THE ROLES OF FORKHEAD TRANSCRIPTION FACTORS IN STEM CELLS AND MYOGENESIS

APPROVED BY SUPERVISORY COMMITTEE

_____

Joseph A. Garcia MD, PhD – Mentor

_____

Jane E. Johnson PhD – Committee Chair

_____

Eric N. Olson PhD – Committee Member

_____

Robert E. Hammer PhD – Committee Member

Dedication

Dedicated to my mother, for all of her guidance and love.

THE ROLES OF FORKHEAD TRANSCRIPTION FACTORS IN STEM CELLS AND MYOGENESIS

by

MATTHEW SCOTT ALEXANDER

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2007

Acknowledgements

I would like to wholeheartedly thank all those who supported me throughout my graduate years on a professional and personal level. I am grateful to my mentor Dr. Daniel J. Garry for his wisdom, guidance, and generosity in allowing me to work on the most exciting projects a graduate student could ask for. I am equally grateful for my current mentor Dr. Joseph A. Garcia for taking me under his wing, and opening up my eyes to the wonderful word of biochemistry. I would also like to extend my gratitude to Dr. Robert E. Hammer for all of his advice and encouragement. I would also like to thank my collaborator and friend Dr. Ronald E. Allen whose passion for science and teaching inspires all those around him. I thank Dr. Cyprian Weaver for the very same reasons, and for bringing much-welcomed humor to the lab.

I owe a great amount of gratitude towards my dissertation committee members, Drs. Eric N. Olson, Jane E. Johnson, and Robert E. Hammer. In addition to providing reagents, my committee members have given me invaluable advice and guidance throughout my graduate years. Special thanks to Dr. James A. Richardson and John M. Shelton for their teachings and technical advice in the world of histology, which without I would be lost.

I would like to thank all the past and present members of the Garry lab. A special note of thanks to Dr. Annette P. Meeson, the pioneer behind the Foxk1 project. Personal thanks to Sean C. Goetsch and his wizardry in making high-quality figures. Additional thanks to Dr. Shane Kanatous, Dr. Cindy M. Martin, Dr. Amanda Masino, Dr. Anwarul Ferdous, Dr. Pradeep P. Mammen, Dr. Arianna Caprioli, Dr. Jamie L. Russell, Dr. Shuaib Latif, Dr. Xiaozhong Shi, Nan Jiang, Caroline Humphries, Dr. L. Jon Embree, Elizabeth Bhoj, Katie Tunison, Jennifer Sherrell, Justin Kallhoff and anyone else in the lab whom I might have left off.

Personal thanks is directed towards all of my friends that have supported me through the good times and the bad, in particular, Sarah, Kristen P., Kristen E., Kelly, Kacey, Kerra, Mike, Bobbie Jo, Erin, Jenn, Candy, Jacquie, Travis, Lillian, Toni, and Chris. Thanks guys for always being there when I needed you.

Finally, I am extremely grateful towards my family, especial my mom, to whom I dedicate this dissertation. Her unconditional love and support has kept me going through the good times and bad. I could not have done this without her belief in me.

THE ROLES OF FORKHEAD TRANSCRIPTION FACTORS IN STEM CELLS AND MYOGENESIS

MATTHEW SCOTT ALEXANDER

The University of Texas Southwestern Medical Center at Dallas, 2007

Supervising Professor: JOSEPH A. GARCIA M.D., Ph.D.

Vertebrate myogenesis is a highly conserved process that involves the formation, activation, proliferation, and overall regulation of myogenic progenitor cells (MPCs) that are essential for muscle formation, growth, and regeneration following injury. While the process of skeletal muscle development and

regeneration has been well-described on a physiological level, the molecular mechanisms that govern the regulation of these cells are poorly understood. Through the utilization of murine transgenic models and gene disruption strategies, I have been able to elucidate important pathways involved in the regulation of MPCs during embryonic myogenesis and adult regeneration following injury.

The experiments performed in satisfaction of my dissertation were aimed at defining the biological regulation of Foxk1 and characterization of a novel *forkhead* factor, Foxj3. Previous studies from our lab have identified the *forkhead*/winged helix transcription factor, Foxk1, as an essential regulator of MPC activation and quiescence. Firstly, I have undertaken a series of molecular, biochemical, and genetic studies to define the upstream regulation of the *Foxk1* gene promoter. Based on evolutionary conservation of the 5' Foxk1 upstream promoter among mouse, rat, and human, I identified a conserved Sox Binding Element (SBE) that I hypothesized as being essential for the transcriptional regulation of Foxk1. I undertook a candidate-based approach, from which I identified Sox15 as being a potent transcriptional activator of Foxk1. Through cell culture, transcriptional assays, electrophorectic mobility shift assays, and transgenic founder analyses, I confirmed that this SBE is essential for the activation of *Foxk1* transcription by Sox15. I demonstrated that Sox15 is

essential for normal myoblast cell cycle kinetics through siRNA knockdown of endogenous Sox15 and the characterization of the Sox15 mutant mouse model.

Secondly, I characterized the *in vivo* functional role of an important binding partner of Foxk1, Sin3a. I have further elucidated the expression of a transgenic mouse model that I utilized to generate Sin3a skeletal muscle-deficient mice. Through the usage of the Sin3a conditional knockout mouse, I have shown that Sin3a expression is essential for mouse viability and normal myogenesis. Additionally, I have characterized the lethality phenotype observed in Sin3a-deficient skeletal muscle as a consequence of dysregulated mitochondrial mass. I have observed that several mitochondrial genes that are dysregulated in Sin3a-deficient mouse embryonic fibroblasts are also deficient in Sin3a-deficient skeletal muscle.

Finally, I have characterized the novel *forkhead* transcription factor Foxj3. I have generated a Foxj3 mutant mouse model. I have observed that Foxj3 mutant mice are growth retarded, have impaired skeletal muscle regeneration following injury, and have a significant decrease in the total percentage of Type I oxidative myofibers. When Foxj3 expression is decreased significantly, myoblasts have perturbed cell cycle kinetics and proliferate at a faster rate. Additionally, I demonstrated that Foxj3 is a direct upstream transactivator of Mef2c in skeletal

muscle and an essential upstream regulator of the Mef2c-signaling pathway. In conclusion, these studies have elucidated the functional regulation of Foxk1 and its binding partner, Sin3a, in the MPC population. Additionally, I have identified a novel regulator of skeletal muscle myogenesis and myofiber identity, Foxj3, through cell culture assays and generation of the Foxj3 mutant mouse model.

Table of Contents

Publications

Shi, X., **Alexander, M.S.**, DePinho, R.A., and Garry D.J. Sin3a is essential for normal myoblast kinetics and mitochondrial function. *Mol. Cell. In preparation*.

Alexander, M.S. and Garry, D.J. Taking the *forkhead* on the stem cell road. Review. *Development*. *In preparation*.

Alexander, M.S., Voekler, K.A., Grange, R.W., Hammer, R.E., and Garry, D.J. Foxj3 transcriptionally activates Mef2c, and is essential for adult skeletal muscle fiber type identity. *J. Clin. Invest*. *In preparation*.

Moody, L.A., **Alexander, M.S.**, Mooney, J.M., Gehlbach, K.A., Garry, D.J., Schatzle, J.D., and Bennett, M. Foxk1 is required for Natural Killer cell development and function. *J. Immun*. *In revision*.

***Alexander, M.S.**, *Meeson, A.P., *Shi, X., Williams, R.S., Allen, R.E., Jiang, N., Adham, I.M., Goetsch, S.C., Hammer, R.E., and Garry, D.J. Sox15 and Fhl3 transcriptionally coactivate Foxk1 and regulate myogenic progenitor cells. 2007. *EMBO. J.* 26(7):1902-1912.

* denotes equal contribution

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Supplemental Data

Abbreviations

aa – amino acids

ATP – adenosine triphosphate

BMT – bone marrow transplant

BrdU – 5-Bromo-2'-deoxyuridine

ChIP – chromatin immunoprecipitation

cdk – cyclin dependent kinase

cdki – cyclin dependent kinase inhibitor

ctx – cardiotoxin

D – day

DMD – Duchenne's Muscular Dystrophy

ds – double-stranded

E – embryonic day

EDL – extensor digitorum longus

EMSA – electrophorectic mobility shift assay

FACS – fluorescence activated cell sorting

FBS – *forkhead* binding site

FHA – *forkhead*-associated domain

Fox – *forkhead* box

G/P – gastrocnemius/plantaris

GFP – green fluorescent protein

HA – hemagglutin

H&E – hematoxylin and eosin

HAT – histone actyltransferase

HDAC – histone deactylase

HMG – high mobility group

HSA – *human skeletal actin*

HSC – hematopoetic stem cell

ICM – inner cell mass

IgG – immunoglobulin G

IHC – immunohistochemistry

MCK – *muscle creatine kinase*

mdx – *dystrophen* mutant mouse model

MEF – myocyte enhancer factor

MNF – *myocyte nuclear factor*

MPC – myogenic progenitor cell

MRF – myogenic regulatory factor

MSC – muscle satellite cell

NFR – nuclear fast red

ORF – open reading frame

P – postnatal day

Pax – paired and homeodomain box

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PGC – primordial germ cell

QPCR – quantitative RT-PCR

ROS – reactive oxygen species

RT – reverse transcriptase

SAP – Sin3a/b-interacting protein

SBE – Sox binding element

SELEX – systematic evolution of ligands by exponential enrichment

shRNA – small hairpin RNA

siRNA – small interfering RNA

SP – side population

SRF – serum response factor

Sox – SRY-homology box

SRY – sex determining region of chromosome Y

SUMO – small ubiquitin-like modifier

TA – tibialis anterior

TEM – transmission electron microscopy

TUNEL – terminal deoxynucleotidal transferase dUTP Nick End Labeling

Chapter 1

Introduction to Myogenic Progenitor Cells

Mammalian adult skeletal muscle has a remarkable amount of plasticity in its ability to respond to injury due to a population of myogenic progenitor cells (MPCs) that normally reside in a quiescent state. These myogenic progenitor cells, also referred to as muscle satellite cells due to their position in a "satellite" area adjacent to the skeletal muscle myofiber, were first identified by Alexander Mauro in 1961 (Mauro, 1961; Muir *et al*, 1965). Upon injury, diverse signaling pathways are activated to induce expression of myogenic and DNA-damage response factors while repressing others in a highly coordinated fashion (Figure 1). It is important to note that while the physiological response of myogenic progenitor cells in response to injury has been well described, the molecular factors that regulate this process are poorly understood (reviewed in Hawke and Garry, 2001). The major focus of my doctoral dissertation is the characterization of two *forkhead* factors, Foxk1 and Foxj3 that function to regulate myogenic progenitor cells and myogenesis.

Early factors that regulate myogenesis and the formation of MPCs

Myogenic Regulator Factors (MRFs)

Early studies identifying the factors involved in myogenesis involved the use of quail-chick chimeras and heterokaryons of muscle and non-muscle cell types (Buckingham *et al*, 2003; Wright, 1984). The discovery of a factor that could cause fibroblasts to transdifferentiate into myoblasts, MyoD, marked the emergence of the identification of factors essential for the early formation of muscle (Davis *et al*, 1987). Soon after, other factors involved in the initial determination of skeletal muscle were uncovered using similar differentially-expressed cDNA screening assays including Myf5, Myogenin, Mrf4, and Mef2c (Braun *et al*, 1989; Rhodes and Konieczny, 1989; Wright *et al*, 1989; Martin *et al*, 1993). With the advent of mouse transgenic technology, new insights into the signaling pathways that were essential for muscle formation were further elucidated. Gene disruption strategies revealed, rather surprisingly, that MyoD and Myf5 were not essential for muscle formation. Although Myf5 was required for viability, there existed an implied incomplete functional redundancy between the early muscle differentiation genes (Rudnicki *et al*, 1992; Braun *et al*, 1992). The epistatic relationship among myogenic regulator factors was not clearly defined until recently, when studies validated that Mrf4 (Myf6) determined

skeletal muscle identity in the absence of MyoD and Myf5, results obtained from a triple knockout of the three myogenic regulatory factors (MRFs) (Kasser-Duchossoy *et al*, 2004)

Pax Genes are Essential Regulators of Myogenic Progenitor Cells

The homeobox transcription factor Pax7 was first identified as a molecular marker of muscle satellite cells by Seale and colleagues in 2000 and was thought to be essential for the specification of the satellite cell population (Seale *et al*, 2000). However, later studies from the Braun lab demonstrated that Pax7 mutant mice did have satellite cells, and that Pax7 was essential for the maintenance and self-renewal of satellite cells, but not their specification (Oustanina *et al*, 2004). More recent studies demonstrated that the earliest formation of the myogenic progenitor cell population occurs with the formation of a $Pax3^{+}/Pax7^{+}$ cell population that arises from the dermomyotome at E10.75 (Relaix *et al*, 2005). These studies from Buckingham and colleagues were conducted using a Pax3-*GFP* knock-in mouse in which a green fluorescent protein open-reading frame (ORF) is inserted into the Pax3 locus. Additional experiments demonstrated that cells isolated, using FACS sorting of adult skeletal muscle from Pax3-*GFP* heterozygote mice, were quiescent muscle progenitors and could contribute to muscle regeneration in injured and myopathic

(*mdx*) mice (Montarras *et al*, 2005). In studies involving the replacement of the Pax3 gene by a Pax7 ORF, Pax7 "knock-in" mice were observed as capable of forming initial myogenic progenitor cells in the neural crest and somites, but not in long-range progenitors of the limb buds (Relaix *et al*, 2004). It is evident that Pax3 and Pax7 both play a critical role in the early specification of muscle progenitor cells; however, they do not play redundant functional roles in other, non-myogenic derived lineages. In recent studies from Relaix and colleages, it was demonstrated that Pax7 was dispensible for the formation of the limb bud progenitors characterized by Pax3 expression (Relaix *et al*, 2006). Additional studies in quail chimeras confirmed that it was Pax3 that drove initial formation and expansion of myogenic progenitors, and that Pax7 was subsequently essential for maintenance of the MPC population (Ben-Yair and Kalcheim, 2005). The downstream targets of Pax3 and Pax7 that are activated in early MPC formation are currently not known.

