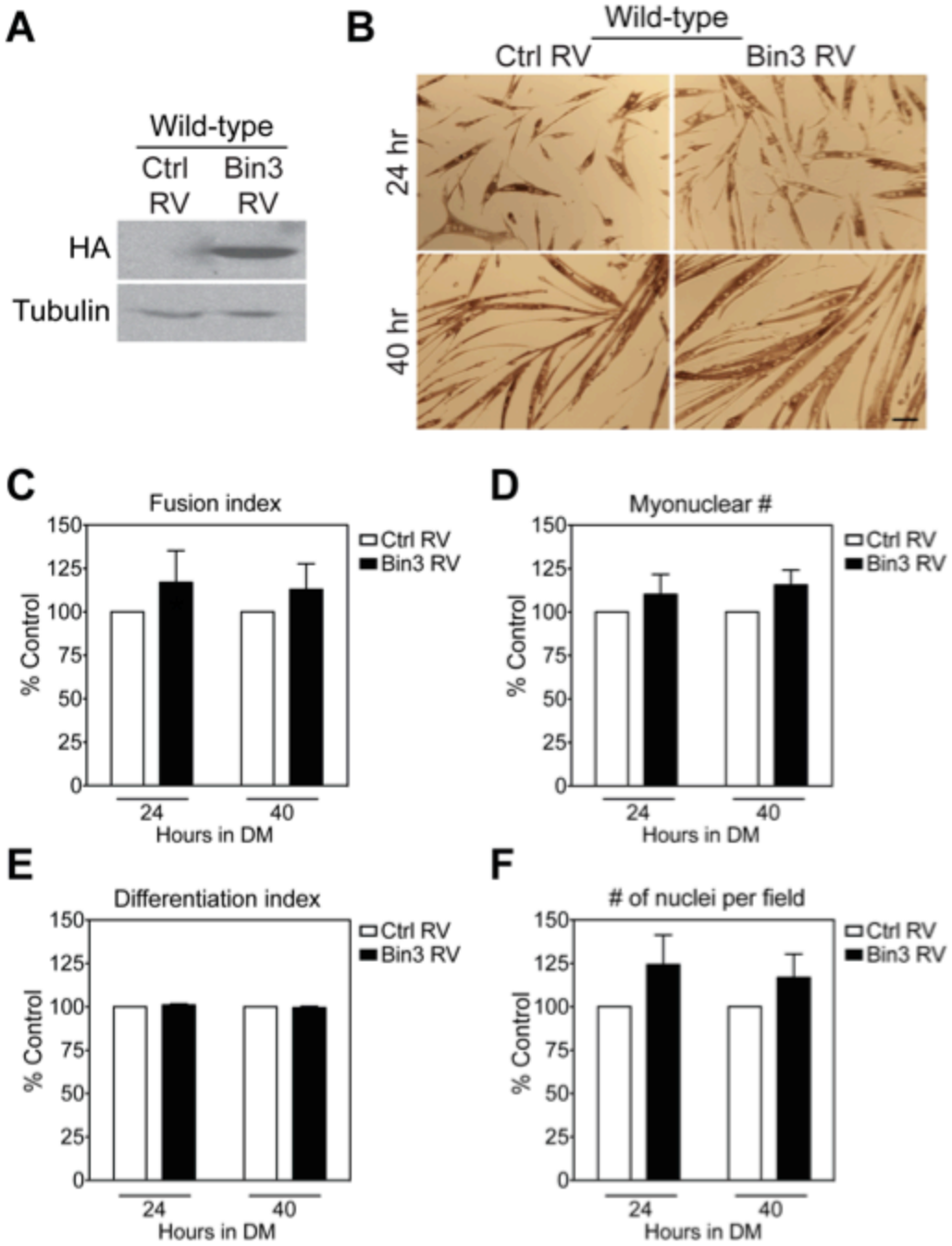

**Figure 5.4.10: Bin3 overexpression is not sufficient to enhance myotube size**

**(A)** Immunoblot demonstrating levels of recombinant HA-Bin3 (Bin3 RV) in WT muscle cells after retroviral infection. Ctrl RV = empty vector. Tubulin was used as a loading control. **(B)** WT muscle cells infected with either HA-Bin3 or empty vector were immunostained for eMyHC at 24 and 40 h in DM. Bar, 150 μm. Retroviral-mediated expression of recombinant HA-Bin3 in WT muscle cells did not significantly enhance **(C)** fusion index, **(D)** myonuclear number, **(E)** differentiation index or **(F)** number of nuclei per field. Data are mean ± s.e.m., *n*=3 independent isolates for each condition.

**Figure 5.4.11: Bin3 is not required for receptor-mediated endocytosis in myocytes**

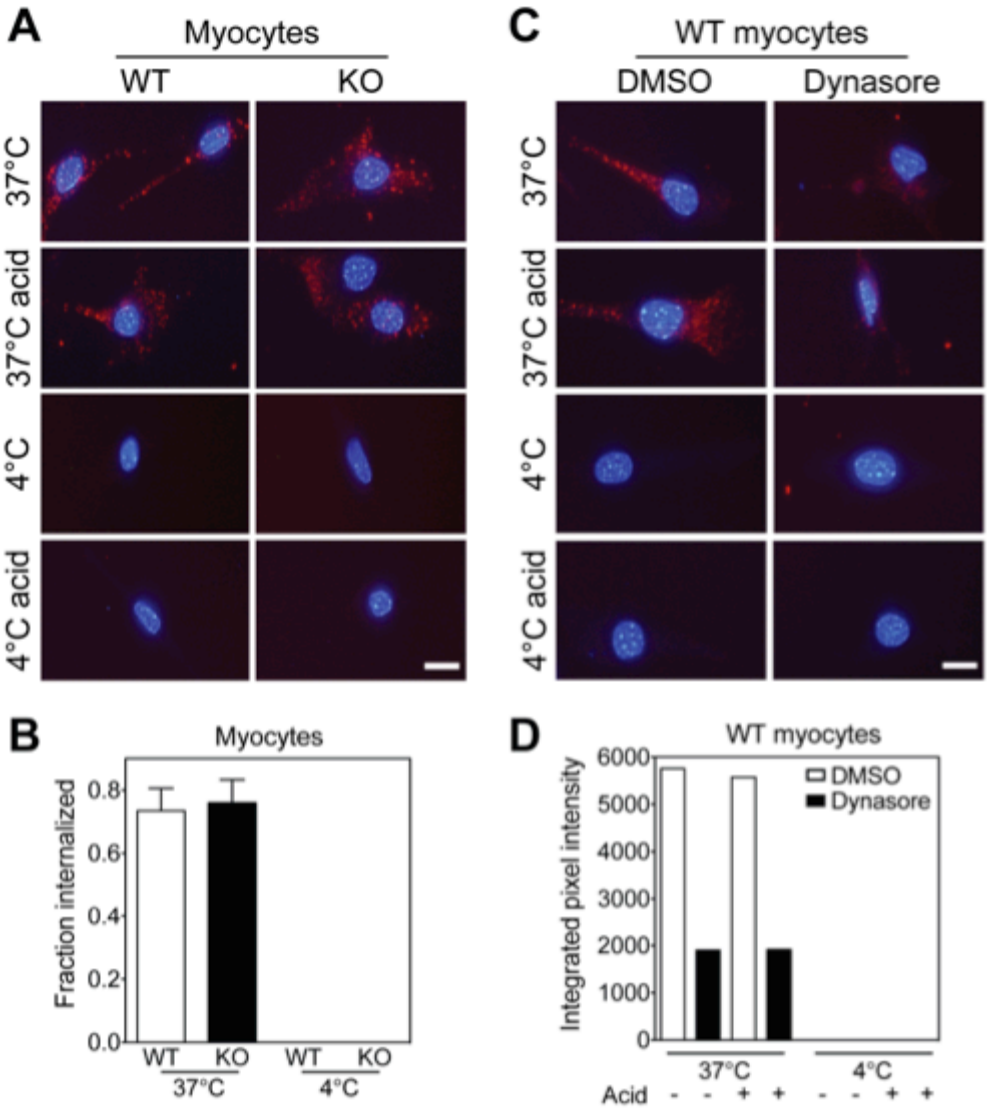

**Figure 5.4.11: Bin3 is not required for receptor-mediated endocytosis in myocytes** **(A)** WT and Bin3 KO myocytes were incubated with Alexa-594 conjugated transferrin at 37°C or 4°C (± acid). Nuclei were counterstained with DAPI. Bar, 50 μm. **(B)** The internalized transferrin fraction (37°C) was similar in WT and Bin3 KO myocytes. No internalization of transferrin was observed in the control 4°C conditions. **(C)** WT myocytes were treated with Dynasore or DMSO (vehicle) and incubated with Alexa-594 conjugated transferrin at 37°C or 4°C (± acid). Nuclei were counterstained with DAPI. Bar, 50 μm. **(D)** Both the total (- acid) and internalized (+ acid) transferrin levels were decreased at 37°C in the presence of Dynasore. No internalization of transferrin was observed in the control 4°C conditions. Data in panel A are mean ± s.e.m., *n*=3 independent isolates for each genotype. Data in panel B are mean, *n*=1 isolate.

**Figure 5.4.12: Absence of Bin3 minimally affects myoblast migration**

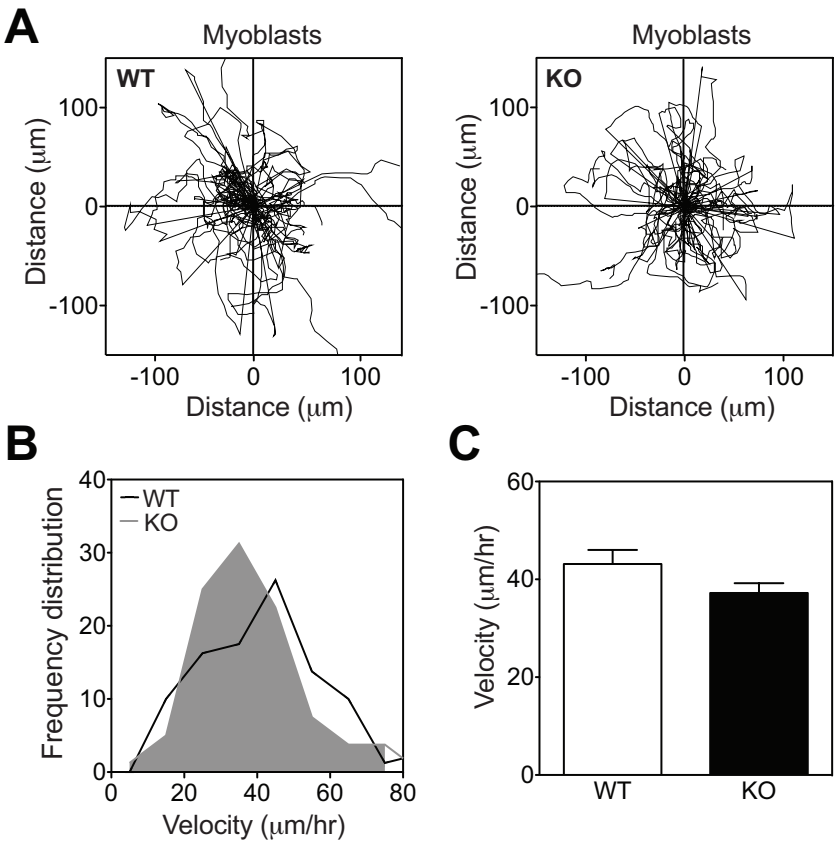

**Figure 5.4.12: Absence of Bin3 minimally affects myoblast migration**

**(A)** Time-lapse microscopy revealed only small differences in the migratory cell paths of Bin3 KO myoblasts compared to WT. The migratory paths of 30 individual cells per genotype are shown. **(B)** Histogram illustrating the absence of a very small population of rapidly moving cells in Bin3 KO myoblasts. **(C)** No significant difference in cell velocity was noted between genotypes. In panels B and C, 60 individual cells were analyzed per genotype. Data in panel C are mean ± s.e.m., $n$=4 independent isolates for each genotype.

**Table 5.4.1: Sequence similarity of Amphiphysin/Bin family members to Bin3 by protein BLAST analysis**

| N-BAR domain protein family | N-BAR domain protein (*Mus musculus*) | Accession number | Similarity (%) |
|---|---|---|---|
| Amphiphysins /Bins | Amphiphysin | NP_778172.1 | 47% |
| | Bin1 | NP_033798.1 (isoform 1)<br>NP_001076803.1 (isoform 2) | 42%<br>46% |
| | Bin2 | NP_001257466.1 | 46% |
| | Bin3 | NP_067303.1 | 100% |

**Table 5.4.2: Average $C_t$ values of Amphiphysin/Bin family members in myocytes by real-time PCR analysis**

| Target gene (*Mus musculus*) | Accession number | Average $C_t$ |
|---|---|---|
| *AMPH* | NM_175007.1 | N/D |
| *BIN1* | NM_009668.2 | 22.83 ± 0.05 |
| *BIN2* | NM_001270537.1 | N/D |
| *BIN3* | NM_021328.3 | 25.02 ± 0.04 |

AMPH = Amphiphysin. N/D = not detected ($C_t$ >35). Data are mean ± s.d., *n*=3 independent isolates. All genes were expressed in control tissues: *AMPH*, *BIN1*, *BIN2* (brain); *BIN3* (muscle).

# Chapter 6: Discussion

# Chapter 6: Discussion

## 6.1 Introduction

Skeletal muscle formation and growth require a series of ordered steps, including satellite cell activation, differentiation, migration, adhesion and fusion, leading to the formation of multinucleated myotubes/myofibers. Several steps of myogenesis are highly dependent on regulation of the actin cytoskeleton. However, the mechanisms controlling actin dynamics in muscle cells are not well understood. The overall goal of this dissertation was to discover novel proteins involved in myotube formation through regulation of actin polymerization.

The first part of this dissertation focused on finding novel binding partners for the brain isoform of Creatine kinase (CKB), a cytosolic CK enzyme involved in local ATP production. We hypothesized that CKB provides ATP for actin polymerization during myogenesis. In Chapter 4, we show α-skeletal-actin and α-cardiac-actin, two predominant skeletal muscle-specific actin isoforms, as well as several actin regulatory proteins, are novel CKB interacting proteins. Interestingly, we also found that the activity of cytosolic CK enzymes is necessary for regulating actin polymerization in muscle cells, as well as for myotube formation. Together, these studies show for the first time that cytosolic CK enzymes play a critical role in regulating actin dynamics during myotube formation.

The second part of this dissertation focused on the N-BAR domain protein Bridging integrator 3 (Bin3), which we initially identified as an actin regulatory protein interacting with CKB. In Chapter 5, we show Bin3 is a novel regulator of myofiber size *in vivo* and myotube formation *in vitro*. Importantly, we found that, in differentiated muscle cells, a Bin3-dependent pathway regulates the activity of Rac1 and Cdc42, small

GTPases associated with actin regulation. These results provide insights into the regulation of actin dynamics by BAR domain proteins during myotube formation.

The research presented in this dissertation identifies CKB and its interacting partners, α-skeletal-actin, α-cardiac-actin and Bin3, as novel regulators of myotube formation. Our findings further suggest CKB may provide ATP for α-skeletal-actin, α-cardiac-actin and/or Bin3-dependent regulation of myotube formation. Overall, this work could have important implications in finding therapies for muscle diseases in which myotube formation is impaired.

### 6.2 Specificity of CKB Binding to Muscle-Specific Actin Isoforms

Our studies showed CKB interacts with and modulates the actin cytoskeleton. Six actin isoforms exist in cells, with no isoform sharing less than 93% identity with any other isoform (Perrin and Ervasti 2010). Interestingly, we demonstrate preferential CKB binding to the predominant muscle-specific actin isoforms (α-skeletal-actin and α-cardiac-actin), but not to the cytoplasmic actin isoforms (β-cyto-actin and γ-cyto-actin) (Perrin and Ervasti 2010). However, we show the interaction between CKB and α-skeletal-actin is indirect, indicating that intermediary proteins, such as actin-binding proteins, could mediate the interaction between CKB and the actin cytoskeleton.

Most actin-binding proteins to date interact specifically with non-muscle actin isoforms, specifically with the cytoplasmic β-actin isoform, by co-sedimentation assays. For example, ezrin, an actin-binding protein of the ezrin/radixin/moesin (ERM) family of cytoskeleton-membrane linker proteins (Yonemura et al. 1993), selectively co-pellets with cytoplasmic β-actin and very poorly with skeletal α-actin (Yao X et al. 1996).

Similarly, βCAP73, an actin-binding protein co-precipitating with both erzin and β-actin (Shuster and Herman 1995), also binds directly to β-actin, but not to α-actin (Shuster et al. 1996). Finally, L-plastin, a leukocyte-specific protein that cross-links actin filaments into tight bundles (Morley 2012), acts on β-actin, but not on α-actin or γ-actin, in T-cells (Namba et al. 1992). Whereas these proteins interact with non-muscle actin isoforms, our studies show for the first time that CKB interacts specifically with muscle-specific actin isoforms. Currently, how the specificity of this interaction occurs is unknown. β-actin and γ-actin isoforms are 93% identical to the muscle-specific actin isoforms (Kashina 2006). In fact, β-actin and γ-actin isoforms differ in sequence from α-actin isoforms by only a few amino acids in the N-terminus (Perrin and Ervasti 2010). Thus, very small differences among actin isoforms are sufficient to confer differential binding affinities to various proteins.

### 6.3 Mechanisms of CKB and CKM Localization in Differentiated Muscle Cells

Myoblasts preferentially fuse with myotube ends in culture (Peckham 2008), however the molecular mechanisms driving fusion at these sites are not well understood. Latrunculin-A, a drug used to disrupt the actin cytoskeleton (Spector et al. 1983) by preventing repolymerization of monomers into filaments (Coue et al. 1987), impairs myofibril assembly at myotube ends, but does not affect mature myofibrils in the center of myotubes (Wang et al. 2005), suggesting the actin pool in the center of myotubes may be more stable, whereas the actin pool near myotube ends may be more dynamic. Given the importance of actin polymerization for myoblast fusion (Gruenbaum-Cohen et al. 2012, Kim et al. 2007, Nowak SJ et al. 2009, Vasyutina et al. 2009), the dynamic nature

of the actin cytoskeleton at myotube ends could facilitate myoblast fusion, and may explain in part why fusion preferentially occurs at these sites. Thus, studies of actin dynamics in myotubes could provide insights into the pathways regulating myoblast fusion at the ends of multinucleated muscle cells.