Notch Signaling Maintains Myogenic Progenitor Cells Pleuripotency

Notch signaling occurs from a series of cell-to-cell interactions that result in the recognition of an extracellular signal (notably *jagged* and *delta* ligands) to the nucleus via the cleavage of the Notch receptor to the Notch intracellular domain (NICD). The Notch intracellular domain is then shuttled to the nucleus where it

transcriptionally activates genes, such as *Hes1* (reviewed by Ehebauer *et al*, 2006). It is the cell-to-cell interactions that allow Notch signaling to determine the cell fate of the cells surrounding the signaling cell and the cell of the signal's origin.

The Notch signaling pathway was first identified as a critical regulator in *Drosophila* myogenic progenitor cells (Giebel, 1999; Brennan *et al*, 1999). The Notch signaling pathway regulates key morphogens and genes essential for the maintenance of an adult stem cell in its defined niche. Studies performed by Rando and colleagues first identified Notch and its agonist Numb as being differentially regulated in myogenic progenitor cells (Conboy and Rando, 2002). Further studies identified the concept that Notch and Numb were asymmetrically regulated dependant on whether or not the myogenic progenitor cell was dividing in a parallel fashion along the basal lamina or directly into the muscle myofiber (Shinin *et al*, 2006). Conditional deletion in the myogenic progenitor lineage of the Notch-interacting co-transcription factor RBP-J (*Rbpsuh*), resulted in the loss of myogenic progenitors by E17.5, but not the initial formation of a Pax3/Pax7 double-positive muscle satellite cell population (Vasyutina *et al*, 2007). The authors of this study noted that the Pax3/Pax7 double-positive myogenic progenitor cell pool was reduced in the RBP-J muscle mutants, and that the progenitor cells themselves, differentiated early. In summary, these studies

indicate that the Notch signaling pathway and its components are essential for the maintenance and differentiation of myogenic progenitor cells during the early stages of embryogenesis and adult skeletal muscle regeneration.

miRNAs Regulate Myogenic Progenitor Cells

Recent advances in microarray techniology (cDNA, protein, and miRNA) offer new insight in the complex regulation and coordinated signaling pathways involved in myogenesis. miRNA1 and miRNA133a have been implicated as post-transcriptional regulators of the myogenic transcription factors MyoD, SRF, and MEF2 during early myogenesis (Zhao *et al*, 2005; Chen *et al*, 2006). Myogenic transcription factors have been implicated as direct transcriptional regulators of miRNAs. MyoD inhibits the translation of follistatin-like 1 (*fstl1*) and utrophin during skeletal muscle differentiation through transcriptional activation of miRNA 206's 5' upstream promoter (Rosenberg *et al*, 2006).

Future experiments are necessary to elucidate the specific functions of the tissue-restricted miRNAs using *in vivo*, animal-based approaches. In summary, miRNAs appear to play an essential function for the regulation of the levels of transcription of essential myogenic factors in myogenic progenitor cells.

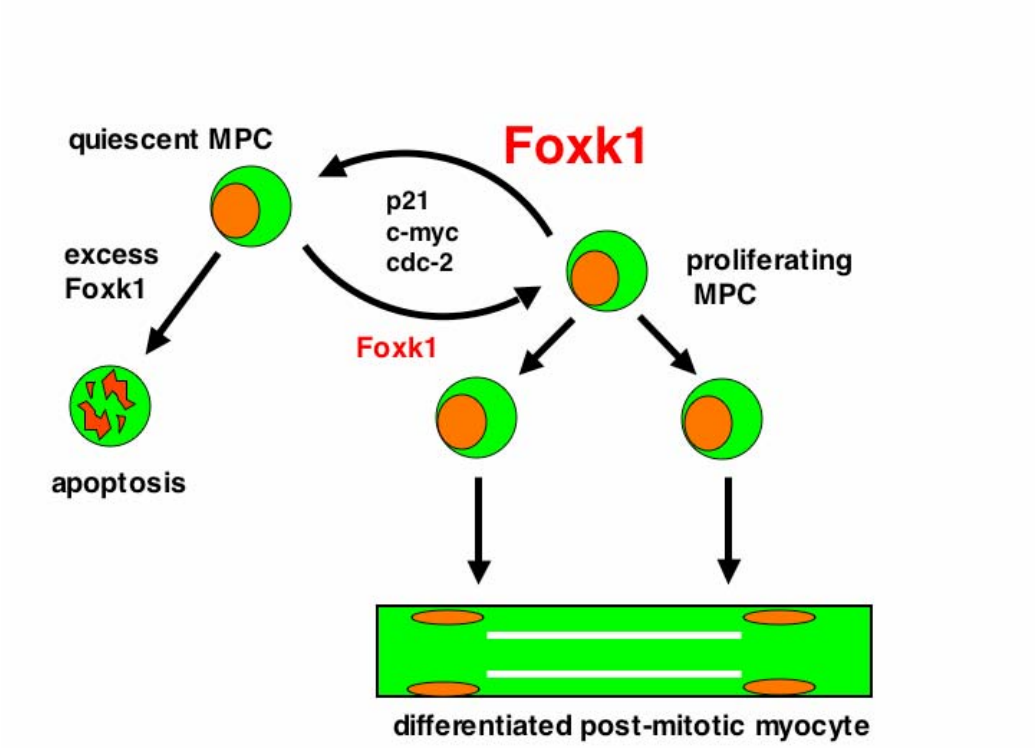


Figure 1. Schematic illustrating the function of MPCs and Foxk1 in muscle regeneration. Myogenic progenitor cells (MPCs) exist in a quiescent state on the periphery of the muscle myofiber and are enriched with Foxk1. High levels of Foxk1 (large red letters) result in a return/maintenance of a quiescent state for the MPC. Excessively high levels of Foxk1 activate the apoptotic pathway resulting in MPC death. Upon injury, Foxk1 levels decrease (small red letters) resulting in increased MPC proliferation and migration to the site of the damaged myofibers. Additional Foxk1 downstream target genes, such as p21, c-myc, and cdc-2, also are essential regulators of MPC proliferation and activation.

Classification and Nomenclature of Forkhead Transcription Factors

Forkhead proteins have been identified in all eukaryotes with the total number of subclasses and genes increasing in concordance with species evolution. The original *forkhead* mutant was identified in *Drosophila* and given the name *fork head* due to its characteristic misshapen head resulting in embryonic lethality (Weigel *et al*, 1989). Since the initial identification of the *fork head* (now classified as *forkhead*) mutant in *Drosophila*, over 40 mammalian *forkhead* genes have been identified that all share a characteristic 110 amino acid DNA-binding domain (Kaestner *et al*, 2000). Currently, there are 19 subfamilies of *forkheads* (Foxa1 through Foxs1) with paralogues that can differ greatly in function (Table 2). A mammalian homolog of the *Drosophila fork head* gene, Foxa1 (previously called HNF-3α), along with two other mammalian *forkhead* genes, Foxa2 and Foxa3 (previously called HNF-3β and HNF-3γ) (Lai *et al*, 1991) were identified, and found to have similar lethality phenotypes in mouse Conventional murine knockouts of the FoxA subfamily, have revealed non-redundant functions in gastrulation, metabolism, and overall mouse viability (Duncan *et al*, 1998).

Additionally, many *forkhead* genes have been implicated in various disease states, particularly with the regulation of immunological progenitor cells of the immune system (reviewed by Jonsson and Peng, 2005). Many *forkhead*

transcription factors have been shown to function as critical regulators of stem cell populations. Foxd3 (formerly called *genesis*) is an important regulator of embryonic stem cell pleuripotency (Hanna *et al*, 2002). Previous work from our laboratory has shown that the *forkhead* transcription factor Foxk1 is a myogenic progenitor cell marker, and is essential for normal skeletal muscle regeneration (Garry *et al*, 1997; Garry *et al*, 2000).

Forkhead factors as regulators of stem cell cycle kinetics

Stem cell proliferation is an important function in separating stem cells from somatic cells. The regulation of the cell cycle is tightly regulated in stem cells by the modulation of key cyclins, cyclin-dependent kinases (cdks), cyclin-dependent kinase inhibitors (cdkis), and forkhead transcription factors (Figure 17). Embryonic stem cells have a short G1 phase, and a prolonged S-phase under proliferative conditions, which is then inverted under differentiation conditions (reviewed by White and Dalton, 2005).

Adult stem cells are arrested in a quiescent state, characterized by prolonged time in the G0/G1 state, until activated through the interaction of various extracellular signaling mechanisms. Recent studies have demonstrated that two cyclin-dependent kinase inhibitors p21 (cdkn1a) and p27 (cdkn1b) are essential

for the normal regulation of adult stem cell cycle kinetics (Dijkers *et al*, 2000). Loss of function studies involving the p21 mutant mouse model reveals that p21 is necessary for the regulation of myogenic progenitor cells under normal and regenerative conditions (Hawke *et al*, 2003). p21 is a critical regulator of adult stem cell populations in other mesodermal-derived lineages. For example, Foxo1 binds to the *p21* upstream promoter in response to insulin signaling, and functions as a modulator of adipocyte differentiation (Nakae *et al*, 2003). It remains unclear whether or not Foxk1 and Foxo1 are functionally redundant in myogenic progenitor cell lineages.

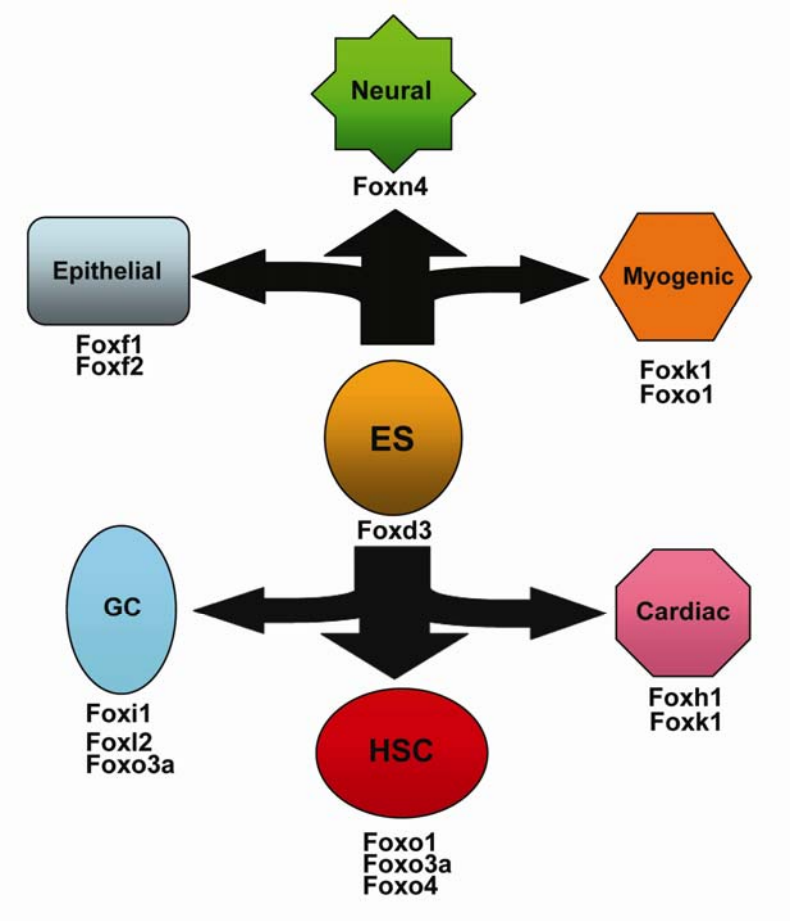


Figure 2. Validated *forkhead* factors that regulate stem and progenitor cells. Schematic of various stem and progenitor cell types and the *forkhead* transcription factors that modulate their activity or function (listed below the each cell). ES, embryonic stem cell. GC, germ cell. HSC, hematopoetic stem cell.

The *forkhead* transcription factor Foxm1 regulates the cell cycle and translation of cyclin-dependent kinase inhibitors through modulation of their degradation. In

elegant studies conducted by the lab of the late Robert Costa, Foxm1 was shown to modulate the transcription of *Skp2* and *Cks1*, components of the SCF ubiquitin ligase complex in hepatocytes (Wang *et al*, 2005). The authors of the study noted that in the absence of Foxm1, p21 and p27 accumulated in the nucleus, resulting in decreased cellular proliferation. Foxm1 plays a broad role in the regulation of normal cell division during development and regeneration, and the prevention of cancerous growth. The generation of conditional knockout mice for the *Foxm1* gene revealed that selected deletion of Foxm1 in hepatic lineages resulted in impaired liver regeneration following partial hepatectomy (Wang *et al*, 2002). Through these and other studies, Foxm1 has an important function in the regulation of any non-somatic cell through the regulation of cyclin dependent kinase inhibitors and other genes involved in the DNA damage repair pathway.

In addition to the modulation of p21, Foxo1 also appears to repress p27 transcripitonal in myogenic progenitor cells, resulting in increased cell proliferation (Machida *et al*, 2003). It remains unclear whether or not Foxo1 is a direct upstream regulator of *p27* or if it indirectly represses *p27*'s transcriptional activity through the modulation of other transcription factors. Interleukin signaling in haematopoetic stem cells results in an upregulation of Foxo3a (also called FKHRL1), an induction of p27, and cell cycle arrest (Dijkers *et al*, 2002). Interestingly, overexpression of Foxo3a results in an induction of Bim and the

Apaf-1/Caspase-9 cell death pathway, while downregulation of Foxo3 resulted in enhanced cell survival. Consequently, the maintenance of normal FoxO levels appears to be essential for normal cell turnover and the prevention of cancerous tumors and cell death.

Forkheads partner with chromatin remodeling complexes in stem cells

Chromatin remodeling complexes, most notably histone deactylases (HDACs), histone acetyl transferases (HATs), and histone methyl transferases (HMTs), are essential regulators of gene transcription during critical developmental time periods (reviewed by Kouzarides, 2007). Indeed, chromatin remodeling during early differentiation from embryonic stem cells in blastocysts is essential for the regulation of the transition to a more differentiated cell type (reviewed by Surani *et al*, 2007).

Several *forkhead* transcription factors have been shown to either directly or indirectly interact with chromatin remodeling proteins to regulate gene transcription. Foxk1 directly interacts with the transcriptional repressor Sin3b to repress the transcription of p21 in myogenic progenitor cells (Yang *et al*, 2000). The ability of the Foxo family of transcription factors to act as a repressor or activator of gene transcription is dependent on its acetylation state. Interaction

with the mammalian deactylase SIRT1 under oxidative stress conditions results in a cell cycle arrest, while inhibiting apoptosis (Brunet *et al*, 2004). Reciprocally, FoxO interactions with cAMP-response element binding protein (CREB)-binding protein (CBP) attenuates FoxO ability to act as a transcriptional activator (Matsuzaki *et al*, 2005). Foxp3, a marker of regulatory T-lymphocytes (also called "Tregs"), directly interacts with the class II histone deactylases HDAC7 and HDAC9 to repress the transcription of the cytokine *interleukin-2* (*IL-2*) (Li *et al*, 2007). A recent paper identified direct downstream target genes of Foxp3 using an *in vivo* ChIP-to-chip strategy with a Foxp3-GFP knock-in mouse model (Zheng *et al*, 2007). In the exact same population of $Foxp3^{+}/GFP^{+}$ regulatory T-lymphocytes, genes were both transcriptionally upregulated and downregulated at the same time and in the same population. This implied a mechanism involving the interactions with other transcriptional repressors and activators that specified the function of Foxp3 and may be a general mechanism for the transcriptional activity of other *forkheads*.

Forkheads as Regulators of Myogenic and Non-myogenic Cell Populations

Embryonic Stem Cells

Embryonic stem cells (or ES cells) have the capacity for self-renewal, unlimited proliferation, and the ability to differentiate into multiple lineages including all three germ layer derivatives. The cell cycle kinetics and chromatin state of embryonic stem cells are tightly regulated, and one well-studied *forkhead* transcription factor was identified as a critical regulator of ES cells. Foxd3 (originally termed *genesis*) was identified in a cDNA library screen as a *forkhead* transcription factor whose expression pattern was spatially and temporally restricted to ES cells and embryonic carcinoma (or EC cells) lineages (Sutton *et al*, 1996). Mice lacking Foxd3, fail to develop beyond E6.5 due to massive defects in the formation of the extraembryonic layers and absence of a primitive streak (Hanna *et al*, 2002). Foxd3-/- blastocysts grown in cultures failed to maintain their inner cell mass (ICM), and underwent massive apoptosis. Additionally, it was observed that Foxd3-/- embryos had large amounts of giant trophoblast progenitor cells that failed to proliferate (Tompers *et al*, 2005). Wild type ES cells failed to fully rescue Foxd3-/- embryos, and chimeric embryos failed to survive beyond E9.5. *In vivo* transcriptional assays have determined that Foxd3 alone acts as a transcriptional activator of two other *forkhead* genes,

Foxa1 and Foxa2, in the co-regulation of two endodermal-specific enhancers. Foxd3 also recruits the pluripotency factor Oct-4 (also referred to as Pou5f1) to repress the gene transcription of *Foxa1* and *Foxa2* in ES cells (Guo *et al*, 2002). Together, these studies establish Foxd3 as an essential regulator of ES cell pluripotency and giant trophoblast progenitor maintenance.

Myogenic Progenitor Cells

The *forkhead* transcription factor Foxk1 (previously referred to as myocyte nuclear factor or MNF) was identified as a key molecular marker of myogenic progenitor cells (also referred to as myogenic progenitor cells) in adult skeletal muscle (Garry *et al*, 1997). Mice lacking Foxk1 are growth retarded and have impaired muscle regeneration following skeletal muscle injury (Garry *et al*, 2000). Foxk1-/- myogenic progenitor cells have perturbed cell cycle kinetics, and a reduced proliferative capacity (Meeson *et al*, 2004). Further studies have identified Foxk1 to act as a transcriptional repressor of the cyclin-dependent kinase inhibitor p21 (cdkn1a), and a critical regulator of the myogenic progenitor cell population throughout development (Hawke *et al*, 2003; Meeson *et al*, 2007).

Several *in vitro* cell culture experiments have identified Foxo1 as playing an important role in the regulation myoblast fusion during myogenic differentiation

(Bois and Grosveld, 2003). Members of the insulin growth factor (IGF) pathway and Akt pathway modulate the function of Foxo1 through the regulation of its cytoplasm-nucleus translocation upon serum withdrawal (Hribal *et al*, 2003). Overexpression of Foxo1 in rat myogenic progenitor cells leads to an upregulation of p27 (cdkn1b), but the affect is attenuated by the IGF-1 (Machida *et al*, 2003). Together, these studies demonstrate that Foxo1 is a critical component of myogenic progenitor cell regulation through upstream IGF-1 signaling and the downstream transcriptional regulation of p27.

Mouse Forkhead Knockouts

Fox Name	Alternative Names	Knockout Phenotype	Reference
Foxa1	HNF-3α, Tcf-3α	postnatal lethal, neural malformations, hypoglycemia	Duncan et al, 1998 *Science*
Foxa2	HNF-3β, Tcf-3β	embryonic lethal E11, absence of a notochord	Ang and Rossant, 1994 *Cell*
Foxa3	HNF-3γ, Tcf-3γ	viable, mild phenotype	Kaestner et al, 1998 *MCB*
Foxb1	Fkh5, Foxb1a/b	viable, growth retarded, CNS and midbrain defects	Wehr et al, 1997 *Development*
Foxc1	Fkh1, Mf1, FREAC3	perinatal lethal,congential heart defects, skeletal defects, hindbrain defects	Kume et al, 1998 *Cell*
Foxc2	Fkh14, MFH-1	perinatal lethal, congenital heart defects	Iida et al, 1997 *Development*
Foxd1	FREAC4, Hfh10, Bf-2	perinatal lethal, kidney malformations	Hatini et al, 1996 *Genes Dev.*
Foxd2	Mf2	viable, renal abnormalities, kidney hyperplasia	Kume et al, 2000 *MCB*
Foxd3	genesis, Hfh2	embryonic lethal E6.5, failure to form primitive streak	Hanna et al, 2002 *Genes Dev*.
Foxe1	Titf2	perinatal lethal, cleft palate, thyroid dysgenesis	De Felice et al, 1998 *Nat. Genet*.
Foxe3	FREAC8	viable, eye lens defects	Medina-Martinez et al, 2005 *MCB*
Foxf1	FREAC1, Hfh8	embryonic lethal E10, extra-embryonic malformations, abnormal yolk sac	Mahlapuu et al, 2001 *Development*
Foxf2	Fkh20, FREAC2, LUN	perinatal lethal, cleft palate	Wang et al, 2003 *Dev. Biol.*
Foxg1	BF-1, Hfh9	perinatal lethal, telencephalon malformations	Xuan et al, 1995 *Neuron*
Foxh1	FAST, FAST2	embryonic lethal E11.5, patterning malformations	Yamamoto et al, 2001 *Genes Dev.*
Foxi1	Pds, Fkh10, Hfh3	viable, deafness, auditory defects	Hulander et al, 1998 *Nat. Genet.*
Foxj1	Hfh4, Fkhl13	perinatal lethal, lung cilia defects, *situs inverses*	Brody et al, 2000 *Am. J. Respir. Cell Mol. Biol.*
Foxj3		viable, growth retarded, myofiber switch, skeletal defects	Alexander et al, 2007, *JCI* Submitted
Foxk1	MNF	viable, growth retarded, skeletal muscle progenitor cell defects	Garry et al, 2000 *PNAS*
Foxk2	ILF1	viable, kidney and bone defects, slight neurological deficits	Alexander, M.S. *unpublished data*
Foxl1	Fkh6, FREAC7	viable, abnromal gastrointestinal tract	Kaestner et al, 1997 *Genes Dev.*
Foxl2	Pfrk	viable, eyelid malformations, premature follicular genesis	Schmidt et al, 2004 *Development.*
Foxm1	Foxm1b, Trident, Fkh16, Winm, MPP2	viable, postnatal lethality due to hepatocyte failure	Korver et al, 1998 *Curr. Biol.*
Foxn1	Hfh11, whn, nude	viable, lack of hair (*nude* mouse)	Flanagan, 1966 *Genet. Res. Camb.*
Foxn4		viable, retinal progenitor cell defects	Li et al, 2004 *Neuron*
Foxo1	Foxo1a, FKHR	embryonic lethal E10.5, vascularization defects	Hosaka et al, 2004 *PNAS*
Foxo3a	FKHRL1, FKHR2	viable, abnormal follicule formation, female sterility	Castrillion et al, 2003 *Science*
Foxo4	AFX, Mllt7	viable, mild phenotype	Hosaka et al, 2004 *PNAS*
Foxp1		embryonic lethal E14.5, cardiovascular failure	Wang et al, 2004 *Development*
Foxp2	CAG-16	perinatal lethal, altered ultrasonic vocalization, cerebellar defects	Shu et al, 2005 *PNAS*
Foxp3	JM2, scurfin	viable, immunological defects (*scurfy* mouse)	Lyon et al, 1990 *PNAS*
Foxp4		embryonic lethal E12.5, bilateral heart formation	Li et al, 2004 *Science*
Foxq1	Hfh1, satin	viable, silky fur coat	Major, 1955 *Mouse News Letter*
Foxs1	Fkh3, FKHL18	viable, motor skills defects	Hegland et al, 2005 *MCB*

Table 1. List of all current *forkhead* knockout mice. List of all currently published and unpublished (verified) mouse forkhead knockout mice and their respective observed phenotypes. Foxk2 knockout mouse phenotypic description based on observations of the currently unpublished Foxk2 mutant mouse identified by Matthew Alexander and Professor Jeromé Rossért (jerome.rossert@egp.aphp.fr)

Cardiac Stem Cells

Foxh1 (previously called FAST2) activation is essential for the formation of anterior heart field, during the looping stage of heart development (von Both *et al*, 2004). It was noted by Hoodless and colleagues that the Mef2c-/- hearts had a striking resemblance to those observed in Foxh1-/- mice in which both knockout mice failed to form an outflow tract and right ventricle (Hoodless *et al*, 2001). In latter experiments, it was demonstrated that Foxh1 and the cardiac stem cell transcription factor Nkx2-5 functionally interact and together regulate TGF-β's activity on the *Mef2c* gene promoter. Nkx2-5 is one of the earliest molecular markers for cardiac stem cells and functions as a transcription factor in the heart at E7.75 in the cardiac crescent (Masino *et al*, 2004). The functional interaction between Nkx2-5 and Foxh1 is downstream of Nodal signaling, thus by functional association assigning Foxh1 a critical role in the regulation of early cardiac stem

cell proliferation, differentiation, and formation of the 4-chambered heart by regulating *Mef2c* gene transcription.

Forkheads as regulators of Hematopoetic Stem Cells

Adult hematopoetic stem cells (HSCs) are capable of repopulating the entire bone marrow following gamma-irradiation and serve as a stem cell therapy for the treatment of specific types of leukemias (reviewed by Garry *et al*, 2003). Additionally, their plasticity and ability to migrate throughout various tissues has made HSCs attractive candidate for cellular-based therapies in myopathic diseases. Hematopoietic stem cells are thought to arise from the common progenitor found in the yolk sac called the "hemangioblast". As the embryo develops, the hemangioblast differentiates into hematopoietic stem cells that will eventually reside in the liver and bone marrow (Lensch and Daley, 2004).

Members of the FoxO subfamily of *forkhead* transcription factors have recently been identified as essential regulators of HSC maintenance (Paik *et al*, 2007). While the loss of Foxo3a alone does not affect the formation of HSCs, Foxo3a-deficent HSCs are incapable of long-term hematopoiesis and fail to rescue lethally-irradiated mice through bone marrow transplants (BMTs) (Miyamoto *et al*, 2007). The triple conditional deletion of Foxo1/3a/4 in the hematopoetic lineages

resulted in significant defects in cell culture-based hematopoiesis and an induction of reactive oxygen species (ROS) in response to oxidative stress (Tothova *et al*, 2007). The interaction with FoxOs and the histone deactylase Sirt1 (also referred to as Sir2), appears to be essential for FoxO transcriptional activity to transcriptionally activate stress-response genes under oxidative stress (Brunet *et al*, 2004; Yang *et al*, 2005). Thus, FoxO factors have a critical role in the regulation of hematopoietic stem cells under conditions of growth and oxidative stress.

Background on Sox Proteins

Sox Proteins Regulate Stem Cell Differentiation and Pluripotency

Sox proteins were first identified in a screen for novel proteins containing the High Mobility Group (HMG) DNA-binding domain of the *SRY* gene, the gene essential for mammalian sex determination (Denny *et al*, 1992). Sox proteins can bind directly to DNA containing the Sox Binding Element (SBE), consisting of the nucleotide sequence (A/T)ACAATG, directly through their HMG DNA-binding domain (Laudet *et al*, 1993). Over twenty separate members of the Sox transcription factor family including the *SRY* gene have been identified in mammals (Schepers *et al*, 2002). Sox proteins can act as either transcriptional activators or repressors depending on their direct-interacting binding partners (reviewed by Wilson and Koopman, 2002; Figure 3). Recent studies have revealed that SUMOylation modification of Sox proteins result in a transcriptional repression at critical times of embryogenesis, one of which is neurogenesis of the neural crest in addition to inner ear hair cell development (Savare *et al*, 2005; Taylor and Labonne, 2005).