CKB becomes progressively localized near the ends of myocytes (Simionescu-Bankston, et al., unpublished) and myotubes (O'Connor et al. 2008). Given the importance of actin polymerization, a highly ATP-dependent process (Lodish et al. 2000), for myoblast fusion, as well as the ability of CKB to replenish local ATP (Wyss and Kaddurah-Daouk 2000), we hypothesized that CKB may provide ATP for actin polymerization at myotube ends. Treatment of muscle cells with cyclocreatine, which leads to decreased ATP generation by cytosolic CK enzymes (Wyss and Kaddurah-Daouk 2000), caused actin depolymerization. However, CKB localization in myotubes was unaffected by cyclocreatine treatment, suggesting F-actin does not provide a structural role in localizing CKB to myotube ends. In contrast, CKB localization in astrocytes was F-actin-dependent, as treatment with cytochalasin D, an inhibitor of actin polymerization (Casella et al. 1981), reduced the accumulation of both F-actin and CKB around fibronectin beads (Kuiper et al. 2009). These results suggest CKB localization is likely controlled by distinct mechanisms in different cell types. However, what localizes CKB to myotube ends is unknown. A possible explanation for CKB localization at myotube ends is the presence and/or the modification of CKB interacting proteins, such as actin regulatory proteins, at these sites. These proteins could recruit CKB to myotube ends, enabling it to subsequently provide local ATP for actin polymerization at these sites. Thus, CKB could influence actin dynamics indirectly by providing ATP for actin

regulatory proteins. Alternatively, as CKB is prone to covalent modifications, such as phosphorylation (Chida et al. 1990), oxidation (Aksenov et al. 2000), methylation (Iwabata et al. 2005) and ubiquitination (Zhao et al. 2007), these modifications of the CKB enzyme itself could also influence the ability of CKB to bind actin regulatory proteins. However, whether CKB provides ATP for actin regulatory proteins is unknown and is a warranted future direction, which would provide insights into whether CKB promotes actin polymerization in muscle cells directly or indirectly via other proteins.

Besides CKB, muscle cells also express the muscle-type creatine kinase (CKM), which localizes to the contractile apparatus (Schafer and Perriard 1988) and exhibits functional redundancy with CKB in replenishing ATP (Renema et al. 2007). In addition, inhibition of CKM activity in neonatal rat cardiomyocytes results in depolymerized actin (Diguet et al. 2011), suggesting that CKB and CKM may exhibit some common functions in regulating actin dynamics during myogenesis. However, our studies showed that CKB and CKM exhibit reciprocal localization in differentiated muscle cells (Simionescu-Bankston, et al., unpublished), indicating potentially differential functions for the two cytosolic CK enzymes during myogenesis. As previously described, the actin pool in the center of myotubes may be more stable, whereas the actin pool near myotube ends may be more dynamic. Thus, our findings that CKB localized to the ends of myocytes and myotubes suggest that CKB could provide ATP for a dynamic actin cytoskeleton at these sites, in order to drive the various steps of myoblast fusion forward. This hypothesis is further strengthened by the colocalization of CKB and sarcomeric actin near the ends of myotubes, indicating that CKB could regulate actin dynamics at these sites. In contrast, CKM was absent from the ends of differentiated muscle cells (Simionescu-Bankston, et

al., unpublished), suggesting that CKB and CKM may exhibit differential functions during myogenesis. Future studies should address how the reciprocal localization of CKB and CKM in differentiated muscle cells is regulated. Testing whether and how altering the expression levels and localization of CKB in differentiated muscle cells would affect CKM levels and localization, and vice versa, would address whether any functional overlap exists between the two cytosolic CK enzymes during myogenesis.

### 6.4 Interactions of CKB with Actin Regulatory Proteins

CKB could provide ATP for actin regulatory proteins, which would, in turn, regulate actin polymerization in muscle cells. One potential mechanism for this regulation is profilin-dependent. Profilin is an actin-binding protein which functions by binding to G-actin at the opposite end of ATP-binding, and increases the rate of nucleotide exchange on the bound actin monomer, leading to the addition of the ATP-bound G-actin to the growing filament to promote actin filament assembly (Witke 2004). Interestingly, profilin also binds to proteins involved in actin dynamics (palladin, dynamin1, VASP and N-WASP), as well as membrane components, and may therefore control actin assembly at the plasma membrane (Lodish et al. 2000). Future studies should address whether CKB and profilin interact in mammalian cells. Alternatively to a profilin-dependent mechanism, CKB could also regulate actin dynamics via other types of actin regulatory proteins, including the molecules we discovered as novel CKB interacting proteins, namely Bin3, Hsbp3, Septin-8. These molecules regulate actin dynamics in various cell types, however the exact mechanisms of actin regulation by these molecules are unknown. Bin3 could link membrane dynamics to the actin

cytoskeleton (Frost et al. 2009, Ren et al. 2006), Hsbp3 could be involved in inhibiting actin polymerization (Mounier and Arrigo 2002), and Septin-8 could help organize actin bundles (Kinoshita et al. 2002). However, whether these molecules are required for actin regulation downstream of CKB in muscle cells is unknown. CKB localization near myotube ends was unchanged in the absence of Bin3 (Fig. 6.9.1), suggesting that Bin3 is not structurally involved in localizing CKB in myotubes. Whether Hsbp3 and/or Septin-8 are responsible for CKB localization near myotube ends is unknown. Moreover, whereas Bin3 localizes throughout the myotube (Fig. 6.9.5), the localization of Hsbp3 or Septin-8 is myotubes is unknown. Finally, the molecules found in our screen using an embryonic day 17 library may not be responsible for localizing CKB to myotube ends, however future studies using a muscle-specific library might reveal better candidates for this role.

### 6.5 Roles of Bin3 in Myogenesis

Muscle cell migration is crucial for proper myotube formation *in vitro* (Bae et al. 2008, Jansen and Pavlath 2006, Kang et al. 2004, Mylona et al. 2006, O'Connor et al. 2007) and regeneration *in vivo* (Bischoff 1997). We show the N-BAR domain protein, Bin3, is a novel cytoplasmic molecule regulating muscle cell migration. In addition, Bin3 specifically regulates migration of differentiated muscle cells. Many molecules differentially regulate the migration of myoblasts and myocytes, however the exact reasons are not well understood. The migratory responses in myoblasts and myocytes may be elicited by different chemoattractants, which act via different receptors in the two cell types, including growth factor receptors for myoblasts, and chemokine receptors for myocytes (Griffin et al. 2010). Another possibility is that, for example, in the case of

Bin3, the partner necessary for Bin3 heterodimerization may only be present in myocytes; however, whether Bin3 heterodimerization is required for muscle cell migration is unknown.

Given the function of Bin3 in regulating muscle cell migration, the fact that Bin3 was also involved in lamellipodia formation was not unexpected. Lamellipodia contain branched actin filaments (Abercrombie et al. 1971, Small et al. 2002), as well as a variety of actin regulatory proteins involved in generating plasma membrane protrusions at the leading edge of cells (Ridley 2011). These proteins include actin-binding proteins that provide actin monomers for actin polymerization (profilin), actin nucleators (Arp2/3) that generate a branched actin filament network, proteins involved in extending Arp2/3 complex-generated filaments (formins, VASP), upstream regulators of Arp2/3 (WAVE), and small GTPases (Rac1, Cdc42) (Ridley 2011). However, the mechanism by which all of these molecules, as well as Bin3, become localized to the lamellipodia is unknown. Similar to most proteins found at lamellipodia, Bin3 colocalized with F-actin at this location, suggesting a possible role for Bin3 in actin regulation during muscle cell migration. In the absence of Bin3, we observed decreased levels of active Rac1 and Cdc42, small GTPases of the Rho family associated with actin regulation, suggesting that Bin3 may be involved in regulating actin dynamics in muscle cells via Rho GTPases. Using biosensors, active Rho GTPases have been shown to localize in lamellipodia of mouse embryonic fibroblasts during protrusion (Machacek et al. 2009). Due to the unavailability of appropriate antibodies, we were unable to examine whether active Rac1 and Cdc42 localize to the lamellipodia together with Bin3 in muscle cells. However, this finding would provide mechanistic insights into how Bin3 could control myocyte

migration. Although the levels of active Rac1 and Cdc42 correlated with Bin3 levels, future studies should also test whether active Rac1 and Cdc42 are required for myocyte migration downstream of Bin3. In addition, whether Bin3 is recruited to the lamellipodia prior to these small GTPases is also unknown. These studies would further dissect the details of actin regulation by Bin3 in differentiated muscle cells.

Most of our data suggest Bin3 plays critical roles during early stages of myotube formation, when myocytes fuse to one another to form nascent myotubes. However, we also noted decreases in myotube size in Bin3 KO cells during later fusion stages, when myocytes fuse with nascent myotubes to give rise to mature myotubes. Therefore, we tested whether Bin3 also functions at this later stage of fusion by performing cell mixing experiments. We separately created and labeled nascent myotubes (Mt) with a green dye and myocytes (Mc) with an orange dye, cocultured the cells for 24 h, and quantified the number of dual labeled myotubes with ≥3 nuclei (Fig. 6.9.2A,B). The WT Mc/KO Mt coculture exhibited 38% fewer dual labeled myotubes, which is very similar to the 43% decrease observed in the KO Mc/KO Mt coculture; in contrast, the percentage of dual labeled myotubes in the KO Mc/WT Mt coculture did not differ significantly from the WT Mc/WT Mt coculture (Fig. 6.9.2C). These data indicate that Bin3 is primarily required in nascent myotubes during later stages of myotube formation. However, the function of Bin3 in myotubes is unknown. BAR domain proteins are known regulators of endocytosis (Ren et al. 2006). Given the importance of endocytosis for myotube formation, Bin3 could also regulate endocytosis in myotubes. We examined whether receptor-mediated endocytosis, the most common and well-studied pathway utilized for internalization (Qualmann et al. 2011), was affected in Bin3 KO myotubes (24 h in DM)

labeled with Alexa-594 conjugated transferrin (Fig. 6.9.3A). However, no difference in the internalized transferrin fraction was observed between WT and Bin3 KO myotubes (Fig. 6.9.3B). In COS-7 cells, Bin2 overexpression has no effect on receptor-mediated-endocytosis, while Bin1 overexpression inhibits this process (Ge K. and Prendergast G. C. 2000, Wigge et al. 1997), indicating that Bin family proteins may have differential functions in regulating receptor-mediated endocytosis. Interestingly, one of the Bin3 yeast orthologs, Rvs161p, regulates both receptor-mediated and fluid-phase endocytosis in yeast cells (Ren et al. 2006), while the BAR-PH domain protein, GRAF1, regulates clathrin-independent endocytosis in fibroblasts (Lundmark et al. 2008). These data suggest that Bin3 may regulate fluid-phase endocytosis in muscle cells, although this pathway is utilized much less by BAR domain proteins for internalization. Alternatively, the ability of BAR domain proteins to bend the membrane is also important for myotube formation, and this function was previously shown to occur via key lysine residues in the BAR domain of the BAR-PH domain protein, GRAF1 (Doherty JT et al. 2011). As these lysine residues also exist in the BAR domain of Bin3, Bin3 could bend membranes necessary for myotube formation. However, the ability of Bin3 to bend membranes *in vitro* has not yet been shown. Alternatively, as BAR domain proteins regulate actin polymerization, which is crucial for myotube formation, Bin3 could regulate actin dynamics in myotubes. However, no differences in F-actin localization were observed between WT and Bin3 KO nascent or mature myotubes (Fig. 6.9.4), suggesting that Bin3 either does not regulate F-actin localization in myotubes, or alternatively live cell imaging may be necessary to detect subtle differences in actin localization in multinucleated muscle cells. If differences in F-actin localization between WT and Bin3

KO myotubes are detected by these more sensitive methods, future studies should address whether the expression and localization of known actin regulatory proteins is also altered in the absence of Bin3. These studies would explain in part the mechanisms of Bin3 action on the actin cytoskeleton in multinucleated muscle cells.

### 6.6 Molecular Mechanisms of Bin3 Action in Muscle Cells

Our data provide the first example of an N-BAR domain protein regulating actin dynamics in mammalian muscle cells in a Rac1- and Cdc42-dependent manner. Most studies on actin regulation in muscle cells have been performed in *Drosophila melanogaster* (Abmayr and Pavlath 2012). However, common mechanisms for controlling this process likely occur in mammalian muscle cells via orthologs of *Drosophila melanogaster* actin regulatory proteins. These mechanisms involve the small GTPases Rac1 and Cdc42, which control the actin regulatory proteins N-WASP and WAVE, leading to Arp2/3-dependent actin polymerization. Many of these actin regulatory proteins are required for mammalian myoblast fusion. For example, the small GTPases Rac1 and Cdc42 are essential for myoblast fusion in the mouse, as well as in cultured primary mouse muscle cells (Vasyutina et al. 2009). Similarly, the actin-regulator N-WASP is required for myoblast fusion in mouse embryos, as well as in primary mouse muscle cells (Gruenbaum-Cohen et al. 2012) and in the mouse myoblast cell line C2C12 (Kim et al. 2007). In addition, WASP-interacting protein (WIP) is also required for myoblast fusion in C2C12 cells (Kim et al. 2007). Finally, shRNA-mediated knockdown of Nck-associated protein 1 (Nap1), a member of the WAVE actin-remodeling complex, blocks myoblast fusion in C2C12 myoblasts (Nowak SJ et al.

2009). However, the signaling pathways that activate changes in the actin cytoskeleton during myoblast fusion have not been studied in depth in mammalian cells. As yeast orthologs of Bin1 and Bin3 function as heterodimers (Ren et al. 2006), and heterodimerization is required for their cellular function, the mammalian counterparts may also regulate actin dynamics as a heterodimer. Given that Bin3 contains only an N-BAR domain, an interaction with Bin1 could provide additional pathways for regulating actin polymerization via the SH3 domain of Bin1, which could then interact with various downstream molecules (Ren et al. 2006). Alternatively, Bin3 could also regulate actin dynamics in muscle cells via GEF proteins. Hob3p, one of the Bin3 yeast orthologs, facilitates the interaction between Gef-1, a Cdc42GEF, and Cdc42 (Coll et al. 2007), suggesting the mammalian Bin3 protein could also interact with a GEF molecule in muscle cells. Nevertheless, extensive knowledge about the expression of GEF molecules in myogenesis is lacking. To date, only a few GEF molecules regulating small GTPase signaling are expressed in muscle, namely Trio, Dedicator of cytokinesis 180/1 (Dock180/Dock1) and Guanine nucleotide exchange protein 100 (GEP100/Brag2). Trio is found in a complex with M-cadherin and Rac1 at the onset of myoblast fusion, which mediates Rac1 activation and myoblast fusion in the muscle cell line C2C12 (Charrasse et al. 2007). Dock180/Dock1 and Brag2, which act as GEFs for small GTPases, also regulate myoblast fusion the mouse muscle cell line C2C12 (Pajcini et al. 2008). Whether Bin3 interacts with any of these GEF molecules during myogenesis is unknown. In addition, Bin3 could also interact with a BAR-domain protein which also has a RhoGEF-domain containing protein, thereby allowing Bin3 to indirectly activate small GTPases or take part in additional signaling pathways via its heterodimer partner molecule containing

the RhoGEF domain. Finally, Bin3 could also interact with a RhoGAP-containing protein (de Kreuk and Hordijk 2012), leading to spatial or temporal inactivation of small GTPases when no longer needed in their active form. In order to gain deeper insights into how Bin3 may regulate actin dynamics in muscle cells, future studies should consist of mass spectrometry analyses to discover the molecules interacting with Bin3 during myogenesis. In addition to actin regulation via small GTPases, BAR domain proteins can also bend membranes (Ren et al. 2006), a function important for myotube formation, as previously shown for the BAR-PH domain protein, GRAF1 (Doherty JT et al. 2011). However, whether membrane bending and small GTPase-binding are exclusive or sequential for BAR domain proteins (Habermann 2004), such as Bin3, is unknown and a warranted future direction which could shed light onto how BAR domain proteins regulate a multitude of cellular functions.

### 6.7 Bin3 Is Involved in Myofiber Branching

Myofiber branching is a phenomenon occurring during muscle regeneration after extensive injury. To date, only two molecules are known to control myofiber branching. The first molecule is mouse odorant receptor 23 (MOR23), which also regulates muscle cell migration and adhesion *in vitro* (Griffin et al. 2009). Our studies show that Bin3 is the second molecule, and the first BAR domain protein, found to be involved in myofiber branching. Interestingly, MOR23 and Bin3 both control muscle cell migration *in vitro* (Griffin et al. 2009, Simionescu-Bankston et al. 2013), which is also necessary for myofiber formation, suggesting that aberrant cell migration is a possible mechanism for myofiber branching. In this model, muscle cells initially migrate to inappropriate places

in the muscle, where they subsequently fuse and form small myofibers; these myofibers can then fuse to one another, forming a small (branched) myofiber, and this small myofiber may only partially fuse with a larger (parent) myofiber, generating a gap. Therefore, this gap could also be due to incomplete fusion, another potential mechanism giving rise to myofiber branching. Whether myofiber branching occurs as a result of aberrant cell migration and/or incomplete fusion is not understood. Currently, we can only infer that migration and fusion are involved in myofiber branching, given the similarity of the muscle repair process *in vivo* to myotube formation *in vitro*.

Myofiber branching results in various patterns that can easily be observed and classified into different categories, including bifurcated, split, and process, however what causes these different patterns of myofiber branching to occur is unknown. For example, are some branching patterns caused by a defect in migration, whereas others are caused by a defect in fusion, or does a combination of defects in these two processes give rise to a particular branching type? As different types of molecules regulate migration and fusion *in vitro*, specific molecules could be responsible for each of the various branching patterns observed. Future loss of function studies of these molecules would determine whether they are required for myofiber branching *in vivo*. These studies should further attempt to correlate the type of molecule found to regulate either migration or fusion *in vitro* with a particular branching pattern observed *in vivo*. At the same time, since branching is observed in patients with muscular dystrophy, determining which branching patterns are present in these patients would enable targeted therapies using a certain type of molecule responsible for that particular branching type. In the absence of Bin3, we observed a branching phenotype characterized by a combination of various branching

patterns (mix), as well as several branches per myofiber, suggesting a greater myofiber branching complexity. This complexity suggests a pattern likely more difficult to repair than for example a simple bifurcation; however, why and how the mixed branching phenotype occurs, is also unknown.

While most injury models for studies of myofiber branching utilize a toxic compound, such as $BaCl_2$ or cardiotoxin, the relevance of these models to physiological injury is sometimes called into question. For example, could branching be an artificial phenotype caused by the particular type of agent used to induce injury? Although myofiber branching occurs in patients and mouse models of muscular dystrophy (Pavlath 2010a), whether the mechanisms regulating branching following dystrophy are similar to those elicited by various myotoxic compounds has not been shown. Therefore, testing whether other injury models, such as crush injury, give rise to similar branching patterns as the established myotoxic injury models is a warranted future direction. Our studies utilized a general Bin3 KO mouse, which lacks Bin3 in all cell types; however, since myofiber branching occurs mainly after injury, and the repair process involves many cell types, the presence of Bin3 in cell types other than muscle cells likely also contributes to the branching phenotype observed in mice devoid of Bin3. Thus, determining which cell types express Bin3 *in vivo* would be beneficial in gaining further insights into possible ways that Bin3 may contribute to muscle regeneration, including myofiber branching. However, current methods do not allow *in vivo* imaging of the branching process. Thus, the development of such methods would be a promising avenue in the future, likely providing insights into the mechanisms of myofiber branching following extensive injury. Generating a muscle-specific Bin3 KO mouse model would also be worthwhile in

examining the contribution of muscle cells to the myofiber branching process; in addition, this mouse model would facilitate knocking out Bin3 at different stages during regeneration, in order to determine the timing of Bin3 requirement during this process. Overall, these techniques would greatly advance our understanding of how the branching process occurs *in vivo*, and may also shed light onto possible strategies to resolve myofiber branching and potentially provide therapies for muscular dystrophy.