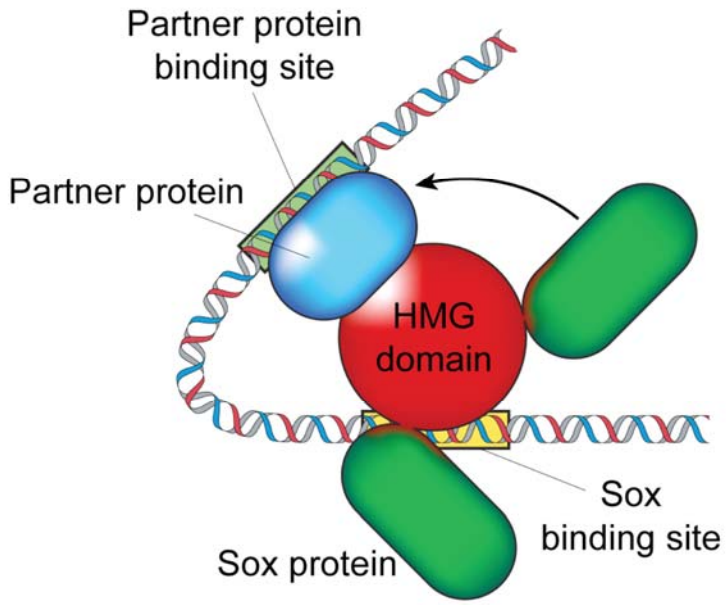


Figure 3. Sox proteins bind directly to DNA and modulate gene transcription through their HMG domain interactions. This schematic illustrates the Sox protein mechanism of action in which Sox proteins bind directly to DNA through their HMG DNA-binding domain (red). Additional Sox proteins (green) can act as transcriptional cofactors to modulate gene transcription. Additional transcriptional cofactors (light blue) have been previously demonstrated to bind to Sox proteins through their HMG domain and modulate Sox transcriptional activity (Wilson and Koopman, 2002).

Sox proteins were first identified as regulators of stem cell pluripotency with the generation of Sox4 mutant mice which have impaired cardiogenesis and loss of B- and T-lymphocyte progenitors (Schilham *et al*, 1996). Several Sox proteins play essential roles in the maintenance of neuronal progenitor cells. Sox10 is essential for the maintenance of an undifferentiated state of neural crest cells through the induction of the transcription factors *Mash1* and *Phox2b* (Kim *et al*, 2003). Sox4 and Sox11 play redundant roles in regulating the cell cycle of undifferentiated neural stem cells through its repression by the REST/NRSF complex and its activation of *NeuroD* (Bergsland *et al*, 2006). Interestingly, a recent study revealed that one Sox factor, Sox21, was capable of transcriptionally repressing three neuronal Sox factors that stimulate neurogenesis (Sox1-3) and thereby maintaining an undifferentiated state in the adult mammalian brain (Sandberg *et al*, 2005).

The most widely studied Sox factor is the one that regulates the pluripotency of embryonic stem cells (ES), Sox2. Targeted inactivation of the *Sox2* gene results in a complete absence of gastrulation and embryonic lethality before E5.5 due to arrest and premature differentiation of the ES cells in the inner cell mass (Avilion *et al*, 2003). Sox2 can transcriptionally activate the *Pou5f1* (also referred to as Oct4) promoter, while Oct4 reciprocally activates the *Sox2* promoter to maintain pluripotency in ES cells (Catena *et al*, 2004; Chew *et al*, 2005). Both Sox2 and

Oct4 bind to a cis-element in the *nanog* promoter to repress its transcription and maintain ES cell pluripotency (Rodda *et al*, 2005). High throughput ChIP-on-chip (ChIP:chip) identification of Sox2 downstream target genes in ES cells revealed a feedforward transcriptional circuit in which Sox2 and Oct4 repress *nanog* and thereby inhibit activation of genes essential for neuronal, cardiac, and germ layer induction (Boyer *et al*, 2005).

Chapter 2

Sox15 Transcriptional Activation of Foxk1 in Myogenic Progenitor Cells

Background

Foxk1 as a Regulator of Myogenic Progenitor Cells

Foxk1 (previously called *myocyte nuclear factor* or *MNF*) was first identified by Bassel-Duby and colleagues in a screen of a skeletal muscle library for proteins that bound to the CCAC box of the human *myoglobin* promoter (Bassel-Duby *et al*, 1994). Foxk1 was the first molecular marker of quiescent muscle satellite cells (MSCs), and is upregulated in skeletal muscle D2-D7 following a cardiotoxin-induced injury (Garry *et al*, 1997). Foxk1 is expressed in early embryonic stem cells formation, and is upregulated in the developing somites, brachial arches, limb buds, and cardiac and neuronal progenitors (Meeson *et al*, 2007).

Murine Foxk1 consists of 719 amino acids, and is highly conserved in vertebrates from zebrafish to humans. Less conservation exists in lower eukaryotes, as the yeast forkhead *fkh2* has significant homology (around 30% amino acid homology) with murine Foxk1 (Freddie *et al*, 2007). Foxk1 is expressed solely in

the nucleus of isolated muscle satellite cells and not in differentiated myofibers; although recent evidence suggests that Foxk1 is shuttled to the nucleus from the cytoplasm upon myogenic differentiation (Garry *et al*, 1997; unpublished data). In a yeast-two hybrid screen for binding partners of Foxk1, the transcriptional repressor Sin3b was identified (Yang *et al*, 2000). A new aspect of Foxk1 modulation of gene transcription was recently identified by Freddie and colleagues who demonstrated that Foxk1 binds to serum response factor (SRF) to transcriptionally repress *SM22α* (*transgelin*) gene transcription and is independent of DNA-binding (Freddie *et al*, 2007). Further questions remain regarding whether or not Foxk1 DNA-independent modulation of gene transcription is its predominant mechanism of transcriptional repression and whether DNA-binding of Foxk1 can modulate Foxk1 transcriptional activity.

Additional biochemical studies have identified a unique region known as the *forkhead* associated domain (FHA). The FHA domain is conserved from prokaryotes to eukaryotes and is present in only Foxk1 and its paralog Foxk2 of the FoxK *forkhead* transcription factor subfamily (Huang and Lee, 2004). It remains unclear what the function of the FHA domain is for Foxk1. However, studies relating to other proteins (such as Rad53, Nbs1, and KIF1Bβ) containing the FHA domain has demonstrated that it is an essential for phosphoprotein interactions and cellular signaling (Durocher and Jackson, 2002).

Recent studies have identified Foxk1 as a global regulator of stem and progenitor cell function (Meeson *et al*, 2007). Mice lacking Foxk1 are growth-retarded and have impaired skeletal muscle regeneration in normal and myopathic (*mdx*) mouse models (Garry *et al*, 2000). Additionally, myogenic progenitor cells and SP cells isolated from Foxk1-/- mice have perturbed cell cycle kinetics with over 80% of myogenic progenitors arrested in the G0/G1 phase of the cell cycle (Hawke *et al*, 2003; Meeson *et al*, 2004). Foxk1 modulates the cell cycle kinetics of MPCs through the gene transcription of the cyclin-dependent kinase inhibitor *cdkn1a* (also called $p21^{WAF}$) through its binding to a consensus *forkhead* binding site (FBS) located in the *p21* 5' upstream promoter region (Hawke *et al*, 2003). Foxk1 levels remain high in the quiescent progenitors, but decrease during muscle progenitor cell activation as p21 expression increases. In the mammalian brain, Foxk1 is strongly expressed in the dopaminergic regions of the subventricular zone (SVZ) essential for the maintenance and formation of neuronal progenitors (Wijchers *et al*, 2006). Under hypoxia-ischemia in rat forebrains, *Foxk1* transcript levels are correspondingly downregulated while *p21* transcript levels are induced (Shimamura *et al*, 2005; GEOmnibus http://www.ncbi.nlm.nih.gov/geo). Consequently, Foxk1 is an essential regulator of the adult progenitor cell populations through its transcriptional repression of *p21*, thereby modulating cell cycle kinetics under conditions of injury or damage.

Sox Proteins as Regulators of Myogenic Progenitor Cells

Two Sox proteins have been directly implicated in the regulation of myogenic progenitor cells and myogenesis. Sox8 has been identified as a marker of quiescent myogenic progenitor cells, and overexpression of Sox8 inhibits C2C12 myoblast differentiation (Schmidt *et al*, 2003). Sox8 mutant mice are growth retarded but show no striking physiological abnormalities (Sock *et al*, 2001). Most mice with defects in the maintenance of the MPC population, such as the Foxk1 mutant, have significant neurological deficits in addition to muscular abnormalities (Hawke and Garry, 2001). The lack of gross neurological deficits, leads to the hypothesis that a functional redundancy exists with Sox8 in the neurological progenitors. Complete replacement of the *Sox10* gene by a *Sox8* ORF revealed a partial rescue of the neuronal abnormalities observed in the Sox10 mutant mice, and a functional redundancy for the class E Sox proteins in neurogenesis, but not in the maintenance of melanocyte progentors (Kellerer *et al*, 2006). One possible explanation for the mild phenotype of the Sox8 mutant mice is that either Sox9 or Sox10 (both members of the SoxE subfamily) are functionally redundant with Sox8 in specific cell populations.

Sox15 is the sole member of the class G Sox transcription factor family and though its expression is ubiquitous, it is enriched in both ES cells and myogenic

progenitor cells (Béranger *et al*, 2001). Sox15 mutant mice have impaired skeletal muscle regeneration following a freeze/crush injury, but otherwise appear to be phenotypically normal (Lee *et al*, 2004). DNA-binding SELEX assays have revealed that Sox15 can bind to the same DNA sequences in ES cell gene promoters as Sox2, but at a much weaker affinity (Maruyama *et al*, 2005). Consequently, it is likely that Sox15 transcriptionally regulates a different set of genes in ES cells and myogenic progenitor cells that is independent of Sox2 function.

Rationale and Aim

It is the aim of my dissertation research to fully characterize the function of Sox15 transcriptional activation of the *Foxk1* gene in myogenic progenitor cells through the use of gene knockdown techniques and the full characterization of the Sox15 mutant mouse model. As mentioned above, Foxk1 is one of the few molecular markers of the MPC population that has been identified, let alone extensively studied. Further chacterization of the components that regulate *Foxk1* transcription would no doubt give a greater understanding of the cell cycle proliferative capacity of MPCs under the condition of skeletal muscle regeneration. Recent studies in mice and canines from the Buckingham and Cossu labs have demonstrated that specific MPC populations have the capacity

to regenerate skeletal muscle in animal models of Duchenne's Muscular Dystrophy (DMD) (Montarras *et al*, 2005; Sampaolesi *et al*, 2006). Through a better understanding of *Foxk1* transcriptional regulation, direct isolation of these Foxk1^{+} MPCs would allow complete examination of their regenerative capacity.

Materials and Methods

Transgenic mice. The transgene constructs (4.6 kb, 1.6 kb and 0.6 kb Foxk1 fragments) were subcloned into a β-*galactosidase* reporter cassette (generously provided by E. Olson) (Cheng *et al,* 1993; Masino *et al,* 2004). Transgenic mice were generated following microinjection of the respective constructs into fertilized F2 eggs (B6SJLFF1; Jackson Lab), which were reimplanted into pseudopregnant F1 foster ICR mothers (Harlan). Identification of transgenic mice was undertaken using PCR for lacZ and β-*galactosidase* expression was assessed using whole mount and histological/histochemical techniques at developmental time points ranging from E9.5 to E15.5 (Cheng *et al,* 1993; Masino *et al,* 2004).

Founder analyses were undertaken for the mutagenesis of the Sox binding element within the 4.6 kb Foxk1 upstream fragment. The Sox binding element (SBE) was mutated from the sequence AA**CAA**TG to AA**TCC**TG in the 4.6 kb Foxk1 promoter-*lacZ* (wildtype) vector using PCR-based site-directed mutagenesis and ligated into the 4.6kb Foxk1-lacZ plasmid using Bgl II and Afl II restriction enzyme cuts that flanked the Sox mutation. As described above, embryos were harvested at E13.5 and processed for β-*galactosidase* staining. The yolk sacs from each embryo were genotyped as outlined above.

Sox15 null mice were engineered as previously described (Lee *et al,* 2004) and mated into the C57BL/6 strain for over 6 generations to ensure isogenicity. The Sox15 null mouse consists of homozygous alleles in which exon 1 of the Sox15 gene has been replaced by a *neomyocin* (*neo*) cassette.

Immunohistochemistry of Tissue Sections. Immunohistochemistry was performed as previously described (Garry *et al,* 1997). Primary antisera utilized in this study included: an affinity purified Foxk1 rabbit polyclonal antibody (1:200 dilution) a rabbit Sox15 polyclonal antibody (Lee *et al*, 2004; 1:50 dilution) and a chicken anti-Fhl3 polyclonal serum (1:500 dilution; Abcam), a monoclonal laminin antibody (1:200; Dako), a monoclonal β-*galactosidase* antibody (1:50; Hybridoma Bank) and a monoclonal desmin antibody (1:250; Dako). Following the incubation with the respective fluorophore conjugated secondary antisera, the sections were coverslipped with Vectashield and imaged using a Zeiss LSM510 META Confocal Microscope.

Electrophoretic Mobility Shift Assay. Sox15 *in vitro* translated protein was generated using the pCS3+MT-Sox15 vector and the Promega TNT kit. 100 μM of a 23-bp oligonucleotide (5'-ATTACTG**AACAATG**GGGTCACAG-3') corresponding to a region in the Foxk1 5' upstream region and its reverse complement and the Sox mutated binding element oligonucleotide

5'ATTACTG**AATCCTG**GGGTCACAG3' and its reverse complement were used as DNA probes.