### 6.8 Perspectives and Therapeutic Strategies

In summary, we report several novel findings for myogenesis. We showed for the first time that CKB specifically binds α-actin isoforms, and that CKB activity is required for actin polymerization during myotube formation. Our findings further revealed that CKB interacts with the N-BAR domain protein Bin3, suggesting that CKB could regulate membrane and actin dynamics, two main functions of BAR domain proteins (Ren et al. 2006). In addition, Bin3 is the first BAR domain protein shown to control both myofiber size and branching *in vivo*, linking BAR domain proteins to regeneration for the first time in any tissue. Finally, Bin3 is a novel cytoplasmic protein regulating *in vitro* muscle cell migration, in a pathway involving Rac1 and Cdc42, small GTPases associated with actin regulation. Overall, our results highlight the importance of CKB and its interacting partners, α-actin and Bin3, in regulating actin dynamics during myogenesis.

Bin3 and CKB regulate different stages of myogenesis, but may also have overlapping functions at some stages. Bin3 is involved in both lamellipodia formation and regulation of active Rac1 and Cdc42 levels (Simionescu-Bankston et al. 2013), providing a possible mechanism for Bin3 to control actin dynamics in myocytes. Whether

CKB also regulates myocyte migration, and whether CKB localizes to the lamellipodia, actin-based protrusions associated with motility, have not been tested. However, CKB regulates motility of tumor cells (Mulvaney et al. 1998) and sea urchin sperm (Tombes and Shapiro 1985), as well as spreading and migration in astrocytes and fibroblasts (Kuiper et al. 2009), suggesting that CKB can regulate migration in cell types other than muscle cells. In addition, CKB may also be important in myotubes. In contrast, whereas Bin3 may have a crucial role in myocytes, its role in myotubes is currently unknown. CKB localized near the ends of differentiated muscle cells, which may be sites of active fusion, whereas Bin3 and F-actin colocalized throughout the myotube (Fig. 6.9.5), suggesting only a partially overlapping function between CKB and Bin3 in myotubes. As CKB localization did not change in Bin3 KO myotubes (Fig. 6.9.1), these studies further suggest that Bin3 is not structurally responsible for CKB localization in differentiated muscle cells. However, CKB could provide ATP for Bin3-dependent regulation of actin dynamics. To determine whether and how CKB could regulate actin polymerization in a Bin3-dependent manner, warranted future directions would be examining whether Rac1 and Cdc42 have a role in multinucleated muscle cells, whether they localize near myotube ends, and whether levels of active Rac1 and Cdc42 are affected when CKB activity is manipulated in muscle cells.

Since Bin3 was identified as a CKB interacting protein, the relevance of this interaction for myogenesis remains to be determined in the future. However, whether this interaction is regulated spatially or temporally during myogenesis is unknown. For example, does CKB recruit Bin3 to lamellipodia of myocytes, where Bin3 can regulate actin dynamics via Rac1 and Cdc42? Also, do CKB and Bin3 interact in lamellipodia and

is this interaction required for myocyte migration? Finally, do CKB and Bin3 interact in myotubes, and what common functions does their partial colocalization in myotubes suggest? Future studies should determine whether a pathway involving CKB, Bin3 and the actin cytoskeleton exists in muscle cells, and how the interactions between these proteins may differ during *in vitro* myogenesis. For example, do some components of this pathway have a function only during certain stages of myogenesis? To test this hypothesis, future studies should determine novel Bin3 binding partners during different stages of myogenesis. The current hypothetical model for actin regulation in muscle cells consists of CKB providing ATP for Bin3-dependent actin regulation, which involves activation of Rac1 and Cdc42, leading to N-WASP/WAVE-dependent regulation of Arp2/3, which then mediates actin polymerization to regulate muscle cell migration/fusion and myotube formation (Fig. 6.9.6). However, this model remains to be thoroughly tested in mammalian skeletal muscle cells.

Several processes described in detail in this dissertation relate to various muscle diseases, and understanding the pathways regulating them would likely enable the development of various therapies for diseases in which these processes are misregulated. For example, myofiber branching has been observed in muscular dystrophy (Pavlath 2010a). The mechanisms regulating myofiber branching are unknown, however aberrant cell migration and/or incomplete fusion are possible contributors. As Bin3 regulates both muscle cell migration and myotube formation *in vitro* (Simionescu-Bankston et al. 2013), Bin3 is a promising therapeutic candidate for myofiber branching. Thus, elevating the levels of Bin3 could be an effective therapeutic strategy for resolving myofiber branching in mice and patients with muscular dystrophy. The second example related to muscle

disease is the regulation of myoblast fusion. Our results do not conclusively show that Bin3 regulates fusion. However, the function of Bin3 during later fusion stages, as well as the ability of BAR domain proteins to regulate both membrane and actin dynamics, which are crucial for the fusion process, suggest that Bin3 could be a possible regulator of the fusion step during myotube formation. Likewise, CKB was also required for myotube formation, and likely provides ATP for actin polymerization in muscle cells. However, whether CKB regulates actin dynamics directly or indirectly via its interacting partners, including Bin3, is unknown. Elucidating the pathways downstream of CKB that involve ATP-dependent regulatory proteins would allow for a deeper understanding of the myoblast fusion process, as well as the potential development of therapies for diseases resulting from fusion defects.

## 6.9 Figures

**Figure 6.9.1: CKB localization is unchanged in Bin3 KO myotubes**

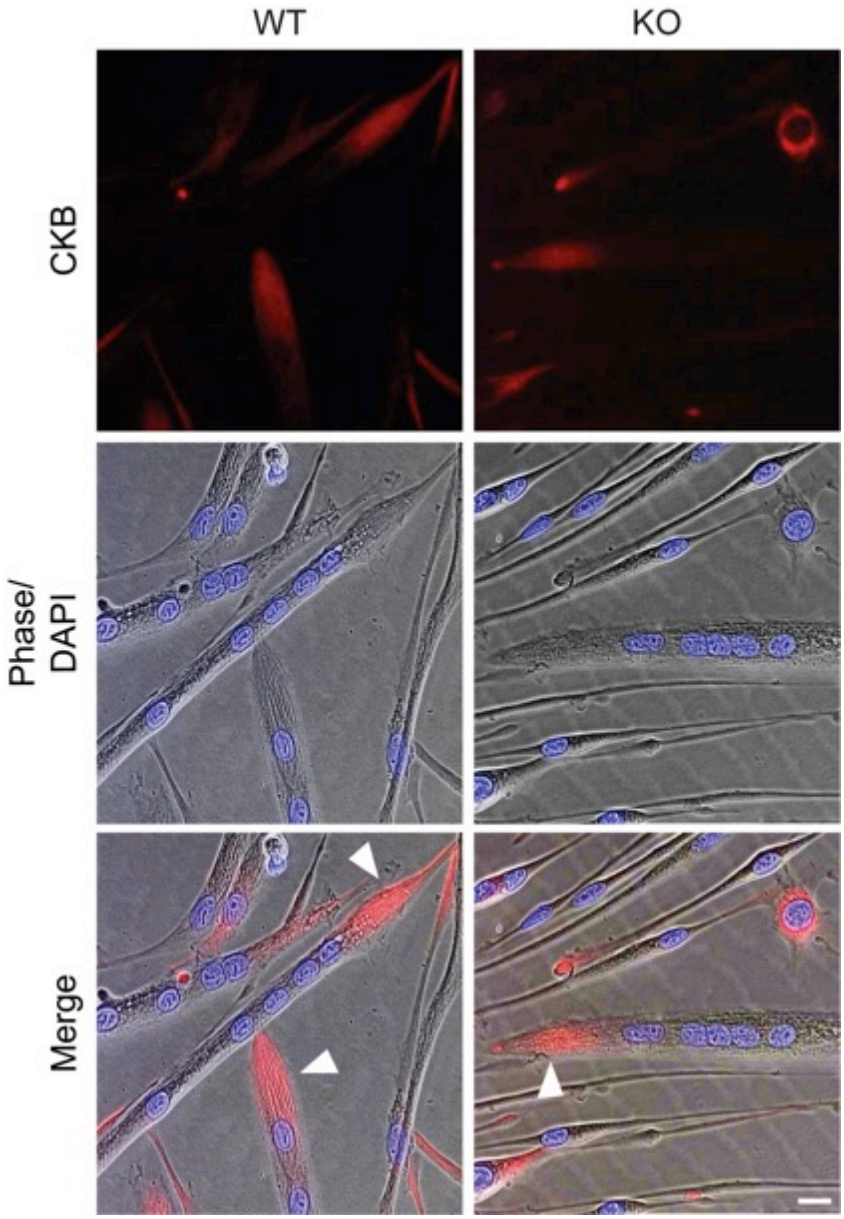

**Figure 6.9.1: CKB localization is unchanged in Bin3 KO myotubes**

Pure cultures of primary mouse muscle cells from WT and Bin3 KO mice were immunostained for CKB at 40 h in differentiation. CKB localized near the ends of myotubes in both WT and Bin3 KO cultures (arrowheads, last panel). Nuclei were counterstained with DAPI. Bar, 50 μm.

**Figure 6.9.2: Bin3 also functions in nascent myotubes for later fusion stages**

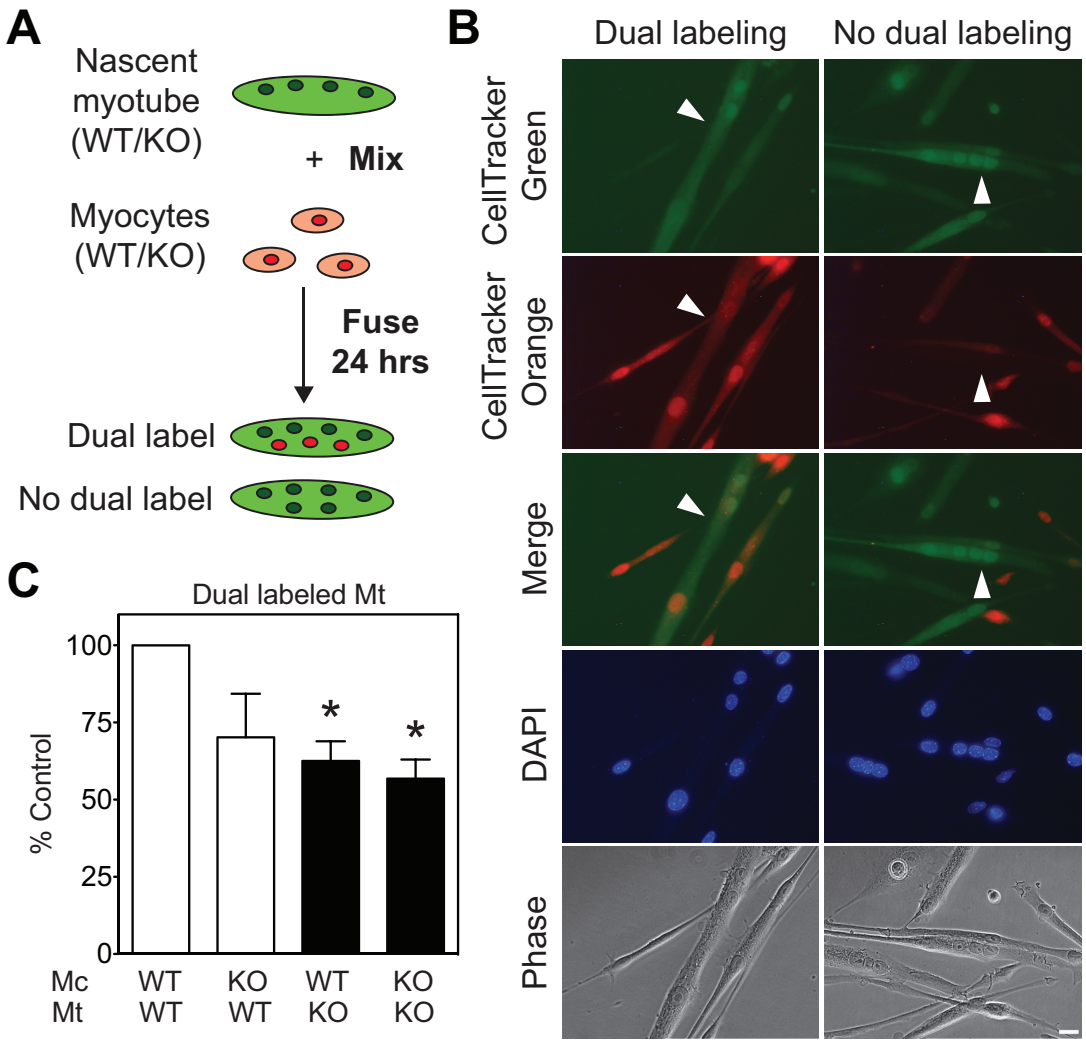

**Figure 6.9.2: Bin3 also functions in nascent myotubes for later fusion stages**

**(A)** Schematic of cell mixing experiment. **(B)** Representative myotubes with and without dual labeling are shown in separate and merged channels (arrowheads). Bar, 50 μm. **(C)** The percentage of myotubes with dual labeling was decreased when mixing Bin3 KO nascent myotubes (Mt) with WT myocytes (Mc), to a similar extent as when mixing Bin3 KO Mt with Bin3 KO Mc (*P<0.05). Data are mean ± s.e.m., *n*=3 independent isolates for each genotype.

**Figure 6.9.3: Bin3 is not required for receptor-mediated endocytosis in myotubes**

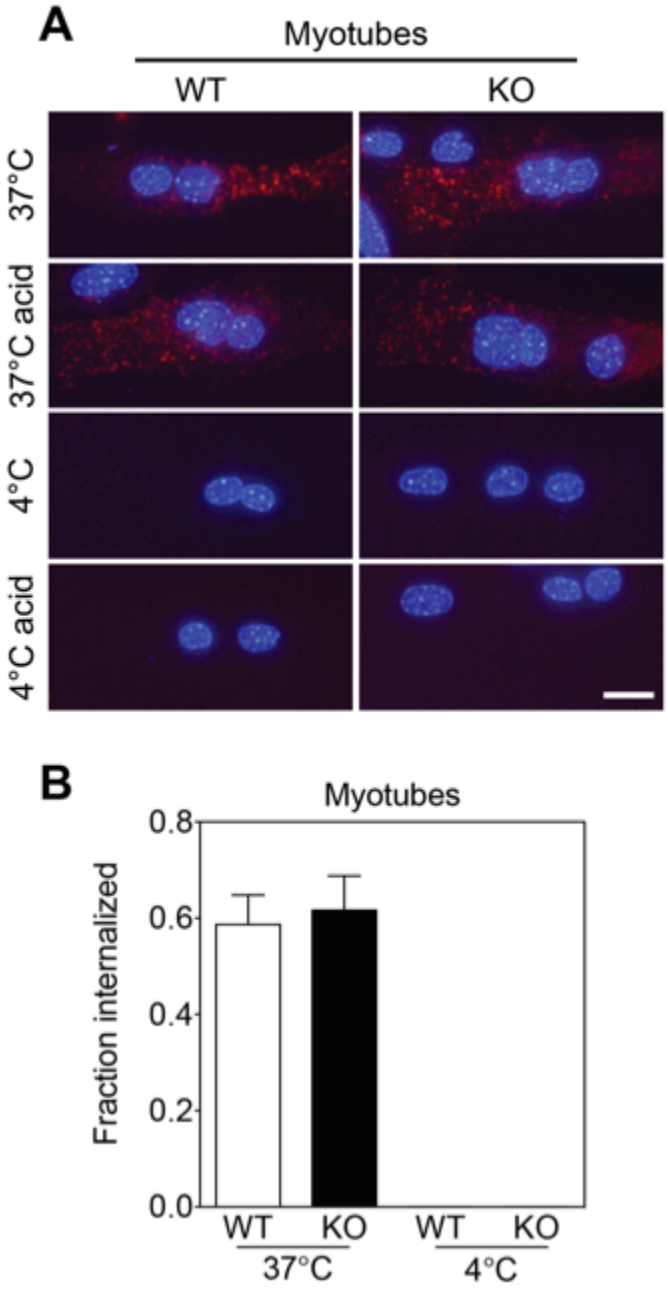

**Figure 6.9.3: Bin3 is not required for receptor-mediated endocytosis in myotubes**

**(A)** WT and Bin3 KO myotubes were incubated with Alexa 594-transferrin at 37°C or 4°C (± acid). No internalization of transferrin was observed in the control 4°C conditions. Nuclei were counterstained with DAPI. Bar, 50 μm. **(B)** The internalized transferrin fraction (37°C) was similar in WT and Bin3 KO myotubes. Data are mean ± s.e.m., *n*=3 independent isolates for each genotype.

**Figure 6.9.4: F-actin localization is similar in WT and Bin3 KO myotubes**

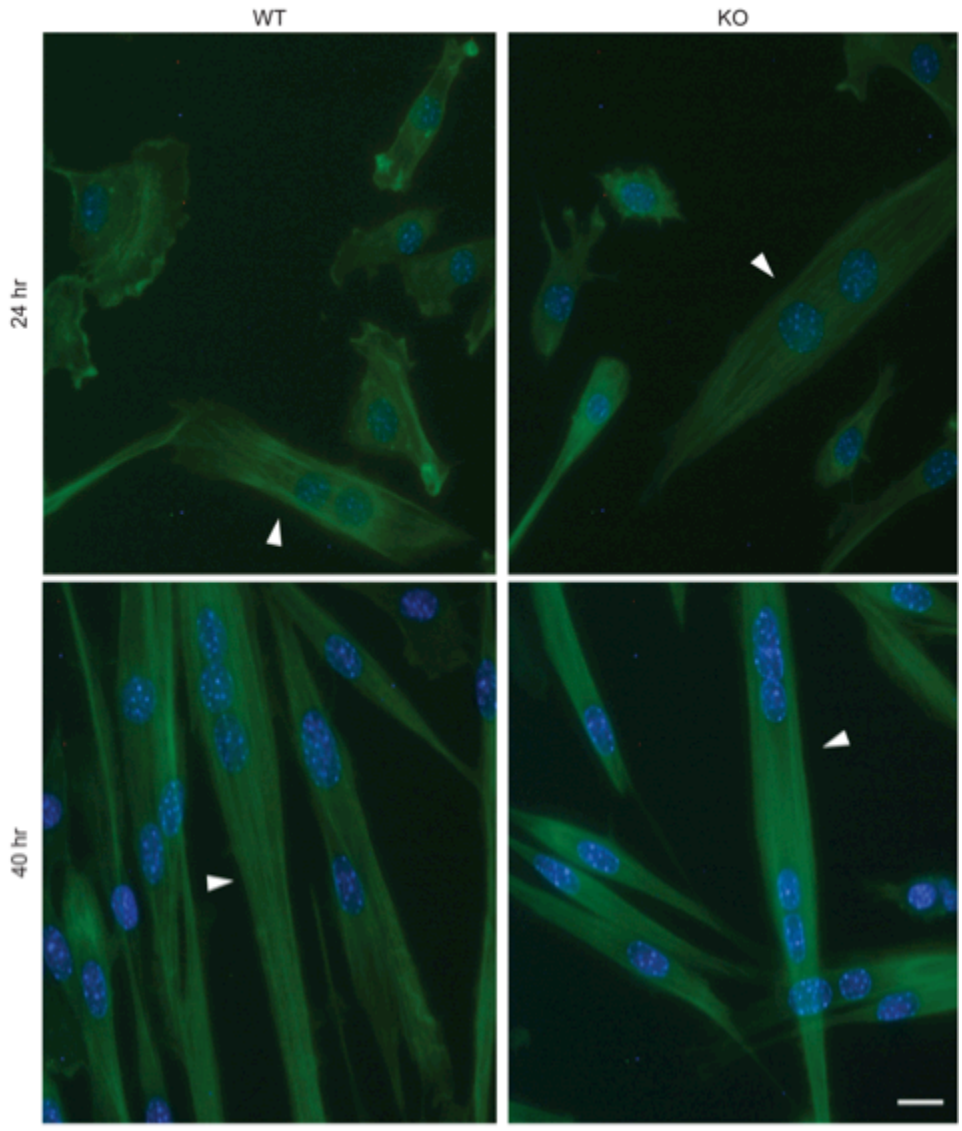

**Figure 6.9.4: F-actin localization is similar in WT and Bin3 KO myotubes**

No difference in F-actin localization (FITC-phalloidin) was noted between WT and Bin3 KO myotubes at 24 and 40 h in DM (arrowheads). Bar, 50 μm.

**Figure 6.9.5: Bin3 and F-actin colocalize in myotubes**

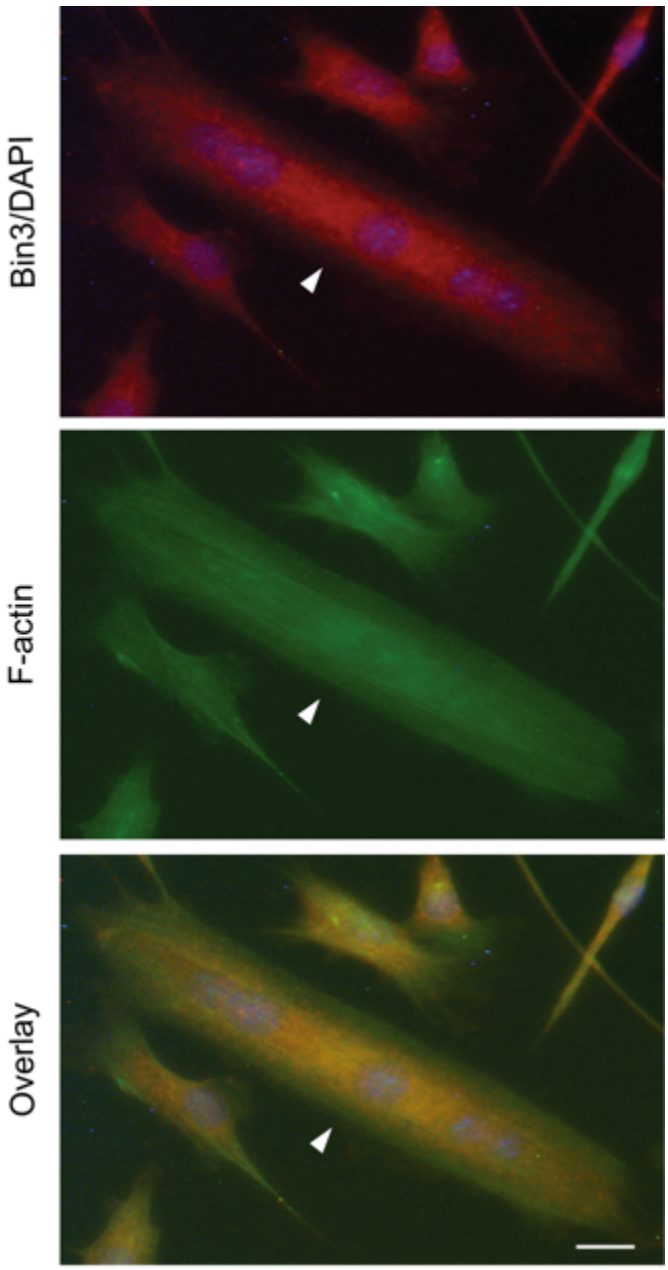

**Figure 6.9.5: Bin3 and F-actin colocalize in myotubes**

Bin3 and F-actin were colocalized throughout the cytoplasm of WT nascent myotubes at 24 h in DM (arrowheads). Nuclei were counterstained with DAPI. Bar, 50 μm.

**Figure 6.9.6: Model of CKB-mediated Bin3 action in myogenesis**

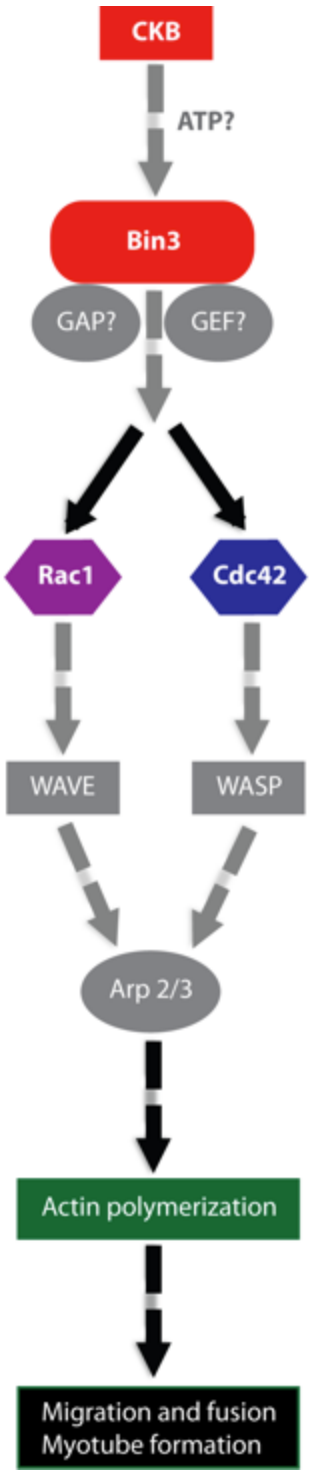

**Figure 6.9.6: Model of CKB-mediated Bin3 action in myogenesis**

CKB may provide ATP for a Bin3-dependent pathway in muscle cells, however the mechanisms by which this regulation occurs remain to be determined. Bin3 may then regulate the activity of Rac1 and Cdc42 in differentiated muscle cells through an unknown molecule containing a GAP or GEF domain. Subsequently, Rac1 and Cdc42 may regulate the actin regulatory proteins WAVE and WASP, respectively, leading to Arp2/3-dependent actin polymerization in muscle cells. Finally, actin dynamics in muscle cells may lead to regulation of muscle cell migration and fusion, and eventually to myotube formation.

**References**

Abbott KL, Friday BB, Thaloor D, Murphy TJ, and Pavlath GK. 1998. Activation and cellular localization of the cyclosporine a-sensitive transcription factor nf-at in skeletal muscle cells. *Mol Biol Cell* 9, no. 10: 2905-16.

Abercrombie M, Heaysman JE, and Pegrum SM. 1970. The locomotion of fibroblasts in culture. Ii. "Rruffling". *Exp Cell Res* 60, no. 3: 437-44.

Abercrombie M, Heaysman JE, and Pegrum SM. 1971. The locomotion of fibroblasts in culture. Iv. Electron microscopy of the leading lamella. *Exp Cell Res* 67, no. 2: 359-67.

Abmayr SM and Pavlath GK. 2012. Myoblast fusion: Lessons from flies and mice. *Development* 139, no. 4: 641-56.

Abramovici H and Gee SH. 2007. Morphological changes and spatial regulation of diacylglycerol kinase-zeta, syntrophins, and rac1 during myoblast fusion. *Cell Motil Cytoskeleton* 64, no. 7: 549-67.

Aksenov M, Aksenova M, Butterfield DA, and Markesbery WR. 2000. Oxidative modification of creatine kinase bb in alzheimer's disease brain. *J Neurochem* 74, no. 6: 2520-7.

Allen DL, Roy RR, and Edgerton VR. 1999. Myonuclear domains in muscle adaptation and disease. *Muscle Nerve* 22, no. 10: 1350-60.

Allen DL, Teitelbaum DH, and Kurachi K. 2003. Growth factor stimulation of matrix metalloproteinase expression and myoblast migration and invasion in vitro. *Am J Physiol Cell Physiol* 284, no. 4: C805-15.

Ames A, 3rd. 2000. Cns energy metabolism as related to function. *Brain Res Brain Res Rev* 34, no. 1-2: 42-68.

Arnaout MA, Mahalingam B, and Xiong JP. 2005. Integrin structure, allostery, and bidirectional signaling. *Annu Rev Cell Dev Biol* 21: 381-410.

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, and Chazaud B. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 204, no. 5: 1057-69.

Bach AS, Enjalbert S, Comunale F, Bodin S, Vitale N, Charrasse S, and Gauthier-Rouviere C. 2010. Adp-ribosylation factor 6 regulates mammalian myoblast fusion through phospholipase d1 and phosphatidylinositol 4,5-bisphosphate signaling pathways. *Mol Biol Cell* 21, no. 14: 2412-24.

Bae GU, Gaio U, Yang YJ, Lee HJ, Kang JS, and Krauss RS. 2008. Regulation of myoblast motility and fusion by the cxcr4-associated sialomucin, cd164. *J Biol Chem* 283, no. 13: 8301-9.

Bains W, Ponte P, Blau H, and Kedes L. 1984. Cardiac actin is the major gene product in skeletal muscle cell differentiation in vitro. *Mol Cell Biol* 4, no. 8: 1449-53.

Balasubramani M, Day BW, Schoen RE, and Getzenberg RH. 2006. Altered expression and localization of creatine kinase b, heterogeneous nuclear ribonucleoprotein f, and high mobility group box 1 protein in the nuclear matrix associated with colon cancer. *Cancer Res* 66, no. 2: 763-9.

Balcerzak D, Poussard S, Brustis JJ, Elamrani N, Soriano M, Cottin P, and Ducastaing A. 1995. An antisense oligodeoxyribonucleotide to m-calpain mrna inhibits myoblast fusion. *J Cell Sci* 108 ( Pt 5): 2077-82.

Balguerie A, Sivadon P, Bonneu M, and Aigle M. 1999. Rvs167p, the budding yeast homolog of amphiphysin, colocalizes with actin patches. *J Cell Sci* 112 ( Pt 15): 2529-37.

Bar-Sagi D and Prives J. 1983. Trifluoperazine, a calmodulin antagonist, inhibits muscle cell fusion. *J Cell Biol* 97, no. 5 Pt 1: 1375-80.

Barr FA. 1999. A novel rab6-interacting domain defines a family of golgi-targeted coiled-coil proteins. *Curr Biol* 9, no. 7: 381-4.

Bartel PL, Chien C, Sternglanz R, and Fields S. 1993. Using the two-hybrid system to detect protein-protein interactions. In *Cellular interactions in development: A practical approach.*, ed. D.A. Hartley. Oxford: IRL Press.

Bauer F, Urdaci M, Aigle M, and Crouzet M. 1993. Alteration of a yeast sh3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol Cell Biol* 13, no. 8: 5070-84.

Becciolini L, Meacci E, Donati C, Cencetti F, Rapizzi E, and Bruni P. 2006. Sphingosine 1-phosphate inhibits cell migration in c2c12 myoblasts. *Biochim Biophys Acta* 1761, no. 1: 43-51.

Behnia R and Munro S. 2005. Organelle identity and the signposts for membrane traffic. *Nature* 438, no. 7068: 597-604.

Bernards A and Settleman J. 2004. Gap control: Regulating the regulators of small gtpases. *Trends Cell Biol* 14, no. 7: 377-85.

Bijlenga P, Liu JH, Espinos E, Haenggeli CA, Fischer-Lougheed J, Bader CR, and Bernheim L. 2000. T-type alpha 1h ca2+ channels are involved in ca2+ signaling during terminal differentiation (fusion) of human myoblasts. *Proc Natl Acad Sci U S A* 97, no. 13: 7627-32.

Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC, Zemni R, Roest Crollius H, Carrie A, Fauchereau F, Cherry M, Briault S, Hamel B, Fryns JP, Beldjord C, Kahn A, Moraine C, and Chelly J. 1998. Oligophrenin-1 encodes a rhogap protein involved in x-linked mental retardation. *Nature* 392, no. 6679: 923-6.

Bintliff S and Walker BE. 1960. Radioautographic study of skeletal muscle regeneration. *Am J Anat* 106: 233-45.

Bischoff R. 1978. Myoblast fusion. In *Membrane fusion*, ed. G. Nicolson:127-79. New York: North-Holland Biomedical Press.

Bischoff R. 1994. The satellite cell and muscle regeneration. In *Myology*, ed. A. G. Engel and C. Franzini-Armstrong:97-118. New York: McGraw-Hill.

Bischoff R. 1997. Chemotaxis of skeletal muscle satellite cells. *Dev Dyn* 208, no. 4: 505-15.

Bitoun M. 2005. Mutations in dynamin 2 cause dominant centronuclear myopathy. *Nat Genet* 37: 1207-09.

Bockhold KJ, Rosenblatt JD, and Partridge TA. 1998. Aging normal and dystrophic mouse muscle: Analysis of myogenicity in cultures of living single fibers. *Muscle Nerve* 21, no. 2: 173-83.

Boettiger D, Enomoto-Iwamoto M, Yoon HY, Hofer U, Menko AS, and Chiquet-Ehrismann R. 1995. Regulation of integrin alpha 5 beta 1 affinity during myogenic differentiation. *Dev Biol* 169, no. 1: 261-72.

Bois PR, Brochard VF, Salin-Cantegrel AV, Cleveland JL, and Grosveld GC. 2005. Foxo1a-cyclic gmp-dependent kinase i interactions orchestrate myoblast fusion. *Mol Cell Biol* 25, no. 17: 7645-56.

Bois PR and Grosveld GC. 2003. Fkhr (foxo1a) is required for myotube fusion of primary mouse myoblasts. *EMBO J* 22, no. 5: 1147-57.

Bondesen BA, Jones KA, Glasgow WC, and Pavlath GK. 2007. Inhibition of myoblast migration by prostacyclin is associated with enhanced cell fusion. *FASEB J* 21, no. 12: 3338-45.

Bondesen BA, Mills ST, Kegley KM, and Pavlath GK. 2004. The cox-2 pathway is essential during early stages of skeletal muscle regeneration. *Am J Physiol Cell Physiol* 287, no. 2: C475-83.

Bourke DL and Ontell M. 1984. Branched myofibers in long-term whole muscle transplants: A quantitative study. *Anat Rec* 209, no. 3: 281-8.

Breton AM and Aigle M. 1998. Genetic and functional relationship between rvsp, myosin and actin in saccharomyces cerevisiae. *Curr Genet* 34: 280-86.

Brizzio V, Gammie AE, and Rose MD. 1998. Rvs161p interacts with fus2p to promote cell fusion in saccharomyces cerevisiae. *J Cell Biol* 141, no. 3: 567-84.

Brzoska E, Bello V, Darribere T, and Moraczewski J. 2006. Integrin alpha3 subunit participates in myoblast adhesion and fusion in vitro. *Differentiation* 74, no. 2-3: 105-18.

Burklen TS, Hirschy A, and Wallimann T. 2007. Brain-type creatine kinase bb-ck interacts with the golgi matrix protein gm130 in early prophase. *Mol Cell Biochem* 297, no. 1-2: 53-64.

Burklen TS, Schlattner U, Homayouni R, Gough K, Rak M, Szeghalmi A, and Wallimann T. 2006. The creatine kinase/creatine connection to alzheimer's disease: Ck-inactivation, app-ck complexes and focal creatine deposits. *J Biomed Biotechnol* 2006, no. 3: 35936.

Butler MH, David C, Ochoa GC, Freyberg Z, Daniell L, Grabs D, Cremona O, and De Camilli P. 1997. Amphiphysin ii (sh3p9; bin1), a member of the amphiphysin/rvs family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of ranvier in brain and around t tubules in skeletal muscle. *J Cell Biol* 137, no. 6: 1355-67.

Campellone KG and Welch MD. 2010. A nucleator arms race: Cellular control of actin assembly. *Nat Rev Mol Cell Biol* 11, no. 4: 237-51.

Casella JF, Flanagan MD, and Lin S. 1981. Cytochalasin d inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature* 293, no. 5830: 302-5.

Cestra G, Kwiatkowski A, Salazar M, Gertler F, and De Camilli P. 2005. Tuba, a gef for cdc42, links dynamin to actin regulatory proteins. *Methods Enzymol* 404: 537-45.

Chan S, Head SI, and Morley JW. 2007. Branched fibers in dystrophic mdx muscle are associated with a loss of force following lengthening contractions. *Am J Physiol Cell Physiol* 293, no. 3: C985-92.

Chang EJ, Ha J, Oerlemans F, Lee YJ, Lee SW, Ryu J, Kim HJ, Lee Y, Kim HM, Choi JY, Kim JY, Shin CS, Pak YK, Tanaka S, Wieringa B, Lee ZH, and Kim HH. 2008. Brain-type creatine kinase has a crucial role in osteoclast-mediated bone resorption. *Nat Med* 14, no. 9: 966-72.

Charge SB and Rudnicki MA. 2004. Cellular and molecular regulation of muscle regeneration. *Physiol Rev* 84, no. 1: 209-38.

Charrasse S, Comunale F, Fortier M, Portales-Casamar E, Debant A, and Gauthier-Rouviere C. 2007. M-cadherin activates rac1 gtpase through the rho-gef trio during myoblast fusion. *Mol Biol Cell* 18, no. 5: 1734-43.

Charrasse S, Comunale F, Grumbach Y, Poulat F, Blangy A, and Gauthier-Rouviere C. 2006. Rhoa gtpase regulates m-cadherin activity and myoblast fusion. *Mol Biol Cell* 17, no. 2: 749-59.

Chazaud B, Sonnet C, Lafuste P, Bassez G, Rimaniol AC, Poron F, Authier FJ, Dreyfus PA, and Gherardi RK. 2003. Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth. *J Cell Biol* 163, no. 5: 1133-43.

Chen EH, Pryce BA, Tzeng JA, Gonzalez GA, and Olson EN. 2003. Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector arf6. *Cell* 114, no. 6: 751-62.

Chen Y, Aardema J, Misra A, and Corey SJ. 2012. Bar proteins in cancer and blood disorders. *Int J Biochem Mol Biol* 3, no. 2: 198-208.

Chida K, Kasahara K, Tsunenaga M, Kohno Y, and Yamada S. 1990. Purification and identification of creatine phosphokinase b as a substrate of protein kinase c in mouse skin in vivo. *Biochem Biophys Res Commun* 173: 351–57.

Chromiak JA and Antonio J. 2008. Skeletal muscle plasticity. In *Essentials of sports nutrition and supplements*, ed. J. Antonio, D. Kalman, J. R. Stout, M. Greenwood, D. S. Willoughby and G. G. Haff, I:21-52. Totowa, NJ: Humana Press.

Coll PM, Rincon SA, Izquierdo RA, and Perez P. 2007. Hob3p, the fission yeast ortholog of human bin3, localizes cdc42p to the division site and regulates cytokinesis. *EMBO J* 26, no. 7: 1865-77.