Whole cell lysates were also collected from C2C12 myoblasts transfected with 4 μg of Sox15. The oligo annealing buffer (0.2M Tris-HCl, pH8.0; 1M NaCl, 0.2M $MgCl_2$, 20mM EDTA, pH8.0) was used to generated double-stranded probes. Double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (Promega) and 5 μL of γ-ATP ^{32}P (GE Healthcare) with a specific activity of 0.01 μCi/μL. 0.5 μL of ^{32}P-labeled dsoligo (approximately 50,000 cpm) were incubated with 15 μg of whole cell lysate, 1 μg poly dI-dC (Sigma), 20 mM DTT, and sterile water up to 20 μL at 4°C for 25 minutes (Molkentin *et al,* 1996; Yang *et al,* 2000). Samples were run on a 5% TBE non-denaturing gel, and imaged on a StormScan860 scanner (GE Healthcare). For the supershift assays, 3 μg of anti-Myc serum (Sigma) were pre-incubated with lysate, before the addition of radiolabeled probe (2 hrs at 4°C), followed by the addition of labeled probe (25 minute incubation) (Yang *et al,* 2000).

Transcriptional assays. C2C12 myoblasts and COS-7 cells were cultured in 35mm dishes containing DMEM (Gibco) supplemented with 20% and 10% fetal bovine serum respectively. Approximately 1.2 x 10^5 of cells were transfected with Lipofectamine (Invitrogen) and assayed for both luciferase and β-

galactosidase activity (Molkentin *et al,* 1996; Yang *et al,* 2000). Cells were transfected with 0.5 μg of the 4.6 kb Foxk1 promoter fused to the *luciferase* reporter along with 0.25 μg human β-*actin* promoter-LacZ (internal control), increasing amounts of transcription factor, and an empty vector (pcDNA3.1) to standardize the total amount of transfected DNA at 2 μg. Cells were incubated in transfected media supplemented with Opti-MEM (Gibco) for 4 hours, and then incubated overnight with normal growth media. Luciferase assays were performed using the Promega Luciferase Assay System. All fold changes in *luciferase* reporter activity were normalized to β-galactosidase activity, and to the vector alone (Molkentin *et al,* 1996; Yang *et al,* 2000). All transfections were performed in triplicate and replicated in three separate experiments.

RNA interference. Small-interfering RNA (siRNA) double-stranded (ds) oligos (SMARTpool, Dharmacon) were transfected in C2C12 myoblasts with modifications as described (Ma *et al,* 2005). Approximately 1.2 X 10^5 C2C12 cells were plated in 6 well plates in 20% FBS/DMEM (n = 3) 24 hours prior to transfection. Cells were transfected 24 hours later with 10 nM dsoligos directed towards mouse Sox15 or mouse Fhl3 with Hyperfect (Qiagen) for 4 hours in serum-free Opti-MEM media (Gibco). After 4 hours, 3 volumes of media with 20% serum were added to each well. Cells were harvested 24 and 48 hours

following transfection and analyzed for gene expression, protein expression and cell cycle analysis (Garry *et al,* 2000, Hawke *et al,* 2003).

Four separate ds oligos were tested for their effectiveness in their ability to knockdown endogenous Sox15 expression in C2C12 myoblasts. We utilized 3 separate dsoligos that were capable of Sox15 knockdown. Untreated cells and siGLO RISC-Free (Dharmacon) were used as both negative controls and indicators of transfection efficiency. Using a siRNA strategy, we evaluated gene expression using Quantitative RT-PCR where primers spanned an intron (see Supplemental Table 2) (Hawke et al, 2003). Cell cycle kinetics, BrdU incorporation and C2C12 culture conditions were performed as previously described (Garry et al, 2000; Hawke et al, 2003).

Wholemount preparation of embryos. Mouse embryos were staged, harvested and immersion fixed in cold 4% paraformaldehyde (30 min to 60 min based on gestational age) as previously described (Garry *et al,* 1997). Staging of the embryos was performed by counting the presence of the vaginal plug as day 0.5 after conception, the number of somites and the presence of developmental anatomical features. Embryos were obtained for all three Foxk1 promoter transgenic lines. Following fixation, embryos were washed in PBS and stained overnight at 37^0C for lacZ expression using a standard lacZ staining protocol

(Cheng *et al*, 1993). Embryos were then washed, post-fixed overnight at 4^0C in 4% paraformaldehyde, cleared in cedar oil (Polysciences Inc) and photographed utilizing an Olympus dissection microscope and Optronics VI-470 CCD camera. Images were captured with Scion imaging software and image processing was completed using Adobe Photoshop 7.0 prior to paraffin embedding and histological analysis.

Primary MPC Isolation and Analyses. As previously described (Hawke *et al,* 2003), primary MPC cultures were established following the isolation of MPCs from hindlimb skeletal muscle of 2 day old neonatal mice. Cells were preplated and cultured in F-10 growth medium supplemented with 25 ng/ml fibroblast growth factor. WT and Sox15 null MPCs were isolated from littermates generated from Sox15 heterozygote breeders. Asynchronously dividing WT and Sox15 null MPCs were fixed for 10 min with 4% paraformaldehyde and immunostained as previously described (Hawke *et al*, 2003). Primary antisera used in these studies include: rabbit anti-Ki67 serum (1:500 dilution; Novocastra), rabbit anti-Foxk1 polyclonal serum (1:200 dilution; Bassel-Duby *et al*, 1994) and β-galactosidase monoclonal antibody (1:50 dilution; Iowa Hybridoma Bank). Secondary antisera included a FITC-conjugated goat anti-mouse serum (1:50 dilution; Jackson ImmunoResearch) and a rhodamine-conjugated goat anti-rabbit serum (1:50 dilution; Jackson ImmunoResearch).

TEM analysis and Quantitation of MPCs. As previously described (Hawke *et al,* 2003), Tibialis anterior muscles from 3-4 month old male adult WT (C57BL/6) and Sox15 (C57BL/6) null mice were harvested, perfusion fixed with 3% glutaraldehyde and postfixed with buffered 1% osmium tetroxide. Samples were then dehydrated with ethanol, embedded in Spurr resin, polymerized, sectioned and placed on copper grids. Sections were examined using a JEOL 1200 EXII TEM. MPCs were quantified using criteria as outlined previously (Hawke *et al,* 2003).

Proliferative Capacity and Cell Cycle Analyses. To assess cellular proliferation, the respective cell populations were grown on collagen-coated coverslips and incubated with 10 μM BrdU (MP Biologicals) for 7 hrs (Hawke *et al,* 2003). Following incubation and fixation (4% paraformaldehyde), the cells were incubated sequentially with anti-BrdU monoclonal serum (1:100 dilution, Roche), which were subsequently detected with a FITC conjugated goat anti-mouse secondary serum (1:50 dilution; Jackson ImmunoResearch) (Hawke *et al*, 2003). Cells were co-stained with propidium iodide (Molecular Probes) at 50 ng/ml concentration to label all nuclei. Cellular proliferation was quantified as a percentage of BrdU positive cells (proliferating MPCs) to propidium iodide-positive nuclei (total cells) (Hawke *et al,* 2003).

Further characterization of the proliferative capacity of the respective MPC populations included the definition of cell cycle kinetics of the asynchronously dividing cell populations. MPCs were permeabilized with 0.3% Triton X-100 and incubated with a propidium iodide (PI) staining solution (1.8 mg/ml RNase A, 50 μg/ml PI) for 3 hrs to label the DNA. Using a MoFlo cytometer, $10{,}000^{+}$ cells were sorted based on DNA content. The percentage of cells in each phase of the cell cycle was quantitated using Cellquest Software as previously described (Hawke *et al,* 2003).

Differentiation capacity of C2C12 myoblasts. Differentiation capacity of MPCs to form multinucleated myotubes was achieved by growing monolayers in DMEM supplemented with 20% fetal bovine serum and antibiotics (Hawke *et al*, 2003). Myotube differentiation was promoted by exposing 80% confluent myoblast cultures to differentiation medium (DMEM supplemented with 2% heat inactivated horse serum, antibiotics, insulin and transferrin) as previously described (Hawke *et al,* 2003).

Cardiotoxin-induced muscle regeneration. Cardiotoxin (150 μL of a 10 μM solution; Calbiochem) was delivered intramuscularly into the gastrocnemius muscle of adult transgenic mice. At specified time periods following injury, mice

were sacrificed and the gastrocnemius/plantaris muscles were harvested, immersion fixed, embedded and sectioned using a Leica CM 3000 cryostat and examined for X-gal expression as previously described (Garry *et al,* 2000).

Plasmids. The myc-Sox15 plasmid constructs were generated by cloning murine Sox15 into the pCS3-MT-myc vector. The Sox8 overexpression plasmid was a generous gift from M. Wegner (Schmidt *et al,* 2003). The HA-Sox15 overexpression plasmids were created by cloning Sox15 ORF into the pcDNA3.1-HA vector.

Statistical analysis. All p values were calculated using Student's t-test analysis (two-tailed).

Results

A 4.6 kb 5' upstream enhancer modulates *Foxk1* gene transcription. It has been previously reported that the *forkhead*/winged helix transcription factor, Foxk1, is expressed in MPCs that are resident in adult skeletal muscle using light microscopic and ultrastructural immunohistochemical techniques (Garry *et al,* 1997). To examine the regulation of the *Foxk1* gene, I characterized a 4.6 kb *Foxk1* promoter fragment that contained evolutionarily conserved regions between mouse and human. Three transgenic lines using this 4.6 kb *Foxk1* promoter fragment, and two other lines consisting of 0.6 kb and 1.6 kb of the 5' *Foxk1* upstream promoter) to drive the *lacZ* reporter gene were subsequently generated. In the 4.6 kb transgenic line, β-*galactosidase* expression was specific to the muscle precursor cells of the developing limbs (E11.5, E13.5 and E14.5) (Figure 4A-F; Supplemental Table 1). These results indicate that the 4.6 kb promoter of the *Foxk1* 5' upstream region contains regulatory elements that control expression in a cell lineage that are destined to become skeletal muscle (the MPC population) in the developing limbs. During midgestational stages of embryogenesis (E13.5), expression of the 4.6 kb Foxk1 promoter was colocalized with Foxk1 in developing muscle using immunohistochemical techniques, a Foxk1 antiserum and a β-galactosidase antiserum (Figure 4G and

3H). While the 4.6 kb Foxk1-*lacZ* expression recapitulated endogenous Foxk1 expression in the developing limbs, trunk muscles (low level) and heart (low level), β-*galactosidase* expression was not observed in the developing somites.

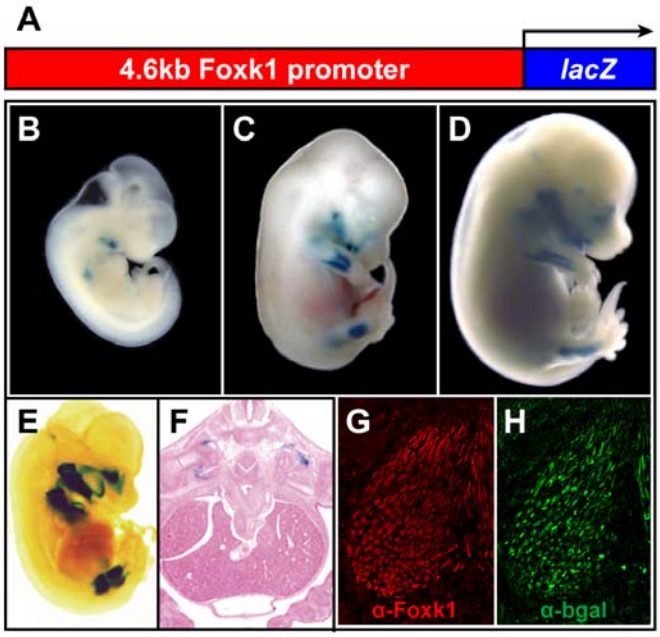


Figure 4. The 4.6 kb Foxk1-lacZ transgenic mouse directs expression to myogenic lineages. **A** Schematic of 4.6 kb Foxk1-*lacZ* transgenic mouse construct. **B-F** X-gal staining of E9.5 transgenic embryo reveals expression of the transgene in the early muscle "pioneer" cells. Later stages (E13.5 and E15.5) reveals expression in the limb buds, brachial arches, and cranio-facial muscles. **E** Cedar-oil cleared E13.5 Foxk1-*lacZ* embryo. **F** H&E stained cross-section of E13.5 Foxk1-*lacZ* embryo. **G-H** Immunohistochemistry of E13.5

Foxk1-*lacZ* embryo limb bud with Foxk1 and β-galactosidase antisera demonstrates Foxk1 and beta-galactosidase co-localization.

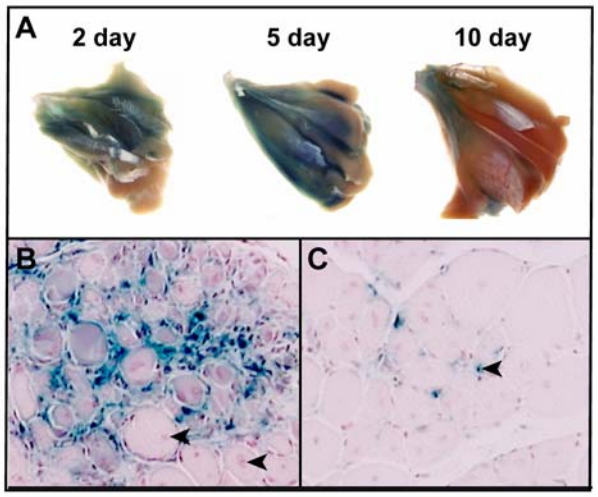


Figure 5. Foxk1 is upregulated following cardiotoxin injury. A X-gal immunostaining of 4.6 kb Foxk1-*lacZ* G/P wholemount skeletal muscle 2 days, 5 days, and 10 days post injury. **B and C** 4.6 kb Foxk1-lacZ G/P skeletal muscle 5 days (**B**) and 10 days (**C**) post cardiotoxin injury shows persistant lacZ (Foxk1) expression in centrally located myonuclei (arrowheads). Sections were counterstained with light eosin.

Following a cardiotoxin-induced injury to hindlimb skeletal muscle, I observed that the expression of the 4.6 kb Foxk1 promoter recapitulates Foxk1 expression during muscle regeneration and identifies both quiescent and proliferating

myogenic progenitor cells MPCs, but no expression was observed in differentiated myotubes using wholemount (Figure 5A) and histological sections of 5 day injured (Figure 5B), 10 day injured (Figure 5C). These experiments are consistant with the hypothesis that *Foxk1* expression regulates the activation of the myogenic progenitor cell activation and proliferation phases.

Sox15 binds to the Foxk1 promoter. Database analysis of this 4.6 kb upstream fragment of the *Foxk1* gene promoter revealed the presence of an evolutionarily conserved Sox binding element (SBE) that was conserved between mouse, rat and human genomes (Figure 6A). Using electrophoretic mobility shift assays (EMSA) with a 23 oligonucleotide probe of the *Foxk1* promoter that contains the SBE, I examined the capacity of a myc-Sox15 fusion protein to bind to this DNA probe (Figure 6B). I demonstrated that Sox15 binds to this site (i.e. SBE) in the *Foxk1* upstream promoter and that the myc-Sox15 fusion protein-oligonucleotide complex could be competed with cold competitor. I further demonstrated that mutagenesis of three nucleotides within the SBE of the radiolabeled oligonucleotide probe precluded formation of the protein-DNA complex. These three nucleotides have been previously shown to be essential for Sox transcription factor activity (Bridgewater *et al*, 2003; Hiraoka *et al*, 1998). Additionally, the incubation of the wildtype oligonucleotide probe that contains the SBE with the myc-Sox15 fusion protein and anti-myc antisera resulted in the

supershift of the protein-DNA complex. Additionally, to address whether or not the binding of Sox15 is conserved, I performed a similar EMSA with the human Foxk1 SBE and mouse recombinant Sox15 which also, as expected, capable of forming a protein DNA complex (Supplemental Figure 1).

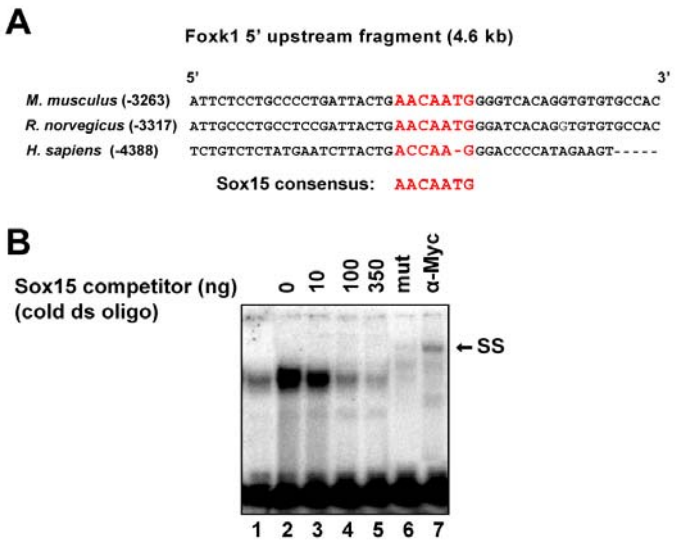


Figure 6. Sox15 binds to an evolutionarily conserved SBE located within the 5' Foxk1 upstream enhancer. A Sequence alignment of the conserved SBE located withing the mouse, rat, and human 5' *Foxk1* upstream promoters. **B** EMSA of recombinant Sox15 binding to the radiolabeled SBE located withing the mouse *Foxk1* upstream enhancer. Lane 1, untransfected (endogenous) Foxk1 protein. Lane 2, recombinant Sox15 binding to the ds oligo. Lanes 3-5, the protein-DNA binding is competed out using unlabeled "cold" ds oligo. Lane 6, mutagenesis of the SBE results in ablation of Sox15-ds oligo binding. Lane 7, α-

myc antibody supershift (SS, arrow) of myc-tagged recombinant Sox15 shifts the protein-DNA complex to run at a higher molecular weight.

Sox15 transcriptionally activates Foxk1 gene expression. I performed transcriptional assays to assess the specificity of Sox transcription factors as regulators of Foxk1 gene expression. I fused the 4.6 kb *Foxk1* promoter region to the pGL3-TATA *luciferase* reporter (Figure 7A). I then transfected C2C12 myoblasts (a cell line generated from myogenic progenitor cells) with the 4.6 kb Foxk1 promoter-*luciferase* reporter construct and Sox factors that have been reported to be expressed in the MPC population (i.e. Sox8 and Sox15) (Schmidt *et al,* 2003; Lee *et al,* 2004). I observed that Sox15, in a dose-dependent fashion, was a potent transcriptional regulator of *Foxk1* gene expression as it resulted in more than a 40-fold activation of reporter expression (Figure 7C). In contrast, Sox8 did not transcriptionally activate the *Foxk1* gene. To further examine the specificity of Sox15 transcriptional activity, I evaluated whether Sox15 was capable of activation of the *myoglobin* gene (a well characterized muscle specific promoter in our laboratory) (Garry *et al,* 1996; Garry *et al,* 1998; Grange *et al,* 2002). I observed that Sox15 is not a transcriptional activator of the 2.0 kb human *myoglobin* promoter-*luciferase* construct (Supplemental Figure 2).

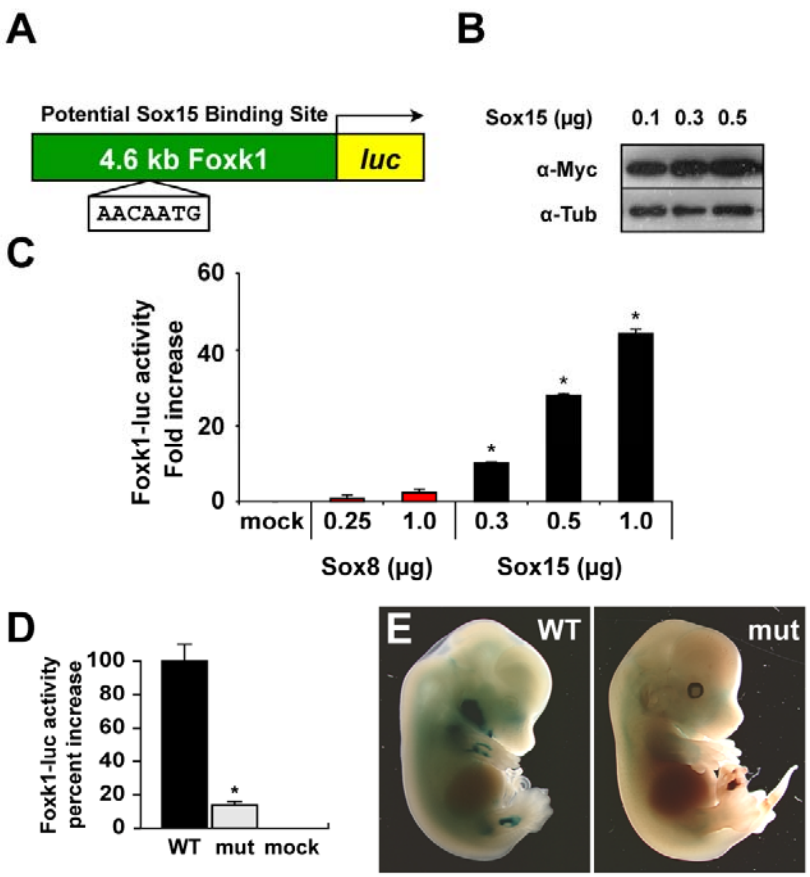


Figure 7. Sox15 transcriptionally activates Foxk1 *in vitro* and *in vivo*. **A** Schematic of the 4.6 kb Foxk1 5' upstream enhancer fused to a *luciferase* (*luc*) reporter. **B** Overexpression of myc-tagged Sox15 protein in C2C12 myoblasts. **C** Transfection of Sox8 and Sox15 in C2C12 myoblasts reveals that only Sox15 can transcriptional activate the 4.6 kb Foxk1-*luc* reporter (n = 3) * p value < 0.005. **D** Mutagenesis of the SBE results in ablation of Sox15 activation of the Foxk1-*luc* reporter. * p value < 0.01. **E** X-gal staining of wild type (WT) and SBE mutated (mut) 4.6 kb Foxk1-*lacZ* transgenic founder embryos (F_0) reveals ablation of reporter expression in the SBE mut embryos.

Mutagenesis of the Sox binding element attenuates Foxk1 gene expression. I undertook *in vitro* and *in vivo* studies to further examine whether Sox15 is an upstream regulator of the *Foxk1* gene. I mutagenized three nucleotides within the Sox binding element located in the 4.6 kb *Foxk1* promoter and performed transcriptional and transgenic analyses. Mutagenesis of the SBE in the 4.6 kb Foxk1-*luc* plasmid and cotransfection with a Sox15 expression plasmid resulted in a 90% reduction of luciferase activity in C2C12 cells compared to the wild type control (Figure 6D; n = 3 for each group). I further examined the endogenous expression of the 4.6 kb (Sox mut) Foxk1-*lacZ* construct using transgenic technologies. Mutant transgenic embryos were harvested in parallel with controls, fixed, stained with X-gal, and examined for β-*galactosidase* expression. Expression was observed in fifteen Sox mutant binding site transgenic embryos at E13.5 (out of 157 embryos screened) and all had a severe reduction of β-*galactosidase* expression in the forelimbs and hindlimbs (Figure 7E). These results further establish that Sox15 is a direct upstream transcriptional regulator of *Foxk1* expression *in vitro* and *in vivo*.

Inactivation of Sox15 results in perturbed cell cycle kinetics. In my initial efforts to further define the functional role of Sox15 in the MPC population, I utilized three distinct Sox15 siRNA double stranded oligomers to knockdown Sox15 gene expression. Sox15-specific siRNA were transfected into

asynchronously dividing C2C12 myoblasts. Forty-eight hours following transfection, I analyzed the experimental and control cells for phenotypic differences in their cell cycle kinetics and gene expression. Transfection with the RISC control (a nonfunctional, nonsense siRNA) had no effect on cellular proliferation, cell cycle kinetics or gene expression. In contrast, transfection of Sox15 siRNA resulted in a severe decrease of Sox15 mRNA. The knockdown of Sox15 expression resulted in decreased cellular proliferation as measured by BrdU incorporation (which labels cells in the S-phase of the cell cycle as a marker of cellular proliferation), impaired cell cycle kinetics (i.e. G0/G1 cell cycle arrest) and decreased Foxk1 transcript expression (Figure 8B, Figure 10A-C). No evidence of increased apoptosis was observed in the experimental vs. the control samples using FACS analysis (Figure 8A; n = 3 for each sample). The impaired cell cycle kinetics (i.e. G0/G1 cell cycle arrest) and decreased *Foxk1* transcript expression were observed using three separate double stranded oligos directed against different regions of the murine *Sox15* transcript. The G0/G1 cell cycle arrest following Sox15 knockdown was associated with an induction of *cdkn1a* (p21) but no change in *cdkn1b* (p27) transcript levels as measured by QRT-PCR analysis (Figure 10C). These results are similar to those observed in the Foxk1 mutant satellite cells (Hawke *et al*. 2003).

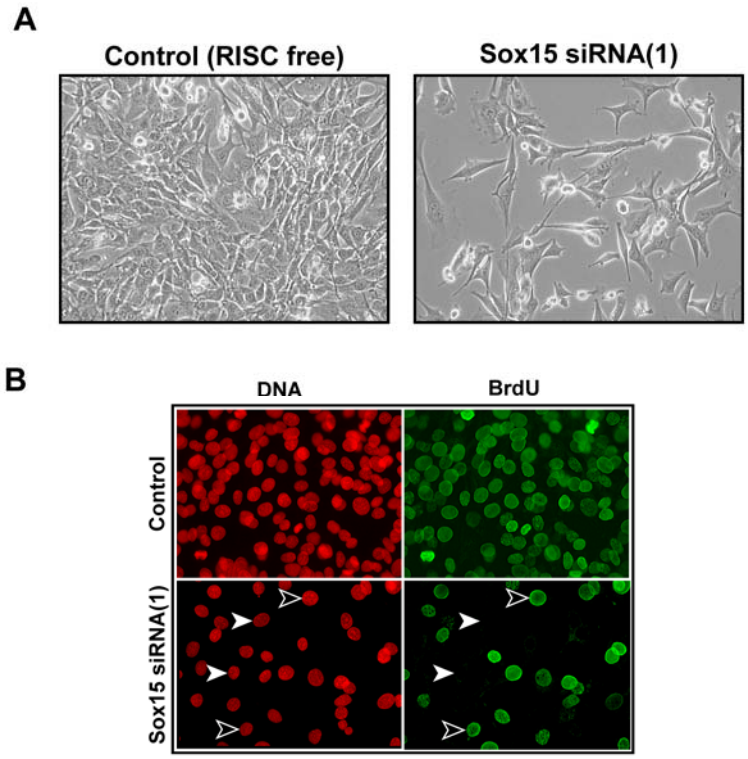
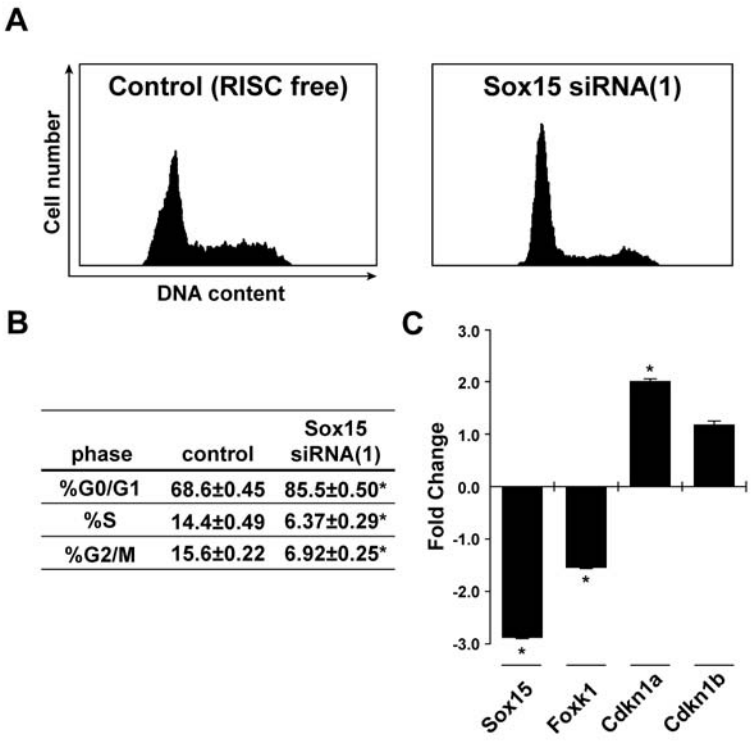