Constantin B, Cognard C, and Raymond G. 1996. Myoblast fusion requires cytosolic calcium elevation but not activation of voltage-dependent calcium channels. *Cell Calcium* 19, no. 5: 365-74.

Conti FJ, Monkley SJ, Wood MR, Critchley DR, and Muller U. 2009. Talin 1 and 2 are required for myoblast fusion, sarcomere assembly and the maintenance of myotendinous junctions. *Development* 136, no. 21: 3597-606.

Corti S, Salani S, Del Bo R, Sironi M, Strazzer S, D'Angelo MG, Comi GP, Bresolin N, and Scarlato G. 2001. Chemotactic factors enhance myogenic cell migration across an endothelial monolayer. *Exp Cell Res* 268, no. 1: 36-44.

Coue M, Brenner SL, Spector I, and Korn ED. 1987. Inhibition of actin polymerization by latrunculin a. *FEBS Lett* 213, no. 2: 316-8.

Crawley S, Farrell EM, Wang W, Gu M, Huang HY, Huynh V, Hodges BL, Cooper DN, and Kaufman SJ. 1997. The alpha7beta1 integrin mediates adhesion and migration of skeletal myoblasts on laminin. *Exp Cell Res* 235, no. 1: 274-86.

Crouzet M, Urdaci M, Dulau L, and Aigle M. 1991. Yeast mutant affected for viability upon nutrient starvation: Characterization and cloning of the rvs161 gene. *Yeast* 7, no. 7: 727-43.

Czuchra A, Wu X, Meyer H, van Hengel J, Schroeder T, Geffers R, Rottner K, and Brakebusch C. 2005. Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells. *Mol Biol Cell* 16, no. 10: 4473-84.

Dalkilic I, Schienda J, Thompson TG, and Kunkel LM. 2006. Loss of filaminc (flnc) results in severe defects in myogenesis and myotube structure. *Mol Cell Biol* 26, no. 17: 6522-34.

Darr KC and Schultz E. 1989. Hindlimb suspension suppresses muscle growth and satellite cell proliferation. *J Appl Physiol* 67, no. 5: 1827-34.

David C, McPherson PS, Mundigl O, and de Camilli P. 1996. A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc Natl Acad Sci U S A* 93, no. 1: 331-5.

David C, Solimena M, and De Camilli P. 1994. Autoimmunity in stiff-man syndrome with breast cancer is targeted to the c-terminal region of human amphiphysin, a protein similar to the yeast proteins, rvs167 and rvs161. *FEBS Lett* 351, no. 1: 73-9.

David JD and Higginbotham CA. 1981. Fusion of chick embryo skeletal myoblasts: Interactions of prostaglandin e1, adenosine 3':5' monophosphate, and calcium influx. *Dev Biol* 82, no. 2: 308-16.

Dawson JC, Legg JA, and Machesky LM. 2006. Bar domain proteins: A role in tubulation, scission and actin assembly in clathrin-mediated endocytosis. *Trends Cell Biol* 16, no. 10: 493-8.

De Camilli P, Thomas A, Cofiell R, Folli F, Lichte B, Piccolo G, Meinck HM, Austoni M, Fassetta G, Bottazzo G, and et al. 1993. The synaptic vesicle-associated protein amphiphysin is the 128-kd autoantigen of stiff-man syndrome with breast cancer. *J Exp Med* 178, no. 6: 2219-23.

de Kreuk BJ and Hordijk PL. 2012. Control of rho gtpase function by bar-domains. *Small Gtpases* 3, no. 1: 45-52.

de Kreuk BJ, Nethe M, Fernandez-Borja M, Anthony EC, Hensbergen PJ, Deelder AM, Plomann M, and Hordijk PL. 2011. The f-bar domain protein pacsin2 associates with rac1 and regulates cell spreading and migration. *J Cell Sci* 124, no. Pt 14: 2375-88.

De Matteis MA and Godi A. 2004. Pi-loting membrane traffic. *Nat Cell Biol* 6, no. 6: 487-92.

Decary S, Mouly V, Hamida CB, Sautet A, Barbet JP, and Butler-Browne GS. 1997. Replicative potential and telomere length in human skeletal muscle: Implications for satellite cell-mediated gene therapy. *Hum Gene Ther* 8, no. 12: 1429-38.

Dhawan J and Rando TA. 2005. Stem cells in postnatal myogenesis: Molecular mechanisms of satellite cell quiescence, activation and replenishment. *Trends Cell Biol* 15, no. 12: 666-73.

Di Paolo G and De Camilli P. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, no. 7112: 651-7.

Di Paolo G, Sankaranarayanan S, Wenk MR, Daniell L, Perucco E, Caldarone BJ, Flavell R, Picciotto MR, Ryan TA, Cremona O, and De Camilli P. 2002. Decreased synaptic vesicle recycling efficiency and cognitive deficits in amphiphysin 1 knockout mice. *Neuron* 33, no. 5: 789-804.

Diguet N, Mallat Y, Ladouce R, Clodic G, Prola A, Tritsch E, Blanc J, Larcher JC, Delcayre C, Samuel JL, Friguet B, Bolbach G, Li Z, and Mericskay M. 2011. Muscle creatine kinase deficiency triggers both actin depolymerization and desmin disorganization by advanced glycation end products in dilated cardiomyopathy. *J Biol Chem* 286, no. 40: 35007-19.

Doherty GJ, Ahlund MK, Howes MT, Moren B, Parton RG, McMahon HT, and Lundmark R. 2011. The endocytic protein graf1 is directed to cell-matrix adhesion sites and regulates cell spreading. *Mol Biol Cell* 22, no. 22: 4380-9.

Doherty GJ and McMahon HT. 2009. Mechanisms of endocytosis. *Annu Rev Biochem* 78: 857-902.

Doherty JT, Lenhart KC, Cameron MV, Mack CP, Conlon FL, and Taylor JM. 2011. Skeletal muscle differentiation and fusion are regulated by the bar-containing rho-gtpase-activating protein (rho-gap), graf1. *J Biol Chem* 286, no. 29: 25903-21.

Doherty KR, Cave A, Davis DB, Delmonte AJ, Posey A, Earley JU, Hadhazy M, and McNally EM. 2005. Normal myoblast fusion requires myoferlin. *Development* 132, no. 24: 5565-75.

Doherty KR, Demonbreun AR, Wallace GQ, Cave A, Posey AD, Heretis K, Pytel P, and McNally EM. 2008. The endocytic recycling protein ehd2 interacts with myoferlin to regulate myoblast fusion. *J Biol Chem* 283, no. 29: 20252-60.

Donaldson JG. 2008. Arfs and membrane lipids: Sensing, generating and responding to membrane curvature. *Biochem J* 414, no. 2: e1-2.

Donovan FM, Pike CJ, Cotman CW, and Cunningham DD. 1997. Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and rhoa activities. *J Neurosci* 17, no. 14: 5316-26.

Dowling JJ, Vreede AP, Kim S, Golden J, and Feldman EL. 2008. Kindlin-2 is required for myocyte elongation and is essential for myogenesis. *BMC Cell Biol* 9: 36.

Duan R and Gallagher PJ. 2009. Dependence of myoblast fusion on a cortical actin wall and nonmuscle myosin iia. *Dev Biol* 325, no. 2: 374-85.

Durante G. 1902. Anatomie pathologique des muscles. In *Manuel d'histologie pathologique*, ed. V. Cornil and L. Ranvier, 2:1. Paris, France: Felix Alcan.

Echtermeyer F, Schober S, Poschl E, von der Mark H, and von der Mark K. 1996. Specific induction of cell motility on laminin by alpha 7 integrin. *J Biol Chem* 271, no. 4: 2071-5.

Endris V, Wogatzky B, Leimer U, Bartsch D, Zatyka M, Latif F, Maher ER, Tariverdian G, Kirsch S, Karch D, and Rappold GA. 2002. The novel rho-gtpase activating

gene megap/ srgap3 has a putative role in severe mental retardation. *Proc Natl Acad Sci U S A* 99, no. 18: 11754-9.

Entwistle A, Zalin RJ, Bevan S, and Warner AE. 1988. The control of chick myoblast fusion by ion channels operated by prostaglandins and acetylcholine. *J Cell Biol* 106, no. 5: 1693-702.

Erb WH. 1891. Dystrophia muscularis progressiva. Klinische und pathologisch anatomische studien. *Dtsch Z Nervenheilk* 1: 173-261.

Even-Ram S, Uziely B, Cohen P, Grisaru-Granovsky S, Maoz M, Ginzburg Y, Reich R, Vlodavsky I, and Bar-Shavit R. 1998. Thrombin receptor overexpression in malignant and physiological invasion processes. *Nat Med* 4, no. 8: 909-14.

Farkas-Bargeton E, Barbet JP, Dancea S, Wehrle R, Checouri A, and Dulac O. 1988. Immaturity of muscle fibers in the congenital form of myotonic dystrophy: Its consequences and its origin. *J Neurol Sci* 83, no. 2-3: 145-59.

Fernando P, Sandoz JS, Ding W, de Repentigny Y, Brunette S, Kelly JF, Kothary R, and Megeney LA. 2009. Bin1 src homology 3 domain acts as a scaffold for myofiber sarcomere assembly. *J Biol Chem* 284, no. 40: 27674-86.

Fielding RA, Manfredi TJ, Ding W, Fiatarone MA, Evans WJ, and Cannon JG. 1993. Acute phase response in exercise. Iii. Neutrophil and il-1 beta accumulation in skeletal muscle. *Am J Physiol* 265, no. 1 Pt 2: R166-72.

Fischer-Lougheed J, Liu JH, Espinos E, Mordasini D, Bader CR, Belin D, and Bernheim L. 2001. Human myoblast fusion requires expression of functional inward rectifier kir2.1 channels. *J Cell Biol* 153, no. 4: 677-86.

Folli F, Solimena M, Cofiell R, Austoni M, Tallini G, Fassetta G, Bates D, Cartlidge N, Bottazzo GF, Piccolo G, De Camilli P, and et al. 1993. Autoantibodies to a 128-kd synaptic protein in three women with the stiff-man syndrome and breast cancer. *N Engl J Med* 328, no. 8: 546-51.

Fornaro M, Burch PM, Yang W, Zhang L, Hamilton CE, Kim JH, Neel BG, and Bennett AM. 2006. Shp-2 activates signaling of the nuclear factor of activated t cells to promote skeletal muscle growth. *J Cell Biol* 175, no. 1: 87-97.

Fortier M, Comunale F, Kucharczak J, Blangy A, Charrasse S, and Gauthier-Rouviere C. 2008. Rhoe controls myoblast alignment prior fusion through rhoa and rock. *Cell Death Differ* 15, no. 8: 1221-31.

Foster R, Hu KQ, Lu Y, Nolan KM, Thissen J, and Settleman J. 1996. Identification of a novel human rho protein with unusual properties: Gtpase deficiency and in vivo farnesylation. *Mol Cell Biol* 16, no. 6: 2689-99.

Friedl P and Gilmour D. 2009. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 10, no. 7: 445-57.

Frost A, De Camilli P, and Unger VM. 2007. F-bar proteins join the bar family fold. *Structure* 15, no. 7: 751-3.

Frost A, Perera R, Roux A, Spasov K, Destaing O, Egelman EH, De Camilli P, and Unger VM. 2008. Structural basis of membrane invagination by f-bar domains. *Cell* 132, no. 5: 807-17.

Frost A, Unger VM, and De Camilli P. 2009. The bar domain superfamily: Membrane-molding macromolecules. *Cell* 137, no. 2: 191-6.

Fulton AB, Prives J, Farmer SR, and Penman S. 1981. Developmental reorganization of the skeletal framework and its surface lamina in fusing muscle cells. *J Cell Biol* 91, no. 1: 103-12.

Galbiati F, Volonte D, Engelman JA, Scherer PE, and Lisanti MP. 1999. Targeted down-regulation of caveolin-3 is sufficient to inhibit myotube formation in differentiating c2c12 myoblasts. Transient activation of p38 mitogen-activated protein kinase is required for induction of caveolin-3 expression and subsequent myotube formation. *J Biol Chem* 274, no. 42: 30315-21.

Ge K and Prendergast GC. 2000. Bin2, a functionally nonredundant member of the bar adaptor gene family. *Genomics* 67, no. 2: 210-20.

Gollnick PD, Parsons D, Riedy M, and Moore RL. 1983. Fiber number and size in overloaded chicken anterior latissimus dorsi muscle. *J Appl Physiol* 54, no. 5: 1292-7.

Gollnick PD, Timson BF, Moore RL, and Riedy M. 1981. Muscle enlargement and number of fibers in skeletal muscle of rats. *J Appl Physiol* 50: 936-43.

Goodman SL, Risse G, and von der Mark K. 1989. The e8 subfragment of laminin promotes locomotion of myoblasts over extracellular matrix. *J Cell Biol* 109, no. 2: 799-809.

Gorza L and Vitadello M. 2000. Reduced amount of the glucose-regulated protein grp94 in skeletal myoblasts results in loss of fusion competence. *FASEB J* 14, no. 3: 461-75.

Grabs D, Slepnev VI, Songyang Z, David C, Lynch M, Cantley LC, and De Camilli P. 1997. The sh3 domain of amphiphysin binds the proline-rich domain of dynamin

at a single site that defines a new sh3 binding consensus sequence. *J Biol Chem* 272, no. 20: 13419-25.

Grand RJ, Turnell AS, and Grabham PW. 1996. Cellular consequences of thrombin-receptor activation. *Biochem J* 313 ( Pt 2): 353-68.

Griffin CA, Apponi LH, Long KK, and Pavlath GK. 2010. Chemokine expression and control of muscle cell migration during myogenesis. *J Cell Sci* 123, no. Pt 18: 3052-60.

Griffin CA, Kafadar KA, and Pavlath GK. 2009. Mor23 promotes muscle regeneration and regulates cell adhesion and migration. *Dev Cell* 17, no. 5: 649-61.

Grounds MD. 1987. Phagocytosis of necrotic muscle in muscle isografts is influenced by the strain, age, and sex of host mice. *J Pathol* 153, no. 1: 71-82.

Gruenbaum-Cohen Y, Harel I, Umansky KB, Tzahor E, Snapper SB, Shilo BZ, and Schejter ED. 2012. The actin regulator n-wasp is required for muscle-cell fusion in mice. *Proc Natl Acad Sci U S A.* 109, no. 28: 11211-6.

Guerrier S, Coutinho-Budd J, Sassa T, Gresset A, Jordan NV, Chen K, Jin WL, Frost A, and Polleux F. 2009. The f-bar domain of srgap2 induces membrane protrusions required for neuronal migration and morphogenesis. *Cell* 138, no. 5: 990-1004.

Guillou H, Depraz-Depland A, Planus E, Vianay B, Chaussy J, Grichine A, Albiges-Rizo C, and Block MR. 2008. Lamellipodia nucleation by filopodia depends on integrin occupancy and downstream rac1 signaling. *Exp Cell Res* 314, no. 3: 478-88.

Habermann B. 2004. The bar-domain family of proteins: A case of bending and binding? *EMBO Rep* 5, no. 3: 250-5.

Hall A. 1994. Small gtp-binding proteins and the regulation of the actin cytoskeleton. *Annu Rev Cell Biol* 10: 31-54.

Hall A. 1998. Rho gtpases and the actin cytoskeleton. *Science* 279, no. 5350: 509-14.

Hall-Craggs EC. 1970. The longitudinal division of fibres in overloaded rat skeletal muscle. *J Anat* 107, no. Pt 3: 459-70.

Hall-Craggs ECB. 1972. The significance of longitudinal fibre division in skeletal muscle. *J Neurol Sci* 15, no. 1: 27-33.

Hawke TJ and Garry DJ. 2001. Myogenic satellite cells: Physiology to molecular biology. *J Appl Physiol* 91, no. 2: 534-51.

He F, Dang W, Abe C, Tsuda K, Inoue M, Watanabe S, Kobayashi N, Kigawa T, Matsuda T, Yabuki T, Aoki M, Seki E, Harada T, Tomabechi Y, Terada T, Shirouzu M, Tanaka A, Guntert P, Muto Y, and Yokoyama S. 2009. Solution structure of the rna binding domain in the human muscleblind-like protein 2. *Protein Sci* 18, no. 1: 80-91.

Head SI, Williams DA, and Stephenson DG. 1992. Abnormalities in structure and function of limb skeletal muscle fibres of dystrophic mdx mice. *Proc Biol Sci* 248, no. 1322: 163-9.

Henderson DM, Lee A, and Ervasti JM. 2010. Disease-causing missense mutations in actin binding domain 1 of dystrophin induce thermodynamic instability and protein aggregation. *Proc Natl Acad Sci U S A* 107, no. 21: 9632-7.

Hildebrand JD, Taylor JM, and Parsons JT. 1996. An sh3 domain-containing gtpase-activating protein for rho and cdc42 associates with focal adhesion kinase. *Mol Cell Biol* 16, no. 6: 3169-78.

Holbert S, Dedeoglu A, Humbert S, Saudou F, Ferrante RJ, and Neri C. 2003. Cdc42-interacting protein 4 binds to huntingtin: Neuropathologic and biological evidence for a role in huntington's disease. *Proc Natl Acad Sci U S A* 100, no. 5: 2712-7.

Hollenberg SM, Sternglanz R, Cheng PF, and Weintraub H. 1995. Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol Cell Biol* 15, no. 7: 3813-22.

Hornemann T, Rutishauser D, and Wallimann T. 2000. Why is creatine kinase a dimer? Evidence for cooperativity between the two subunits. *Biochim Biophys Act* 1480: 365–73.

Horsley V, Friday BB, Matteson S, Kegley KM, Gephart J, and Pavlath GK. 2001. Regulation of the growth of multinucleated muscle cells by an nfatc2-dependent pathway. *J Cell Biol* 153, no. 2: 329-38.

Horsley V, Jansen KM, Mills ST, and Pavlath GK. 2003. Il-4 acts as a myoblast recruitment factor during mammalian muscle growth. *Cell* 113, no. 4: 483-94.

Horsley V and Pavlath GK. 2003. Prostaglandin f2(alpha) stimulates growth of skeletal muscle cells via an nfatc2-dependent pathway. *J Cell Biol* 161, no. 1: 111-8.

Horsley V and Pavlath GK. 2004. Forming a multinucleated cell: Molecules that regulate myoblast fusion. *Cells Tissues Organs* 176, no. 1-3: 67-78.

Hu W, Zhou S, Wang JS, and Meng F. 2011. Computation simulations to predict creatine-kinase associated factors: Protein-protein interaction studies of brain and muscle types of creatine kinases. *Enzyme Research* 2011: 1-12.

Huang D, Patrick G, Moffat J, Tsai LH, and Andrews B. 1999. Mammalian cdk5 is a functional homologue of the budding yeast pho85 cyclin-dependent protein kinase. *Proc Natl Acad Sci U S A* 96, no. 25: 14445-50.

Huddleston HG, Wong KK, Welch WR, Berkowitz RS, and Mok SC. 2005. Clinical applications of microarray technology: Creatine kinase b is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. *Gynecol Oncol* 96, no. 1: 77-83.

Hurme T, Kalimo H, Lehto M, and Jarvinen M. 1991. Healing of skeletal muscle injury: An ultrastructural and immunohistochemical study. *Med Sci Sports Exerc* 23, no. 7: 801-10.