Figure 8. Knockdown of endogenous Sox15 results in decreased myoblast proliferation. **A** Phase microscopy of C2C12 myoblasts 48 hours post-transfection of either a non-sense siRNA control (RISC free) ds oligo or a siRNA directed against murine Sox15 that resulted in decreased cellular proliferation. **B** BrdU pulse labeling of cells transfected with control (top panels) or Sox15 siRNA (bottom panels). Open arrowheads indicate BrdU negative cells. Closed arrowheads indicate BrdU positive cells. DNA, red (propidium iodide). BrdU, green.



phase	control	Sox15 siRNA(1)
%G0/G1	68.6±0.45	85.5±0.50*
%S	14.4±0.49	6.37±0.29*
%G2/M	15.6±0.22	6.92±0.25*

Figure 9. Knockdown of endogenous Sox15 results in perturbed cell cycle kinetics. **A** FACS histograms of control and Sox15 siRNA transfected C2C12 myoblasts. **B** Table summary of cell cycle phase of control and Sox15 siRNA treated cells. Note that Sox15 siRNA treated cells have significantly more cells in the G0/G1 phase of the cell cycle. **C** Quantitative PCR of Sox15 siRNA treated cells demonstrates significant Sox15 transcript knockdown. Foxk1 levels also decrease, while cdkn1a (p21) levels increase. * p value < 0.005.

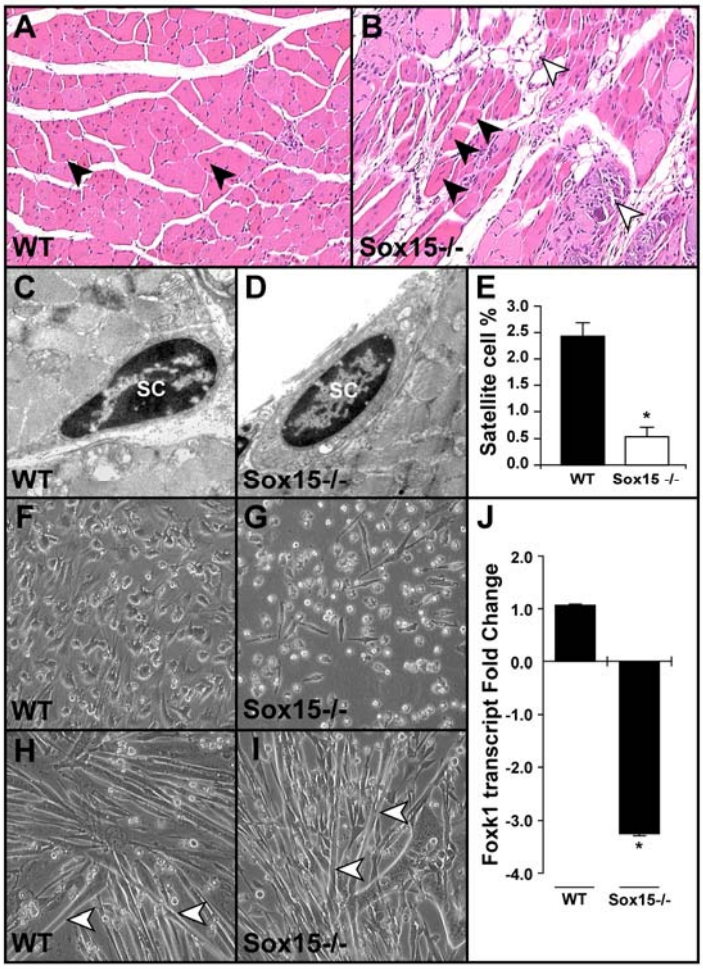


Figure 10. Sox15-/- mice have impaired muscle regeneration and have fewer myogenic progenitor cells. **A-B** WT and Sox15-/- gastrocnemis skeletal muscle 2 weeks post ctx injury. Open arrowheads demarcate regions of adipogenesis and necrosis. Closed arrowheads demarcate centrally-located myonuclei. **C-E** Sox15-/- mice have muscle satellite cells, but at fewer numbers than their WT littermates. Wild type (**F** and **H**) and Sox15-/- (**G** and **I**) primary myoblasts can form myotubes (open arrowheads). **J** Quantitative PCR reveals decreased Foxk1 transcript levels in Sox15-/- myoblasts. * p value < 0.001.

Sox15 null mice have perturbed muscle regeneration. To further examine the role of Sox15 in the MPC population, I analyzed the Sox15 deficient mice. In contrast to previous reports which analyzed the Sox15 mutant phenotype in a mixed 129/C57BL/6 strain (Lee *et al,* 2004), I undertook our studies in the pure C57BL/6 background (backcrossed over 6 generations; >95% isogenic). Two weeks following an intramuscular delivery of cardiotoxin, wild type mice had evidence of complete skeletal muscle regeneration (centronucleated myotubes which are a hallmark of regeneration) whereas the Sox15 mutant mice had a modest impairment with evidence of persistent necrosis and the replacement of myofibers with adipose tissue (Figure 10A and 10B). Using electron microscopy, I observed an 80% reduction in the number of satellite cells located within the Sox15 null tibialis anterior muscles compared to the wild type controls (Figure 10C-E; n = 3 for each sample; p value < 0.005). Sox15 mutant MPCs displayed impaired proliferation as measured by Ki67 immunohistochemical staining and a threefold decrease in *Foxk1* expression without evidence of impaired differentiation (Figure 10J). In contrast to the previous study by Lee and colleagues, Sox15 mutant primary myoblasts were capable of forming myotubes in culture (Figure 10F-I). One explanation for this result may be the increased number of cells (2 x 10^5 versus 10^5 per 35 mm plate) used in this study for differentiation assays.

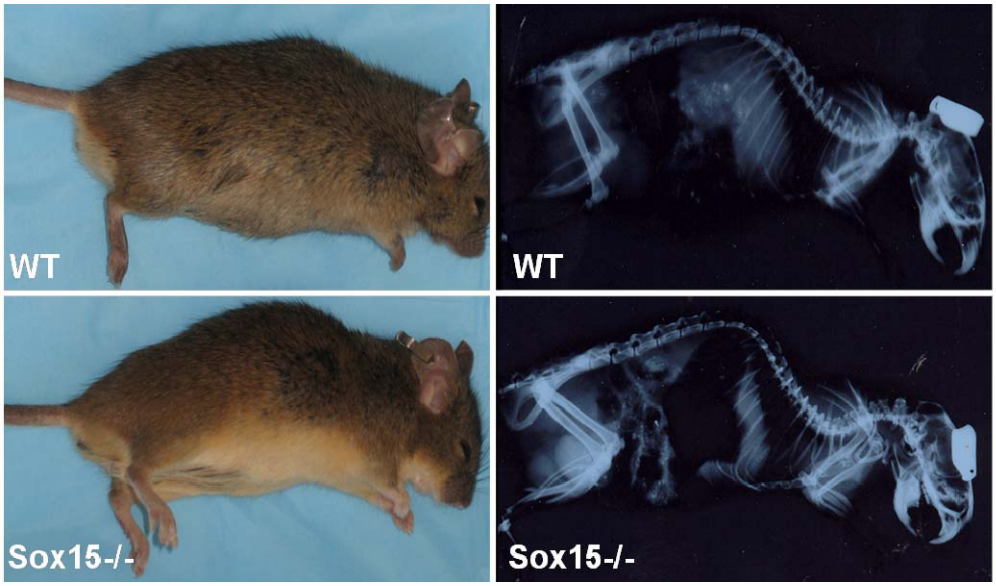


Figure 11. Sox15-/- mice develop kyphosis of the spine as they age. Wild type (top panels) and Sox15-/- (bottom panels) of 10 month old male mice reveals kyphosis of the spine (a hallmark of MPC loss). X-rays shows the kyphosis (abnormal curvature) of the Sox15-/- mutant mice, compared to their wild type littermates. Of importance to note, kyphosis is a hallmark of MPC myopathic mouse models which develop the spine curvature due to a loss of integrity of the intercostal muscles that surround the rib cage and spine.

Discussion

These data establish a role for Sox15 as a regulator of *Foxk1* expression and the myogenic progenitor cell population. The regulation of myogenic progenitor cells by Sox15 is clearly established; however, the exact nature of whether or not this regulation occurs through an exclusive Foxk1-dependent pathway remains unclear. While Foxk1 and several Foxk1-dependent genes (p21 and cdk1) are dysregulated in Sox15 mutant myoblasts, there are additional genes involved in myogenic progenitor cell migration (notably members of the CXCR signaling family) that were also dysregulated (data not shown). Given the relatively mild kyphosis phenotype of Sox15 mutant mice (Figure 11), compared to the severe growth retardation observed in the Foxk1 mutant mice, it remains likely that additional factors regulate *Foxk1* transcription or that Foxk1 is decreased in expression but not completely absent. Questions remain as to what is causing the loss of myogenic progenitor cells in the adult skeletal muscle. While Sox15-/- unperturbed skeletal muscle showed no significant amounts of TUNEL positive apoptotic myogenic progenitors, given the low frequency of quiescent myogenic progenitors normally present (1-3% of skeletal myonuclei), it is difficult to determine the turnover rate using histologically assays. Experiments utilizing single fiber isolation of myogenic progenitor cells from wild type and Sox15 mutant skeletal muscle may address these questions and are clearly warranted.

Chapter 3

Sin3a expression in skeletal muscle is essential for mouse viability and regulation of mitochondrial mass

Background

Sin3 as a Transcriptional Repressor

The *Sin3* gene was first identified in yeast in a screen for anti-supressor mutants that reduce the efficiency of non-suppressor tRNA from adding an adenine at the wobble position at the trancription terminus (Heyer *et al*, 1984). It was later revealed that Sin3 had a post-translational function in the repression of the yeast *HO* gene transcription in a reciprocal relationship with the transactivating SWI/SNF complex (Sternberg *et al*, 1987). Subsequent studies revealed that yeast Sin3 interacts with many components of transcriptional repression complexes, including the Mad1/Max complex that regulates cellular proliferation through the transcriptional regulation of critical components of the cell cycle (Kasten *et al*, 1996).

In mammals, two isoforms of Sin3 (referred to as Sin3a and Sin3b) are present and have redundant as well as unique functional roles in the regulation of gene

transcription. It was in mammalian cells that Sin3a and Sin3b were first identified as binding partners that formed a transcriptional repression complex with each other as well as with Mad to repress genes that were otherwise activated by the Myc-Mad complex (Ayer *et al*, 1995). Sin3a and Sin3b bind with equal affinity to histone deactylases (HDACs) to form large transcriptional repression complexes that tightly modulate gene transcription (Heinzel *et al*, 1997). A recent RNA interference (RNAi) study involving the knockdown of Drosophila HDACs (dHDAC1) in Schneider S2 cells revealed a strikingly similar dysregulated transcriptome to that of cells treated with RNAi against dSin3 (Foglietti *et al*, 2006). The Sin3a/b transcriptional repression complex is quite large, and novel members of the complex, often called SAPs (Sin3a/b-interacting proteins) have been recently identified by using mass-spectrometry (Fleischer *et al*, 2003).

Sin3a and Sin3b are ubiquitously expressed in a variety of cell types, yet the role of Sin3a and Sin3b in myogenic lineages remains unclear. Sin3a/b has been shown to bind directly to MyoD to regulate myogenic pathways (Bailey *et al*, 1999). In myogenic progenitor cells, our lab has previously demonstrated that both Sin3a and Sin3b can bind to Foxk1 to regulate the transcription of the cyclin-dependent kinase inhibitor cdkn1a (also referred to as p21) (Yang *et al*, 2000). While a recent paper implies a novel function for Sin3a/b as a regulator of

the somatic clock, its comprehensive function in myogenic lineages are not fully understood (Sheeba *et al*, 2007).

Sin3a/b Knockdown and Knockout Studies

Advances in transgenic technologies utilizing *in vivo* gene knockdown and knockout of Sin3a and Sin3b have revealed novel functions for this family of proteins. Loss of Sin3 in yeast results in impaired growth, shortened telomeres, and overall decreased galactose metabolism (Sternberg *et al*, 1987). Analogous to yeast, *drosophila* have only one isoform of Sin3 (*dSin3*) *dSin3* mutants are early embryonic lethal due to massive defects in the patterning of the dentical bands and body walls (Pennetta and Pauli, 1998).

Recently, two groups utilized conditional mouse knockout approaches to globally delete Sin3a, these studies revealed a broad role for Sin3a in early embryogenesis and chromosomal segregation (Dannenberg *et al*, 2005; Cowley *et al*, 2005). Global removal of Sin3a results in embryonic lethality by E5.5 due to massive defects in gastrulation and chromosomal breakage. Conditional deletion of Sin3a in T-lymphocyte lineages through the use of lck-*cre* transgenic mice, resulted in massive T-lymphocyte apoptosis and complete disruption of T-lymphocyte homeostasis (Cowley *et al*, 2005). Additional analysis of Sin3a-

deficient mouse embryonic fibroblasts revealed dysregulation of a large number of genes involved in the regulation of mitochondrial respiration and fatty acid oxidation/synthesis.