Iezzi S, Di Padova M, Serra C, Caretti G, Simone C, Maklan E, Minetti G, Zhao P, Hoffman EP, Puri PL, and Sartorelli V. 2004. Deacetylase inhibitors increase muscle cell size by promoting myoblast recruitment and fusion through induction of follistatin. *Dev Cell* 6, no. 5: 673-84.

Isaacs ER, Bradley WG, and Henderson G. 1973. Longitudinal fibre splitting in muscular dystrophy: A serial cinematographic study. *J Neurol Neurosurg Psychiatry* 36, no. 5: 813-9.

Ishikawa J, Taniguchi T, Takeshita A, and Maekawa M. 2005. Increased creatine kinase bb activity and ckb mrna expression in patients with hematologic disorders: Relation to methylation status of the ckb promoter. *Clin Chim Acta* 361, no. 1-2: 135-40.

Itoh T and De Camilli P. 2006. Bar, f-bar (efc) and enth/anth domains in the regulation of membrane-cytosol interfaces and membrane curvature. *Biochim Biophys Acta* 1761, no. 8: 897-912.

Iwabata H, Yoshida M, and Komatsu Y. 2005. Proteomic analysis of organspecific post-translational lysine-acetylation and -methylation in mice by use of anti-acetyllysine and -methyllysine mouse monoclonal antibodies. *Proteomics* 5: 4653–64.

Jane DT, DaSilva L, Koblinski J, Horwitz M, Sloane BF, and Dufresne MJ. 2002. Evidence for the involvement of cathepsin b in skeletal myoblast differentiation. *J Cell Biochem* 84, no. 3: 520-31.

Jansen KM and Pavlath GK. 2006. Mannose receptor regulates myoblast motility and muscle growth. *J Cell Biol* 174, no. 3: 403-13.

Joe MK, Sohn S, Kim TE, Im JE, Choi YR, and Kee C. 2011. Analysis of glucocorticoid-induced myoc expression in human trabecular meshwork cells. *Vision Res* 51, no. 9: 1033-8.

Kadlec L and Pendergast AM. 1997. The amphiphysin-like protein 1 (alp1) interacts functionally with the cabl tyrosine kinase and may play a role in cytoskeletal regulation. *Proc Natl Acad Sci U S A* 94, no. 23: 12390-5.

Kafadar KA, Yi L, Ahmad Y, So L, Rossi F, and Pavlath GK. 2009. Sca-1 expression is required for efficient remodeling of the extracellular matrix during skeletal muscle regeneration. *Dev Biol* 326, no. 1: 47-59.

Kaksonen M, Toret CP, and Drubin DG. 2005. A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell* 123, no. 2: 305-20.

Kang JS, Yi MJ, Zhang W, Feinleib JL, Cole F, and Krauss RS. 2004. Netrins and neogenin promote myotube formation. *J Cell Biol* 167, no. 3: 493-504.

Kashina AS. 2006. Differential arginylation of actin isoforms: The mystery of the actin n-terminus. *Trends Cell Biol* 16, no. 12: 610-5.

Katz FRS. 1961. The termination of the afferent nerve fiber in the muscle spindle of the frog. *Philos Trans R Soc Lond B Biol Sci* 243: 221-25.

Kawamura K, Takano K, Suetsugu S, Kurisu S, Yamazaki D, Miki H, Takenawa T, and Endo T. 2004. N-wasp and wave2 acting downstream of phosphatidylinositol 3-kinase are required for myogenic cell migration induced by hepatocyte growth factor. *J Biol Chem* 279, no. 52: 54862-71.

Kim S, Shilagardi K, Zhang S, Hong SN, Sens KL, Bo J, Gonzalez GA, and Chen EH. 2007. A critical function for the actin cytoskeleton in targeted exocytosis of prefusion vesicles during myoblast fusion. *Dev Cell* 12, no. 4: 571-86.

Kim SJ, Kim S, Shin H, and Uhm CS. 2008. Intercellular interaction observed by atomic force microscopy. *Ultramicroscopy* 108, no. 10: 1148-51.

Kinoshita M, Field CM, Coughlin ML, Straight AF, and Mitchison TJ. 2002. Self- and actin-templated assembly of mammalian septins. *Dev Cell* 3, no. 6: 791-802.

Kitiyakara A and Angevine DM. 1963. A study of the pattern of postembryonic growth of m. Gracilis in mice. *Dev Biol* 8: 322-40.

Klein-Ogus C and Harris JB. 1983. Preliminary observations of satellite cells in undamaged fibres of the rat soleus muscle assaulted by a snake-venom toxin. *Cell Tissue Res* 230, no. 3: 671-6.

Knudsen KA. 1992. Membrane fusion. In *Membrane fusion*, ed. D. Hoekstra:601-26. New York: Dekker.

Knudsen KA and Horwitz AF. 1977. Tandem events in myoblast fusion. *Dev Biol* 58, no. 2: 328-38.

Knudsen KA and Horwitz AF. 1978. Differential inhibition of myoblast fusion. *Dev Biol* 66, no. 2: 294-307.

Knudsen KA, Myers L, and McElwee SA. 1990. A role for the ca2(+)-dependent adhesion molecule, n-cadherin, in myoblast interaction during myogenesis. *Exp Cell Res* 188, no. 2: 175-84.

Krauss RS, Cole F, Gaio U, Takaesu G, Zhang W, and Kang JS. 2005. Close encounters: Regulation of vertebrate skeletal myogenesis by cell-cell contact. *J Cell Sci* 118, no. Pt 11: 2355-62.

Kuiper JW, Pluk H, Oerlemans F, van Leeuwen FN, de Lange F, Fransen J, and Wieringa B. 2008. Creatine kinase-mediated atp supply fuels actin-based events in phagocytosis. *PLoS Biol* 6, no. 3: e51.

Kuiper JW, van Horssen R, Oerlemans F, Peters W, van Dommelen MM, te Lindert MM, ten Hagen TL, Janssen E, Fransen JA, and Wieringa B. 2009. Local atp generation by brain-type creatine kinase (ck-b) facilitates cell motility. *PLoS One* 4, no. 3: e5030.

Kuwano R, Miyashita A, Arai H, Asada T, Imagawa M, Shoji M, Higuchi S, Urakami K, Kakita A, Takahashi H, Tsukie T, Toyabe S, Akazawa K, Kanazawa I, and Ihara Y. 2006. Dynamin-binding protein gene on chromosome 10q is associated with late-onset alzheimer's disease. *Hum Mol Genet* 15, no. 13: 2170-82.

Lafreniere JF, Mills P, Bouchentouf M, and Tremblay JP. 2006. Interleukin-4 improves the migration of human myogenic precursor cells in vitro and in vivo. *Exp Cell Res* 312, no. 7: 1127-41.

Lafreniere JF, Mills P, Tremblay JP, and El Fahime E. 2004. Growth factors improve the in vivo migration of human skeletal myoblasts by modulating their endogenous proteolytic activity. *Transplantation* 77, no. 11: 1741-7.

Lafuste P, Sonnet C, Chazaud B, Dreyfus PA, Gherardi RK, Wewer UM, and Authier FJ. 2005. Adam12 and alpha9beta1 integrin are instrumental in human myogenic cell differentiation. *Mol Biol Cell* 16: 861-70.

Lai FP, Szczodrak M, Block J, Faix J, Breitsprecher D, Mannherz HG, Stradal TE, Dunn GA, Small JV, and Rottner K. 2008. Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J* 27, no. 7: 982-92.

Laurin M, Fradet N, Blangy A, Hall A, Vuori K, and Cote JF. 2008. The atypical rac activator dock180 (dock1) regulates myoblast fusion in vivo. *Proc Natl Acad Sci U S A* 105, no. 40: 15446-51.

Le Clainche C and Carlier MF. 2008. Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev* 88, no. 2: 489-513.

Le Grand F and Rudnicki MA. 2007. Skeletal muscle satellite cells and adult myogenesis. *Curr Opin Cell Biol* 19, no. 6: 628-33.

Lee E, Marcucci M, Daniell L, Pypaert M, Weisz OA, Ochoa GC, Farsad K, Wenk MR, and De Camilli P. 2002. Amphiphysin 2 (bin1) and t-tubule biogenesis in muscle. *Science* 297, no. 5584: 1193-6.

Lee J, Colwill K, Aneliunas V, Tennyson C, Moore L, Ho Y, and Andrews B. 1998. Interaction of yeast rvs167 and pho85 cyclin-dependent kinase complexes may link the cell cycle to the actin cytoskeleton. *Curr Biol* 8, no. 24: 1310-21.

Lee KH, Baek MY, Moon KY, Song WK, Chung CH, Ha DB, and Kang MS. 1994. Nitric oxide as a messenger molecule for myoblast fusion. *J Biol Chem* 269, no. 20: 14371-4.

Lee KK, Wong CC, Webb SE, Tang MK, Leung AK, Kwok PF, Cai DQ, and Chan KM. 1999. Hepatocyte growth factor stimulates chemotactic response in mouse embryonic limb myogenic cells in vitro. *J Exp Zool* 283, no. 2: 170-80.

Lee KS, Smith K, Amieux PS, and Wang EH. 2008. Mbnl3/chcr prevents myogenic differentiation by inhibiting myod-dependent gene transcription. *Differentiation* 76, no. 3: 299-309.

LeGros Clark WE. 1946. An experimental study of regeneration of mammalian striped muscle. *J Anat* 80: 24-36.

Leikina E, Melikov K, Sanyal S, Verma SK, Eun B, Gebert C, Pfeifer K, Lizunov VA, Kozlov MM, and Chernomordik LV. 2013. Extracellular annexins and dynamin are important for sequential steps in myoblast fusion. *J Cell Biol* 200, no. 1: 109-23.

Leprince C, Le Scolan E, Meunier B, Fraisier V, Brandon N, De Gunzburg J, and Camonis J. 2003. Sorting nexin 4 and amphiphysin 2, a new partnership between endocytosis and intracellular trafficking. *J Cell Sci* 116, no. Pt 10: 1937-48.

Lewis AK and Bridgman PC. 1992. Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. *J Cell Biol* 119, no. 5: 1219-43.

Lichte B, Veh RW, Meyer HE, and Kilimann MW. 1992. Amphiphysin, a novel protein associated with synaptic vesicles. *EMBO J* 11, no. 7: 2521-30.

Lila T and Drubin DG. 1997. Evidence for physical and functional interactions among two saccharomyces cerevisiae sh3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol Biol Cell* 8: 367-85.

Lipton BH and Konigsberg IR. 1972. A fine-structural analysis of the fusion of myogenic cells. *J Cell Biol* 53, no. 2: 348-64.

Lodish H, Berk A, Zipursky SL, and al. e. 2000. The dynamics of actin assembly. In *Molecular cell biology. 4th edition.*, ed. W. H. Freeman. New York.

Long JH, Lira VA, Soltow QA, Betters JL, Sellman JE, and Criswell DS. 2006. Arginine supplementation induces myoblast fusion via augmentation of nitric oxide production. *J Muscle Res Cell Motil* 27, no. 8: 577-84.

Lovering RM, Michaelson L, and Ward CW. 2009. Malformed mdx myofibers have normal cytoskeletal architecture yet altered ec coupling and stress-induced ca2+ signaling. *Am J Physiol Cell Physiol* 297, no. 3: C571-80.

Lu Z, Joseph D, Bugnard E, Zaal KJ, and Ralston E. 2001. Golgi complex reorganization during muscle differentiation: Visualization in living cells and mechanism. *Mol Biol Cell* 12, no. 4: 795-808.

Lundmark R, Doherty GJ, Howes MT, Cortese K, Vallis Y, Parton RG, and McMahon HT. 2008. The gtpase-activating protein graf1 regulates the clic/geec endocytic pathway. *Curr Biol* 18, no. 22: 1802-8.

Machacek M, Hodgson L, Welch C, Elliott H, Pertz O, Nalbant P, Abell A, Johnson GL, Hahn KM, and Danuser G. 2009. Coordination of rho gtpase activities during cell protrusion. *Nature* 461, no. 7260: 99-103.

Machesky LM and Insall RH. 1998. Scar1 and the related wiskott-aldrich syndrome protein, wasp, regulate the actin cytoskeleton through the arp2/3 complex. *Curr Biol* 8, no. 25: 1347-56.

Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, and Kirchhausen T. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell* 10, no. 6: 839-50.

Mahajan VB, Pai KS, Lau A, and Cunningham DD. 2000. Creatine kinase, an atp-generating enzyme, is required for thrombin receptor signaling to the cytoskeleton. *Proc Natl Acad Sci U S A* 97, no. 22: 12062-7.

Mauro A. 1961. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9: 493-5.

Meffert G, Gellerich FN, Margreiter R, and Wyss M. 2005. Elevated creatine kinase activity in primary hepatocellular carcinoma. *BMC Gastroenterol* 5: 9.

Millay DP, O'Rourke JR, Sutherland LB, Bezprozvannaya S, Shelton JM, Bassel-Duby R, and Olson EN. 2013. Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* 499, no. 7458: 301-5.

Mitchell PO and Pavlath GK. 2001. A muscle precursor cell-dependent pathway contributes to muscle growth after atrophy. *Am J Physiol Cell Physiol* 281, no. 5: C1706-15.

Modregger J, DiProspero NA, Charles V, Tagle DA, and Plomann M. 2002. Pacsin 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic huntington's disease brains. *Hum Mol Genet* 11, no. 21: 2547-58.

Modregger J, Ritter B, Witter B, Paulsson M, and Plomann M. 2000. All three pacsin isoforms bind to endocytic proteins and inhibit endocytosis. *J Cell Sci* 113 Pt 24: 4511-21.

Moon SY and Zheng Y. 2003. Rho gtpase-activating proteins in cell regulation. *Trends Cell Biol* 13, no. 1: 13-22.

Morgan JE, Coulton GR, and Partridge TA. 1987. Muscle precursor cells invade and repopulate freeze-killed muscles. *J Muscle Res Cell Motil* 8, no. 5: 386-96.

Morgenstern JP and Land H. 1990. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* 18, no. 12: 3587-96.

Morley SC. 2012. The actin-bundling protein l-plastin: A critical regulator of immune cell function. *Int J Cell Biol* 2012: 935173.

Mostowy S and Cossart P. 2012. Septins: The fourth component of the cytoskeleton. *Nat Rev Mol Cell Biol* 13, no. 3: 183-94.

Mounier N and Arrigo AP. 2002. Actin cytoskeleton and small heat shock proteins: How do they interact? *Cell Stress Chaperones* 7, no. 2: 167-76.

Muir AR, Kanji AH, and Allbrook D. 1965. The structure of the satellite cells in skeletal muscle. *J Anat* 99, no. Pt 3: 435-44.

Mukai A and Hashimoto N. 2008. Localized cyclic amp-dependent protein kinase activity is required for myogenic cell fusion. *Exp Cell Res* 314, no. 2: 387-97.

Mukai A, Kurisaki T, Sato SB, Kobayashi T, Kondoh G, and Hashimoto N. 2009. Dynamic clustering and dispersion of lipid rafts contribute to fusion competence of myogenic cells. *Exp Cell Res* 315, no. 17.

Muller AJ, Baker JF, DuHadaway JB, Ge K, Farmer G, Donover PS, Meade R, Reid C, Grzanna R, Roach AH, Shah N, Soler AP, and Prendergast GC. 2003. Targeted disruption of the murine bin1/amphiphysin ii gene does not disable endocytosis but results in embryonic cardiomyopathy with aberrant myofibril formation. *Mol Cell Biol* 23, no. 12: 4295-306.

Mullins RD, Heuser JA, and Pollard TD. 1998. The interaction of arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc Natl Acad Sci U S A* 95, no. 11: 6181-6.

Mulvaney PT, Stracke ML, Nam SW, Woodhouse E, O'Keefe M, Clair T, Liotta LA, Khaddurah-Daouk R, and Schiffmann E. 1998. Cyclocreatine inhibits stimulated motility in tumor cells possessing creatine kinase. *Int J Cancer* 78, no. 1: 46-52.

Mundigl O, Ochoa GC, David C, Slepnev VI, Kabanov A, and De Camilli P. 1998. Amphiphysin i antisense oligonucleotides inhibit neurite outgrowth in cultured hippocampal neurons. *J Neurosci* 18, no. 1: 93-103.

Munn AL, Stevenson BJ, Geli MI, and Riezman H. 1995. End5, end6, and end7: Mutations that cause actin delocalization and block the internalization step of endocytosis in saccharomyces cerevisiae. *Mol Biol Cell* 6, no. 12: 1721-42.

Musa H, Meek S, Gautel M, Peddie D, Smith AJ, and Peckham M. 2006. Targeted homozygous deletion of m-band titin in cardiomyocytes prevents sarcomere formation. *J Cell Sci* 119, no. Pt 20: 4322-31.

Mylona E, Jones KA, Mills ST, and Pavlath GK. 2006. Cd44 regulates myoblast migration and differentiation. *J Cell Physiol* 209, no. 2: 314-21.

Namba Y, Ito M, Zu Y, Shigesada K, and Maruyama K. 1992. Human t cell l-plastin bundles actin filaments in a calcium-dependent manner. *J Biochem* 112, no. 4: 503-7.

Neuhaus P, Oustanina S, Loch T, Kruger M, Bober E, Dono R, Zeller R, and Braun T. 2003. Reduced mobility of fibroblast growth factor (fgf)-deficient myoblasts might contribute to dystrophic changes in the musculature of fgf2/fgf6/mdx triple-mutant mice. *Mol Cell Biol* 23, no. 17: 6037-48.

Nicot AS, Toussaint A, Tosch V, Kretz C, Wallgren-Pettersson C, Iwarsson E, Kingston H, Garnier JM, Biancalana V, Oldfors A, Mandel JL, and Laporte J. 2007. Mutations in amphiphysin 2 (bin1) disrupt interaction with dynamin 2 and cause autosomal recessive centronuclear myopathy. *Nat Genet* 39, no. 9: 1134-9.

Nishiyama T, Kii I, and Kudo A. 2004. Inactivation of rho/rock signaling is crucial for the nuclear accumulation of fkhr and myoblast fusion. *J Biol Chem* 279, no. 45: 47311-9.

Nishizawa M, Kanaya Y, and Toh EA. 1999. Mouse cyclin-dependent kinase (cdk) 5 is a functional homologue of a yeast cdk, pho85 kinase. *J Biol Chem* 274, no. 48: 33859-62.

Nowak KJ, Ravenscroft G, Jackaman C, Filipovska A, Davies SM, Lim EM, Squire SE, Potter AC, Baker E, Clement S, Sewry CA, Fabian V, Crawford K, Lessard JL, Griffiths LM, Papadimitriou JM, Shen Y, Morahan G, Bakker AJ, Davies KE, and Laing NG. 2009. Rescue of skeletal muscle alpha-actin-null mice by cardiac (fetal) alpha-actin. *J Cell Biol* 185, no. 5: 903-15.

Nowak SJ, Nahirney PC, Hadjantonakis AK, and Baylies MK. 2009. Nap1-mediated actin remodeling is essential for mammalian myoblast fusion. *J Cell Sci* 122, no. Pt 18: 3282-93.

O'Connor RS, Mills ST, Jones KA, Ho SN, and Pavlath GK. 2007. A combinatorial role for nfat5 in both myoblast migration and differentiation during skeletal muscle myogenesis. *J Cell Sci* 120, no. Pt 1: 149-59.

O'Connor RS, Steeds CM, Wiseman RW, and Pavlath GK. 2008. Phosphocreatine as an energy source for actin cytoskeletal rearrangements during myoblast fusion. *J Physiol* 586, no. Pt 12: 2841-53.

Ocalan M, Goodman SL, Kuhl U, Hauschka SD, and von der Mark K. 1988. Laminin alters cell shape and stimulates motility and proliferation of murine skeletal myoblasts. *Dev Biol* 125, no. 1: 158-67.

Odemis V, Boosmann K, Dieterlen MT, and Engele J. 2007. The chemokine sdf1 controls multiple steps of myogenesis through atypical pkczeta. *J Cell Sci* 120, no. Pt 22: 4050-9.

Ohtake Y, Tojo H, and Seiki M. 2006. Multifunctional roles of mt1-mmp in myofiber formation and morphostatic maintenance of skeletal muscle. *J Cell Sci* 119, no. Pt 18: 3822-32.

Okazaki K and Holtzer H. 1965. An analysis of myogenesis in vitro using fluorescein-labeled antimyosin. *J Histochem Cytochem* 13, no. 8: 726-39.

Olguin HC, Santander C, and Brandan E. 2003. Inhibition of myoblast migration via decorin expression is critical for normal skeletal muscle differentiation. *Dev Biol* 259, no. 2: 209-24.

Ontell M. 1986. Morphological aspects of muscle fiber regeneration. *Fed Proc* 45: 1461-5.

Ontell M and Feng KC. 1981. The three-dimensional cytoarchitecture and pattern of motor innervation of branched striated myotubes. *Anat Rec* 200, no. 1: 11-31.

Ontell M, Hughes D, and Bourke D. 1982. Secondary myogenesis of normal muscle produces abnormal myotubes. *Anat Rec* 204, no. 3: 199-207.

Orimo S, Hiyamuta E, Arahata K, and Sugita H. 1991. Analysis of inflammatory cells and complement c3 in bupivacaine-induced myonecrosis. *Muscle Nerve* 14, no. 6: 515-20.

Owen DJ, Wigge P, Vallis Y, Moore JD, Evans PR, and McMahon HT. 1998. Crystal structure of the amphiphysin-2 sh3 domain and its role in the prevention of dynamin ring formation. *EMBO J* 17, no. 18: 5273-85.

Padrick SB, Cheng HC, Ismail AM, Panchal SC, Doolittle LK, Kim S, Skehan BM, Umetani J, Brautigam CA, Leong JM, and Rosen MK. 2008. Hierarchical regulation of wasp/wave proteins. *Mol Cell* 32, no. 3: 426-38.

Pajcini KV, Pomerantz JH, Alkan O, Doyonnas R, and Blau HM. 2008. Myoblasts and macrophages share molecular components that contribute to cell-cell fusion. *J Cell Biol* 180, no. 5: 1005-19.

Papadimitriou JM, Robertson TA, Mitchell CA, and Grounds MD. 1990. The process of new plasmalemma formation in focally injured skeletal muscle fibers. *J Struct Biol* 103, no. 2: 124-34.

Park IH and Chen J. 2005. Mammalian target of rapamycin (mtor) signaling is required for a late-stage fusion process during skeletal myotube maturation. *J Biol Chem* 280, no. 36: 32009-17.

Paschou P, Feng Y, Pakstis AJ, Speed WC, DeMille MM, Kidd JR, Jaghori B, Kurlan R, Pauls DL, Sandor P, Barr CL, and Kidd KK. 2004. Indications of linkage and association of gilles de la tourette syndrome in two independent family samples: 17q25 is a putative susceptibility region. *Am J Hum Genet* 75, no. 4: 545-60.

Paterson JM, Ydenberg CA, and Rose MD. 2008. Dynamic localization of yeast fus2p to an expanding ring at the cell fusion junction during mating. *J Cell Biol* 181, no. 4: 697-709.

Pavlath GK. 2010a. A new function for odorant receptors: Mor23 is necessary for normal tissue repair in skeletal muscle. *Cell Adh Migr* 4, no. 4: 502-6.

Pavlath GK. 2010b. Spatial and functional restriction of regulatory molecules during mammalian myoblast fusion. *Exp Cell Res* 316, no. 18: 3067-72.

Peckham M. 2008. Engineering a multi-nucleated myotube, the role of the actin cytoskeleton. *J Microsc* 231, no. 3: 486-93.

Pellegrin S and Mellor H. 2005. The rho family gtpase rif induces filopodia through mdia2. *Curr Biol* 15, no. 2: 129-33.

Pellegrin S and Mellor H. 2007. Actin stress fibers. *J Cell Sci* 120: 3491-99.

Perrin BJ and Ervasti JM. 2010. The actin gene family: Function follows isoform. *Cytoskeleton (Hoboken)* 67, no. 10: 630-4.

Perrin BJ, Strandjord DM, Narayanan P, Henderson DM, Johnson KR, and Ervasti JM. 2013. Beta-actin and fascin-2 cooperate to maintain stereocilia length. *J Neurosci* 33, no. 19: 8114-21.

Peter BJ, Kent HM, Mills IG, Vallis Y, Butler PJ, Evans PR, and McMahon HT. 2004. Bar domains as sensors of membrane curvature: The amphiphysin bar structure. *Science* 303, no. 5657: 495-9.

Phelan JN and Gonyea WJ. 1997. Effect of radiation on satellite cell activity and protein expression in overloaded mammalian skeletal muscle. *Anat Rec* 247, no. 2: 179-88.

Phillips GD, Hoffman JR, and Knighton DR. 1990. Migration of myogenic cells in the rat extensor digitorum longus muscle studied with a split autograft model. *Cell Tissue Res* 262, no. 1: 81-8.

Phillips GD, Lu DY, Mitashov VI, and Carlson BM. 1987. Survival of myogenic cells in freely grafted rat rectus femoris and extensor digitorum longus muscles. *Am J Anat* 180, no. 4: 365-72.

Pichot CS, Arvanitis C, Hartig SM, Jensen SA, Bechill J, Marzouk S, Yu J, Frost JA, and Corey SJ. 2010. Cdc42-interacting protein 4 promotes breast cancer cell invasion

and formation of invadopodia through activation of n-wasp. *Cancer Res* 70, no. 21: 8347-56.

Pietropaolo M, Castano L, Babu S, Buelow R, Kuo YL, Martin S, Martin A, Powers AC, Prochazka M, Naggert J, and et al. 1993. Islet cell autoantigen 69 kd (ica69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. *J Clin Invest* 92, no. 1: 359-71.

Pirozzi F, Di Raimo FR, Zanni G, Bertini E, Billuart P, Tartaglione T, Tabolacci E, Brancaccio A, Neri G, and Chiurazzi P. 2011. Insertion of 16 amino acids in the bar domain of the oligophrenin 1 protein causes mental retardation and cerebellar hypoplasia in an italian family. *Hum Mutat* 32, no. 11: E2294-307.

Pisconti A, Brunelli S, Di Padova M, De Palma C, Deponti D, Baesso S, Sartorelli V, Cossu G, and Clementi E. 2006. Follistatin induction by nitric oxide through cyclic gmp: A tightly regulated signaling pathway that controls myoblast fusion. *J Cell Biol* 172, no. 2: 233-44.

Posey AD, Jr., Pytel P, Gardikiotes K, Demonbreun AR, Rainey M, George M, Band H, and McNally EM. 2011. Endocytic recycling proteins ehd1 and ehd2 interact with fer-1-like-5 (fer1l5) and mediate myoblast fusion. *J Biol Chem* 286, no. 9: 7379-88.

Powell JA. 1973. Development of normal and genetically dystrophic mouse muscle in tissue culture. I. Prefusion and fusion activities of muscle cells: Phase contrast and time lapse study. *Exp Cell Res* 80, no. 2: 251-64.

Powers SK and Howley ET. 1997. *Exercise physiology: Theory and application to fitness and performance*: Brown and Benchmark.

Prehoda KE, Scott JA, Mullins RD, and Lim WA. 2000. Integration of multiple signals through cooperative regulation of the n-wasp-arp2/3 complex. *Science* 290, no. 5492: 801-6.

Prendergast GC, Muller AJ, Ramalingam A, and Chang MY. 2009. Bar the door: Cancer suppression by amphiphysin-like genes. *Biochim Biophys Acta* 1795, no. 1: 25-36.

Przybylski RJ and Blumberg JM. 1966. Ultrastructural aspects of myogenesis in the chick. *Lab Invest* 15, no. 5: 836-63.

Quach NL, Biressi S, Reichardt LF, Keller C, and Rando TA. 2009. Focal adhesion kinase signaling regulates the expression of caveolin 3 and beta1 integrin, genes essential for normal myoblast fusion. *Mol Biol Cell* 20, no. 14: 3422-35.

Qualmann B, Koch D, and Kessels MM. 2011. Let's go bananas: Revisiting the endocytic bar code. *EMBO J* 30, no. 17: 3501-15.

Quinones GA, Jin J, and Oro AE. 2010. I-bar protein antagonism of endocytosis mediates directional sensing during guided cell migration. *J Cell Biol* 189, no. 2: 353-67.

Quinones GA and Oro AE. 2010. Bar domain competition during directional cellular migration. *Cell Cycle* 9, no. 13: 2522-8.

Rabouille C and Jokitalo E. 2003. Golgi apparatus partitioning during cell division. *Mol Membr Biol* 20, no. 2: 117-27.

Ramalingam A, Duhadaway JB, Sutanto-Ward E, Wang Y, Dinchuk J, Huang M, Donover PS, Boulden J, McNally LM, Soler AP, Muller AJ, Duncan MK, and Prendergast GC. 2008. Bin3 deletion causes cataracts and increased susceptibility to lymphoma during aging. *Cancer Res* 68, no. 6: 1683-90.

Ramjaun AR and McPherson PS. 1998. Multiple amphiphysin ii splice variants display differential clathrin binding: Identification of two distinct clathrin-binding sites. *J Neurochem* 70, no. 6: 2369-76.

Ramjaun AR, Philie J, de Heuvel E, and McPherson PS. 1999. The n terminus of amphiphysin ii mediates dimerization and plasma membrane targeting. *J Biol Chem* 274, no. 28: 19785-91.

Rappolee DA and Werb Z. 1992. Macrophage-derived growth factors. *Curr Top Microbiol Immunol* 181: 87-140.

Rash JE and Fambrough D. 1973. Ultrastructural and electrophysiological correlates of cell coupling and cytoplasmic fusion during myogenesis in vitro. *Dev Biol* 30, no. 1: 166-86.

Razzaq A, Robinson IM, McMahon HT, Skepper JN, Su Y, Zelhof AC, Jackson AP, Gay NJ, and O'Kane CJ. 2001. Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in drosophila. *Genes Dev* 15, no. 22: 2967-79.

Relaix F and Zammit PS. 2012. Satellite cells are essential for skeletal muscle regeneration: The cell on the edge returns centre stage. *Development* 139, no. 16: 2845-56.

Ren G, Vajjhala P, Lee JS, Winsor B, and Munn AL. 2006. The bar domain proteins: Molding membranes in fission, fusion, and phagy. *Microbiol Mol Biol Rev* 70, no. 1: 37-120.

Renema WK, Kan HE, Wieringa B, and Heerschap A. 2007. In vivo magnetic resonance spectroscopy of transgenic mouse models with altered high-energy phosphoryl transfer metabolism. *NMR Biomed* 20, no. 4: 448-67.

Richard JP, Leikina E, Langen R, Henne WM, Popova M, Balla T, McMahon HT, Kozlov MM, and Chernomordik L. 2011. Intracellular curvature-generating proteins in cell-cell fusion. *Biochem J* 440, no. 2: 185-93.

Ridley AJ. 2011. Life at the leading edge. *Cell* 145: 1012-22.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, and Horwitz AR. 2003. Cell migration: Integrating signals from front to back. *Science* 302, no. 5651: 1704-9.

Robertson TA, Grounds MD, Mitchell CA, and Papadimitriou JM. 1990. Fusion between myogenic cells in vivo: An ultrastructural study in regenerating murine skeletal muscle. *J Struct Biol* 105, no. 1-3: 170-82.

Robertson TA, Maley MA, Grounds MD, and Papadimitriou JM. 1993a. The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp Cell Res* 207, no. 2: 321-31.

Robertson TA, Papadimitriou JM, and Grounds MD. 1993b. Fusion of myogenic cells to the newly sealed region of damaged myofibres in skeletal muscle regeneration. *Neuropathol Appl Neurobiol* 19, no. 4: 350-8.

Rohatgi R. 1999. The interaction between n-wasp and the arp2/3 complex links cdc42-dependent signals to actin assembly. *Cell* 97: 221-31.

Rosen GD, Sanes JR, LaChance R, Cunningham JM, Roman J, and Dean DC. 1992. Roles for the integrin vla-4 and its counter receptor vcam-1 in myogenesis. *Cell* 69, no. 7: 1107-19.

Rosenblatt JD and Parry DJ. 1992. Gamma irradiation prevents compensatory hypertrophy of overloaded mouse extensor digitorum longus muscle. *J Appl Physiol* 73, no. 6: 2538-43.

Rossi MJ, Clark MA, and Steiner SM. 1989. Possible role of prostaglandins in the regulation of mouse myoblasts. *J Cell Physiol* 141, no. 1: 142-7.

Rossman KL, Der CJ, and Sondek J. 2005. Gef means go: Turning on rho gtpases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 6, no. 2: 167-80.

Routhier EL, Burn TC, Abbaszade I, Summers M, Albright CF, and Prendergast GC. 2001. Human bin3 complements the f-actin localization defects caused by loss of hob3p, the fission yeast homolog of rvs161p. *J Biol Chem* 276, no. 24: 21670-7.

Routhier EL, Donover PS, and Prendergast GC. 2003. Hob1+, the fission yeast homolog of bin1, is dispensable for endocytosis or actin organization, but required for the response to starvation or genotoxic stress. *Oncogene* 22, no. 5: 637-48.

Rybakova IN, Patel JR, Davies KE, Yurchenco PD, and Ervasti JM. 2002. Utrophin binds laterally along actin filaments and can couple costameric actin with sarcolemma when overexpressed in dystrophin-deficient muscle. *Mol Biol Cell* 13, no. 5: 1512-21.

Saarikangas J, Zhao H, Pykalainen A, Laurinmaki P, Mattila PK, Kinnunen PK, Butcher SJ, and Lappalainen P. 2009. Molecular mechanisms of membrane deformation by i-bar domain proteins. *Curr Biol* 19, no. 2: 95-107.

Sabharanjak S, Sharma P, Parton RG, and Mayor S. 2002. Gpi-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* 2, no. 4: 411-23.

Saclier M, Yacoub-Youssef H, Mackey AL, Arnold L, Ardjoune H, Magnan M, Sailhan F, Chelly J, Pavlath GK, Mounier R, Kjaer M, and Chazaud B. 2012. Differentially activated macrophages orchestrate myogenic precursor cell fate during human skeletal muscle regeneration. *Stem Cells*.

Sakamuro D, Elliott KJ, Wechsler-Reya R, and Prendergast GC. 1996. Bin1 is a novel myc-interacting protein with features of a tumour suppressor. *Nat Genet* 14, no. 1: 69-77.

Sakamuro D and Prendergast GC. 1999. New myc-interacting proteins: A second myc network emerges. *Oncogene* 18, no. 19: 2942-54.

Salazar MA, Kwiatkowski AV, Pellegrini L, Cestra G, Butler MH, Rossman KL, Serna DM, Sondek J, Gertler FB, and De Camilli P. 2003. Tuba, a novel protein containing bin/amphiphysin/rvs and dbl homology domains, links dynamin to regulation of the actin cytoskeleton. *J Biol Chem* 278, no. 49: 49031-43.

Sánchez-Barrena MJ, Vallis Y, Clatworthy MR, Doherty GJ, Veprintsev DB, Evans PR, and McMahon HT. 2012. Bin2 is a membrane sculpting n-bar protein that influences leucocyte podosomes, motility and phagocytosis. *PLoS One* 7, no. 12.

Schafer BW and Perriard JC. 1988. Intracellular targeting of isoproteins in muscle cytoarchitecture. *J Cell Biol* 106: 1161-70.

Schmalbruch H. 1976. The morphology of regeneration of skeletal muscles in the rat. *Tissue Cell* 8, no. 4: 673-92.

Schmalbruch H. 1984. Regenerated muscle fibers in duchenne muscular dystrophy: A serial section study. *Neurology* 34, no. 1: 60-5.

Schmalbruch H and Lewis DM. 2000. Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve* 23, no. 4: 617-26.

Schollmeyer JE. 1986. Role of ca2+ and ca2+-activated protease in myoblast fusion. *Exp Cell Res* 162, no. 2: 411-22.

Schultz E, Albright DJ, Jaryszak DL, and David TL. 1988. Survival of satellite cells in whole muscle transplants. *Anat Rec* 222, no. 1: 12-7.

Schultz E, Jaryszak DL, and Valliere CR. 1985. Response of satellite cells to focal skeletal muscle injury. *Muscle Nerve* 8, no. 3: 217-22.

Schultz E and McCormick KM. 1994. Skeletal muscle satellite cells. *Rev Physiol Biochem Pharmacol* 123: 213-57.

Schwander M, Leu M, Stumm M, Dorchies OM, Ruegg UT, Schittny J, and Muller U. 2003. Beta1 integrins regulate myoblast fusion and sarcomere assembly. *Dev Cell* 4, no. 5: 673-85.

Schwartz MS, M. S, and Swash M. 1976. Longitudinal fibre splitting in neurogenic muscular disorders: Its relation to the pathogenesis of "myopathic" change. *Brain* 99: 617-36.

Seale P and Rudnicki MA. 2000. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol* 218, no. 2: 115-24.

Shafey D, Cote PD, and Kothary R. 2005. Hypomorphic smn knockdown c2c12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Exp Cell Res* 311, no. 1: 49-61.

Shainberg A, Yagil G, and Yaffe D. 1969. Control of myogenesis in vitro by ca 2 + concentration in nutritional medium. *Exp Cell Res* 58, no. 1: 163-7.

Shimada A, Niwa H, Tsujita K, Suetsugu S, Nitta K, Hanawa-Suetsugu K, Akasaka R, Nishino Y, Toyama M, Chen L, Liu ZJ, Wang BC, Yamamoto M, Terada T, Miyazawa A, Tanaka A, Sugano S, Shirouzu M, Nagayama K, Takenawa T, and Yokoyama S. 2007. Curved efc/f-bar-domain dimers are joined end to end into a filament for membrane invagination in endocytosis. *Cell* 129, no. 4: 761-72.

Shin JB, Streijger F, Beynon A, Peters T, Gadzala L, McMillen D, Bystrom C, Van der Zee CE, Wallimann T, and Gillespie PG. 2007. Hair bundles are specialized for atp delivery via creatine kinase. *Neuron* 53, no. 3: 371-86.

Shin OH and Exton JH. 2001. Differential binding of arfaptin 2/por1 to adp-ribosylation factors and rac1. *Biochem Biophys Res Commun* 285, no. 5: 1267-73.

Shuster CB and Herman IM. 1995. Indirect association of ezrin with f-actin: Isoform specificity and calcium sensitivity. *J Cell Biol* 128, no. 5: 837-48.

Shuster CB, Lin AY, Nayak R, and Herman IM. 1996. Beta cap73: A novel beta actin-specific binding protein. *Cell Motil Cytoskeleton* 35, no. 3: 175-87.

Simionescu A and Pavlath GK. 2011. Molecular mechanisms of myoblast fusion across species. *Adv Exp Med Biol* 713: 113-35.

Simionescu-Bankston A, Leoni G, Wang Y, Pham PP, Ramalingam A, Duhadaway JB, Faundez V, Nusrat A, Prendergast GC, and Pavlath GK. 2013. The n-bar domain

protein, bin3, regulates rac1- and cdc42-dependent processes in myogenesis. *Dev Biol* 382: 160-71.

Simpson F, Hussain NK, Qualmann B, Kelly RB, Kay BK, McPherson PS, and Schmid SL. 1999. Sh3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat Cell Biol* 1, no. 2: 119-24.

Sivadon P, Bauer F, Aigle M, and Crouzet M. 1995. Actin cytoskeleton and budding pattern are altered in the yeast rvs161 mutant: The rvs161 protein shares common domains with the brain protein amphiphysin. *Mol Gen Genet* 246, no. 4: 485-95.

Slepnev VI, Ochoa GC, Butler MH, and De Camilli P. 2000. Tandem arrangement of the clathrin and ap-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J Biol Chem* 275, no. 23: 17583-9.

Slepnev VI, Ochoa GC, Butler MH, Grabs D, and De Camilli P. 1998. Role of phosphorylation in regulation of the assembly of endocytic coat complexes. *Science* 281, no. 5378: 821-4.

Small JV and Celis JE. 1978. Filament arrangements in negatively stained cultured cells: The organization of actin. *Cytobiologie* 16, no. 2: 308-25.

Small JV, Stradal T, Vignal E, and Rottner K. 2002. The lamellipodium: Where motility begins. *Trends Cell Biol* 12, no. 3: 112-20.

Sohn RL, Huang P, Kawahara G, Mitchell M, Guyon J, Kalluri R, Kunkel LM, and Gussoni E. 2009. A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. *Proc Natl Acad Sci U S A* 106, no. 23: 9274-9.

Sonnet C, Lafuste P, Arnold L, Brigitte M, Poron F, Authier FJ, Chretien F, Gherardi RK, and Chazaud B. 2006. Human macrophages rescue myoblasts and myotubes from apoptosis through a set of adhesion molecular systems. *J Cell Sci* 119, no. Pt 12: 2497-507.

Sotiropoulos A, Ohanna M, Kedzia C, Menon RK, Kopchick JJ, Kelly PA, and Pende M. 2006. Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor 1 up-regulation. *Proc Natl Acad Sci U S A* 103, no. 19: 7315-20.

Sparks AB, Hoffman NG, McConnell SJ, Fowlkes DM, and Kay BK. 1996. Cloning of ligand targets: Systematic isolation of sh3 domain-containing proteins. *Nat Biotechnol* 14, no. 6: 741-4.

Spector I, Shochet NR, Kashman Y, and Groweiss A. 1983. Latrunculins: Novel marine toxins that disrupt microfilament organization in cultured cells. *Science* 219, no. 4584: 493-5.

Spitzenberger F, Pietropaolo S, Verkade P, Habermann B, Lacas-Gervais S, Mziaut H, Pietropaolo M, and Solimena M. 2003. Islet cell autoantigen of 69 kda is an arfaptin-related protein associated with the golgi complex of insulinoma ins-1 cells. *J Biol Chem* 278, no. 28: 26166-73.

Squillace RM, Chenault DM, and Wang EH. 2002. Inhibition of muscle differentiation by the novel muscleblind-related protein chcr. *Dev Biol* 250, no. 1: 218-30.

Stadler B, Blattler TM, and Franco-Obregon A. 2010. Time-lapse imaging of in vitro myogenesis using atomic force microscopy. *J Microsc* 237, no. 1: 63-9.

Stolz M and Wallimann T. 1998. Myofibrillar interaction of cytosolic creatine kinase (ck) isoenzymes: Allocation of n-terminal binding epitope in mm-ck and bb-ck. *J Cell Sci* 111 (Pt 9): 1207-16.

Strating JR and Martens GJ. 2009. The p24 family and selective transport processes at the er-golgi interface. *Bio Cell* 101, no. 9: 495-509.

Straube A and Merdes A. 2007. Eb3 regulates microtubule dynamics at the cell cortex and is required for myoblast elongation and fusion. *Curr Biol* 17, no. 15: 1318-25.

Suetsugu S. 2010. The proposed functions of membrane curvatures mediated by the bar domain superfamily proteins. *J Biochem* 148, no. 1: 1-12.

Suetsugu S and Gautreau A. 2012. Synergistic bar-npf interactions in actin-driven membrane remodeling. *Trends Cell Biol* 22, no. 3: 141-50.

Suetsugu S, Toyooka K, and Senju Y. 2010. Subcellular membrane curvature mediated by the bar domain superfamily proteins. *Semin Cell Dev Biol* 21, no. 4: 340-9.

Sun Y, Ge Y, Drnevich J, Zhao Y, Band M, and Chen J. 2010. Mammalian target of rapamycin regulates mirna-1 and follistatin in skeletal myogenesis. *J Cell Biol* 189, no. 7: 1157-69.

Suraneni P, Rubinstein B, Unruh JR, Durnin M, Hanein D, and Li R. 2012. The arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *J Cell Biol* 197, no. 2: 239-51.

Suzuki J, Yamazaki Y, Li G, Kaziro Y, and Koide H. 2000. Involvement of ras and ral in chemotactic migration of skeletal myoblasts. *Mol Cell Biol* 20, no. 13: 4658-65.

Suzuki M, Angata K, Nakayama J, and Fukuda M. 2003. Polysialic acid and mucin type o-glycans on the neural cell adhesion molecule differentially regulate myoblast fusion. *J Biol Chem* 278, no. 49: 49459-68.

Svitkina TM and Borisy GG. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol* 145, no. 5: 1009-26.

Svitkina TM, Bulanova EA, Chaga OY, Vignjevic DM, Kojima S, Vasiliev JM, and Borisy GG. 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *J Cell Biol* 160, no. 3: 409-21.

Swailes NT, Colegrave M, Knight PJ, and Peckham M. 2006. Non-muscle myosins 2a and 2b drive changes in cell morphology that occur as myoblasts align and fuse. *J Cell Sci* 119, no. Pt 17: 3561-70.

Swailes NT, Knight PJ, and Peckham M. 2004. Actin filament organization in aligned prefusion myoblasts. *J Anat* 205, no. 5: 381-91.

Tachibana I and Hemler ME. 1999. Role of transmembrane 4 superfamily (tm4sf) proteins cd9 and cd81 in muscle cell fusion and myotube maintenance. *J Cell Biol* 146, no. 4: 893-904.

Tachikawa M, Fukaya M, Terasaki T, Ohtsuki S, and Watanabe M. 2004. Distinct cellular expressions of creatine synthetic enzyme gamt and creatine kinases uck-mi and ck-b suggest a novel neuron-glial relationship for brain energy homeostasis. *Eur J Neurosci* 20, no. 1: 144-60.

Takenawa T and Suetsugu S. 2007. The wasp-wave protein network: Connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol* 8, no. 1: 37-48.

Tamaki T and Akatsuka A. 1994. Appearance of complex branched fibers following repetitive muscle trauma in normal rat skeletal muscle. *Anat Rec* 240, no. 2: 217-24.

Tamaki T, Akatsuka A, Tokunaga M, Uchiyama S, and Shiraishi T. 1996. Characteristics of compensatory hypertrophied muscle in the rat: I. Electron microscopic and immunohistochemical studies. *Anat Rec* 246, no. 3: 325-34.

Tamaki T, Akatsuka A, Uchiyama S, Uchiyama Y, and Shiraishi T. 1997. Appearance of complex branched muscle fibers is associated with a shift to slow muscle characteristics. *Acta Anat (Basel)* 159, no. 2-3: 108-13.

Tamaki T, Sekine T, Akatsuka A, Uchiyama S, and Nakano S. 1992. Detection of neuromuscular junctions on isolated branched muscle fibers: Application of nitric acid fiber digestion method for scanning electron microscopy. *J Electron Microsc (Tokyo)* 41, no. 2: 76-81.

Tamaki T, Sekine T, Akatsuka A, Uchiyama S, and Nakano S. 1993. Three-dimensional cytoarchitecture of complex branched fibers in soleus muscle from mdx mutant mice. *Anat Rec* 237, no. 3: 338-44.

Tarricone C, Xiao B, Justin N, Walker PA, Rittinger K, Gamblin SJ, and Smerdon SJ. 2001. The structural basis of arfaptin-mediated cross-talk between rac and arf signalling pathways. *Nature* 411, no. 6834: 215-9.

Temm-Grove CJ, Wert D, Thompson VF, Allen RE, and Goll DE. 1999. Microinjection of calpastatin inhibits fusion in myoblasts. *Exp Cell Res* 247, no. 1: 293-303.

Tentler D, Gustavsson P, Leisti J, Schueler M, Chelly J, Timonen E, Anneren G, Willard HF, and Dahl N. 1999. Deletion including the oligophrenin-1 gene associated

with enlarged cerebral ventricles, cerebellar hypoplasia, seizures and ataxia. *Eur J Hum Genet* 7, no. 5: 541-8.

Teplova M and Patel DJ. 2008. Structural insights into rna recognition by the alternative-splicing regulator muscleblind-like mbnl1. *Nat Struct Mol Biol* 15, no. 12: 1343-51.

Tian L, Huang K, DuHadaway JB, Prendergast GC, and Stambolian D. 2010. Genomic profiling of mirnas in two human lens cell lines. *Curr Eye Res* 35, no. 9: 812-8.

Tidball JG. 1995. Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc* 27, no. 7: 1022-32.

Tidball JG and Villalta SA. 2010. Regulatory interactions between muscle and the immune system during muscle regeneration. *Am J Physiol Regul Integr Comp Physiol* 298, no. 5: 1173-87.

Tombes RM, Farr A, and Shapiro BM. 1988. Sea urchin sperm creatine kinase: The flagellar isozyme is a microtubule-associated protein. *Exp Cell Res* 178, no. 2: 307-17.

Tombes RM and Shapiro BM. 1985. Metabolite channeling: A phosphorylcreatine shuttle to mediate high energy phosphate transport between sperm mitochondrion and tail. *Cell* 41, no. 1: 325-34.

Torrente Y, El Fahime E, Caron NJ, Del Bo R, Belicchi M, Pisati F, Tremblay JP, and Bresolin N. 2003. Tumor necrosis factor-alpha (tnf-alpha) stimulates chemotactic response in mouse myogenic cells. *Cell Transplant* 12, no. 1: 91-100.

Tsuboi S, Takada H, Hara T, Mochizuki N, Funyu T, Saitoh H, Terayama Y, Yamaya K, Ohyama C, Nonoyama S, and Ochs HD. 2009. Fbp17 mediates a common

molecular step in the formation of podosomes and phagocytic cups in macrophages. *J Biol Chem* 284, no. 13: 8548-56.

Tsutsui K, Maeda Y, Tsutsui K, Seki S, and Tokunaga A. 1997. Cdna cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. *Biochem Biophys Res Commun* 236, no. 1: 178-83.

van den Eijnde SM, van den Hoff MJ, Reutelingsperger CP, van Heerde WL, Henfling ME, Vermeij-Keers C, Schutte B, Borgers M, and Ramaekers FC. 2001. Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation. *J Cell Sci* 114, no. Pt 20: 3631-42.

Vandekerckhove J, Bugaisky G, and Buckingham M. 1986. Simultaneous expression of skeletal muscle and heart actin proteins in various striated muscle tissues and cells. A quantitative determination of the two actin isoforms. *J Biol Chem* 261, no. 4: 1838-43.

Vasyutina E, Martarelli B, Brakebusch C, Wende H, and Birchmeier C. 2009. The small g-proteins rac1 and cdc42 are essential for myoblast fusion in the mouse. *Proc Natl Acad Sci U S A* 106, no. 22: 8935-40.

Wakelam MJ. 1985. The fusion of myoblasts. *Biochem J* 228, no. 1: 1-12.

Wallimann T and Hemmer W. 1994. Creatine kinase in non-muscle tissues and cells. *Mol Cell Biochem* 133-134: 193-220.

Wanderling S, Simen BB, Ostrovsky O, Ahmed NT, Vogen SM, Gidalevitz T, and Argon Y. 2007. Grp94 is essential for mesoderm induction and muscle development because it regulates insulin-like growth factor secretion. *Mol Biol Cell* 18, no. 10: 3764-75.

Wang J, Sanger JM, and Sanger JW. 2005. Differential effects of latrunculin-a on myofibrils in cultures of skeletal muscle cells: Insights into mechanisms of myofibrillogenesis. *Cell Motil Cytoskeleton* 62, no. 1: 35-47.

Watt DJ, Karasinski J, Moss J, and England MA. 1994. Migration of muscle cells. *Nature* 368, no. 6470: 406-7.

Watt DJ, Morgan JE, Clifford MA, and Partridge TA. 1987. The movement of muscle precursor cells between adjacent regenerating muscles in the mouse. *Anat Embryol (Berl)* 175, no. 4: 527-36.

Wechsler DS and Dang CV. 1992. Opposite orientations of DNA bending by c-myc and max. *Proc Natl Acad Sci U S A* 89, no. 16: 7635-9.

Wechsler-Reya RJ, Elliott KJ, and Prendergast GC. 1998. A role for the putative tumor suppressor bin1 in muscle cell differentiation. *Mol Cell Biol* 18, no. 1: 566-75.

Weijer CJ. 2009. Collective cell migration in development. *J Cell Sci* 122, no. Pt 18: 3215-23.

Weirich CS, Erzberger JP, and Barral Y. 2008. The septin family of gtpases: Architecture and dynamics. *Nat Rev Mol Cell Biol* 9, no. 6: 478-89.

Wigge P, Kohler K, Vallis Y, Doyle CA, Owen D, Hunt SP, and McMahon HT. 1997. Amphiphysin heterodimers: Potential role in clathrin-mediated endocytosis. *Mol Biol Cell* 8, no. 10: 2003-15.

Wigge P and McMahon HT. 1998. The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci* 21, no. 8: 339-44.

Wigmore PM and Evans DJ. 2002. Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. *Int Rev Cytol* 216: 175-232.

Wise CA, Gillum JD, Seidman CE, Lindor NM, Veile R, Bashiardes S, and Lovett M. 2002. Mutations in cd2bp1 disrupt binding to ptp pest and are responsible for papa syndrome, an autoinflammatory disorder. *Hum Mol Genet* 11, no. 8: 961-9.

Witke W. 2004. The role of profilin complexes in cell motility and other cellular processes. *Trends Cell Biol* 14, no. 8: 461-9.

Wockel L, Ketelsen UP, Stotter M, Laule S, Meyermann R, and Bornemann A. 1998. Abundant minute myotubes in a patient who later developed centronuclear myopathy. *Acta Neuropathol* 95, no. 5: 547-51.

Wyss M and Kaddurah-Daouk R. 2000. Creatine and creatinine metabolism. *Physiol Rev* 80, no. 3: 1107-213.

Yaffe D and Saxel O. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270, no. 5639: 725-7.

Yagami-Hiromasa T, Sato T, Kurisaki T, Kamijo K, Nabeshima Y, and Fujisawa-Sehara A. 1995. A metalloprotease-disintegrin participating in myoblast fusion. *Nature* 377, no. 6550: 652-6.

Yamada H, Ohashi E, Abe T, Kusumi N, Li SA, Yoshida Y, Watanabe M, Tomizawa K, Kashiwakura Y, Kumon H, Matsui H, and Takei K. 2007. Amphiphysin 1 is important for actin polymerization during phagocytosis. *Mol Biol Cell* 18, no. 11: 4669-80.

Yao CC, Ziober BL, Sutherland AE, Mendrick DL, and Kramer RH. 1996. Laminins promote the locomotion of skeletal myoblasts via the alpha 7 integrin receptor. *J Cell Sci* 109 ( Pt 13): 3139-50.

Yao X, Cheng L, and Forte JG. 1996. Biochemical characterization of ezrin-actin interaction. *J Biol Chem* 271, no. 12: 7224-9.

Yonemura S, Nagafuchi A, Sato N, and Tsukita S. 1993. Concentration of an integral membrane protein, cd43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. *J Cell Biol* 120, no. 2: 437-49.

Yoo J, Jeong MJ, Kwon BM, Hur MW, Park YM, and Han MY. 2002. Activation of dynamin i gene expression by sp1 and sp3 is required for neuronal differentiation of n1e-115 cells. *J Biol Chem* 277, no. 14: 11904-9.

Yoon S, Molloy MJ, Wu MP, Cowan DB, and Gussoni E. 2007. C6orf32 is upregulated during muscle cell differentiation and induces the formation of cellular filopodia. *Dev Biol* 301, no. 1: 70-81.

Youn JY, Friesen H, Kishimoto T, Henne WM, Kurat CF, Ye W, Ceccarelli DF, Sicheri F, Kohlwein SD, McMahon HT, and Andrews BJ. 2010. Dissecting bar domain function in the yeast amphiphysins rvs161 and rvs167 during endocytosis. *Mol Biol Cell* 21, no. 17: 3054-69.

Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, Kreutz R, Wang Y, Maleeff B, Parsons AA, and Ohlstein EH. 2000. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J Biol Chem* 275, no. 48: 37895-901.

Zalin RJ. 1977. Prostaglandins and myoblast fusion. *Dev Biol* 59, no. 2: 241-8.

Zelhof AC, Bao H, Hardy RW, Razzaq A, Zhang B, and Doe CQ. 2001. Drosophila amphiphysin is implicated in protein localization and membrane morphogenesis but not in synaptic vesicle endocytosis. *Development* 128, no. 24: 5005-15.

Zeschnigk M, Kozian D, Kuch C, Schmoll M, and Starzinski-Powitz A. 1995. Involvement of m-cadherin in terminal differentiation of skeletal muscle cells. *J Cell Sci* 108 ( Pt 9): 2973-81.

Zhang J, Wang Y, Zhou Y, Cao Z, Huang P, and Lu B. 2005. Yeast two-hybrid screens imply that ggnbp1, ggnbp2 and oaz3 are potential interaction partners of testicular germ cell-specific protein ggn1. *FEBS Lett* 579, no. 2: 559-66.

Zhang M and McLennan IS. 1995. During secondary myotube formation, primary myotubes preferentially absorb new nuclei at their ends. *Dev Dyn* 204, no. 2: 168-77.

Zhang Y, Li H, Wang X, Gao X, and Liu X. 2009. Regulation of t cell development and activation by creatine kinase b. *PLoS One* 4, no. 4: e5000.

Zhao TJ, Yan YB, Liu Y, and Zhou HM. 2007. The generation of the oxidized form of creatine kinase is a negative regulation on muscle creatine kinase. *J Biol Chem* 282: 12022–29.