Rationale and Aim

As mentioned above, Sin3a is a direct interacting partner with Foxk1 in the MPC population. However, while it is a known general component of a large transcriptional repression complex, its role in skeletal muscle has not been elucidated. To define a functional role for Sin3a in skeletal muscle, I utilized the Sin3a conditional knockout mouse combinatorially mated to the Myo-*cre* transgenic line, and characterized its phenotype. Using a *cre* transgenic line that is expressed in early myogenic progenitors, I am able to examine the role of Sin3a at a critical developmental time point in embryonic myogenesis. Additionally, I will be able to determine if whether or not Sin3a plays a role in the fate determination of MPCs, or similarly to Foxk1, MPC maintance in neonatal and/or adult myogenesis. Together, these studies will additionally determine if Sin3a plays a functional role in chromosomal stability, glycolysis, or both in skeletal muscle development.

Materials and Methods

Transgenic Mice. Sin3a conditional knockout mice (mice homozygous for the targeted alleles referred to as $Sin3a^{L/L}$) were a generous gift from R. DePinho and were generated as previously described (Dannenberg *et al*, 2005). Exon 4, containing the PAH2 domain essential for Sin3a's ability to function as a transcriptional repressor, was floxed with *loxP* sites. The resulting cross with a *cre* recombinase line results in a non-functional truncated protein (Dannenberg *et al*, 2005). $Sin3a^{L/L}$ *cre* recombinase negative littermates were used as controls for all experiments.

Myo-*cre* transgenic mice were a generous gift from E. Olson and were generated as previously described (Li *et al*, 2005). The Myo-*cre* transgenic line consists of a *Mef2c* skeletal muscle enhancer and a minimal *myogenin* promoter fused to *cre* recombinase. *Cre* recombinase negative littermates were used as controls for all experiments. Myo-*cre* mice were mated to the *Rosa26R* transgenic line (a generous gift from M. Tallquist) and have been described elsewhere (Soriano, 1999). The *Rosa26R* construct consists of a β-*galactosidase* open reading frame interrupted by *loxP* sites. Upon expression of *cre* recombinase, the resulting *loxP* excision produces an in-frame, functional lacZ protein that can be detected using X-gal immunohistochemistry.

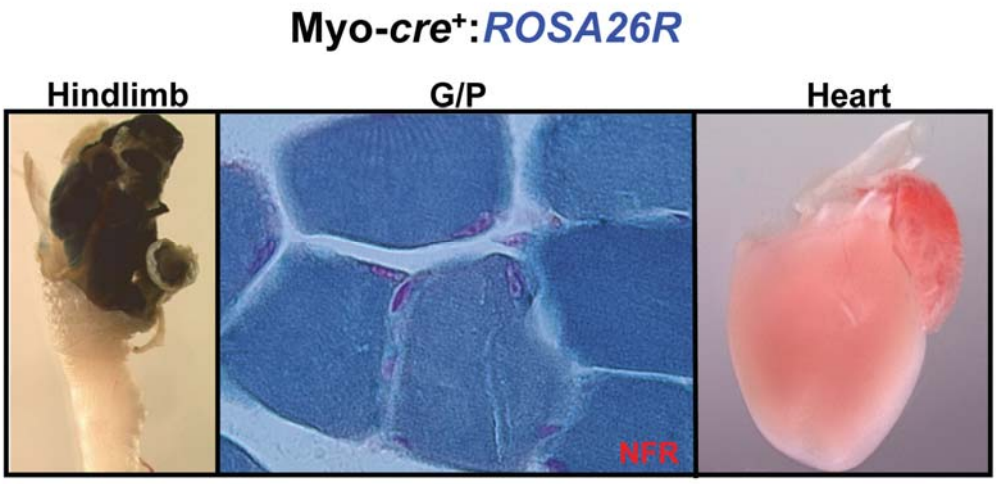


Figure 12. Myo-*cre* transgenic line expression is restricted to skeletal muscle lineages. (Left Panel) Neonatal hindlimb skeletal muscle taken from a P2 Myo-cre^{+}:Rosa26R^{+} pup. (Middle Panel) G/P skeletal muscle X-gal and nuclear fast red (NFR) stained reveals expression in all skeletal myonuclei as evident by the purple (blue and red) coloration. 63x magnification. (Right Panel) No expression of the Myo-*cre* transgene observed in the heart.

Whole Mount Preparation of Mouse Embryos. Mice were mated and observed for vaginal plugs. The indication of a vaginal plug represented an embryo staged at E0.5. Embryos less than E16 were fixed in 0.2% glutarahldehyde/1xPBS overnight at 4^{0}C, followed by 3 washes in 1xPBS for 5

minutes per wash. Yolk sacs were washed 3 times for 5 minutes in 1xPBS and digested in proteinase K (Sigma) for three hours for genotyping purposes. Embryos staged older than E16 were first de-skinned, and then fixed overnight in 0.2% glutarahldehyde/1xPBS overnight at 4^0C, followed by 3 washes for 5 minutes in 1xPBS. Tail clippings were digested overnight in proteinase K, and used for genotyping embryos greater than E16. Embryos were then embedded and sectioned as previously described (Meeson *et al*, 2007).

Transmission Electron Microscopy. Sin3a$^{L/L}$:Myo-*cre*$^{+}$ and Sin3a$^{L/L}$ neonatal pups were harvested 2 hours following birth, decapitated, deskinned, and fixed overnight in a mixture of 5% paraformaldehyde/0.2% glutaraldehyde/0.2M cacodylate buffer (pH 7.4) (Sigma) overnight at 4^0C. Hindlimbs, thoracic sections, and whole diaphragms were removed the next day for submission to the UTSW Electron Microscopy core. Samples were then dehydrated in a series of ethanol washes, embedded in Spurr resin, polymerized, sectioned and placed on copper grids. Sections were examined using a JEOL 1200 EXII transmission electron microscope (TEM). MPCs were quantified using criteria as outlined previously (Garry *et al,* 1997; Hawke *et al,* 2003).

Histological Sections. Embryonic and neonatal Sin3a$^{L/L}$:Myo-*cre*$^{+}$ and Sin3a$^{L/L}$ mice were harvested as described above, and fixed overnight in 4%

paraformaldehyde at 4^0C. Gastrocnemis/Plantaris (G/P) muscle groups were then dissected from neonatal pups; whereas, whole embryos were submitted intact for sectioning. Samples were washed 3 times in 1xPBS the following day, before being submitted for processing by the UTSW Histological Core Facility. Samples were then dehydrated through a series of alcohols, embedded in paraffin, sectioned on a microtome, and placed on frosted slides. Sections were stained with hematoxylin and eosin (H&E, Sigma) or nuclear fast red (NFR, Sigma), or light eosin (Sigma) using standard techniques. Images of all slides were taken on an Optronics digital camera and refined in Adobe Photoshop CS2.

cDNA Microarray Amplification/Analysis. Gastrocnemis/Plantaris skeletal muscles from Sin3a$^{L/L}$:Myo-*cre*$^{+}$ and Sin3a$^{L/L}$ 2-hour-old neonatal pups were placed in cryotubes and snap-frozen in liquid nitrogen. Samples were then homogenized and total RNA was extracted using Tripure (Roche) following the manufacturer's protocol. 1 µg of total RNA was labeled and amplified using the Affymetrix Gene Expression cDNA oligo kit. Samples were run on Affymetrix MGU74v2 chips, and analyzed using the Affymetrix dChip software (adapted from Goetsch *et al*, 2003). Fold changes were calculated using the dChip analysis software, and only genes present in both samples, with an expression value greater than .99 were considered significant (Masino *et al*, 2004).

Western Blots. Gastrocnemis/plantaris skeletal muscle from Sin3a$^{L/L}$:Myo-*cre*$^{+}$, Sin3a*L/*+:Myo-*cre*$^{+}$, and Sin3a$^{L/L}$ 2 hour old pups was extracted, homogenized, and boiled in SDS loading dye at 100^{0}C for 5 minutes. 20 µg of total protein was run out on a 7.5% SDS polyacrylamide gel. Samples were incubated with either anti-Sin3a (rabbit polyclonal, Santa Cruz, 1:1000 dilution) or anti-tubulin (mouse monoclonal, Sigma, 1:2000 dilution) antisera overnight at 4^{0}C on a shaker. Nitrocellulose membranes (GE Healthcare/Amersham) were then incubated with horse-radish peroxidase secondary antiseras (Pierce, 1:200 dilution), and then incubated with chemoluminescent substrate (Pico Substrate Kit, Pierce). Membranes were then exposed on Kodak blue film as per the manufacturer's instructions.

Results

The Myo-*cre* transgene is expressed in all myogenic progenitor lineages. Before determining the role of Sin3a using the *in vivo* Sin3a conditional knockout mouse model, we first needed to identify a mouse line that was capable of expressing *cre* recombinase in all myogenic progenitor lineages. The two widely used skeletal muscle specific lines available, *human skeletal actin* promoter (HSA-*cre*) and muscle creatine kinase promoter (MCK-*cre*), express *cre* recombinase in differentiated muscle fibers and not in the myogenic progenitors (Nicole *et al*, 2003; Huh *et al*, 2004). A MyoD early enhancer (MyoD-*cre*) and the full *Mef2c* promoter (Mef2c-*cre*) transgenic lines were recently generated; however, they could not be obtained for my studies (Chen *et al*, 2005; Heidt and Black, 2005). A Myf5-*cre* "knock-in" transgenic line exists and expression is observed in myogenic progenitors, but *cre* recombinase expression is also present in the brain, vasculature, and other non-myogenic cell types (Tallquist *et al*, 2000). At the time of the writing of this dissertation, another skeletal muscle *cre* transgenic line consisting of the murine *Lbx1* promoter fused to *cre* recombinase (Lbx1-*cre*) was recently generated and its expression appears to be restricted to myogenic progenitors and branchial arch derivatives (Vasyutina *et al*, 2007). I was able to obtain a Myo-*cre* transgenic mouse line from the Olson

lab, which is comprised of a skeletal muscle-specific *Mef2c* enhancer and a minimal myogenin promoter fused to *cre* recombinase.

The Myo-*cre* transgenic mouse line has β-*galactosidase* expression in differentiated myogenic lineages as previously described (Li *et al*, 2005). To confirm those previous studies and to determine if the Myo-*cre* line expression was ubiquitous throughout the skeletal muscle, I mated the Myo-*cre* mice to the *Rosa26R* transgenic mouse line. The *Rosa26R* line consists of a *loxP*-STOP-*loxP* construct in which β-*galactosidase* is expressed upon *cre*-mediated excision of the STOP insertion (Soriano, 1999). I first confirmed embryonic expression of the Myo-*cre* transgene by harvesting Myo-*cre*$^{+}$:*Rosa26R* embryos at embryonic time points E9.5, E10.5, and E13.5. X-gal histochemical staining confirmed expression solely in the somites at E9.5 and E10.5. At E13.5 expression of the Myo-*cre* transgene is observed in the somites, brachial arches, limb muscles, and somatic derivatives, but as predicted is completely absent in the heart (Figure 12).

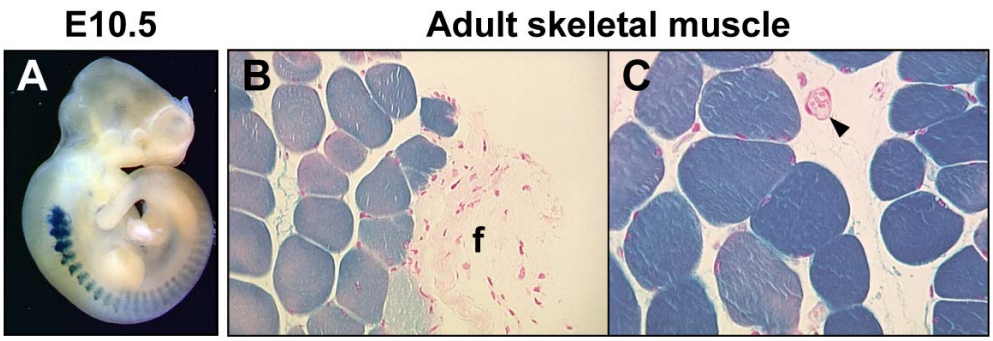


Figure 13. The Myo-*cre* transgene is expressed exclusively in skeletal muscle. **A** E10.5 Myo-*cre*$^{+}$:*Rosa26R*$^{+}$ embryo x-gal stained revealing expression restricted to the somites. 25x magnification. **B-C** Adult TA Myo-*cre*$^{+}$:*Rosa26R*$^{+}$ skeletal muscle reveals no X-gal staining expression of the Myo-*cre* transgene in the adjacent adipocytes (f). **C** Soleus X-gal stained skeletal muscle reveals that expression is restriced to the myofibers, and not the intermingled epithelial-derived blood vessels (arrowhead).

To examine the expression of the Myo-*cre* transgene in adult skeletal muscle, I isolated gastrocnemis/plantaris skeletal muscle from 4-month-old Myo-*cre*$^{+}$:*Rosa26R* male mice. X-gal histochemistry revealed expression of the transgene throughout the neonatal skeletal muscle, including in the nuclei co-stained with nuclear fast red (NFR) (Figure 13). Expression was observed in all myogenic nuclei; however, no expression of the transgene was observed in any

of the vessels or the adult heart. It is important to note that I did not test whether or not expression of the Myo-*cre* transgene was observed earlier than E9.5 or whether it was present in early $Pax3^{+}/Pax7^{+}$ myogenic progenitors. It is possible, that some $Pax3^{+}/Pax7^{+}$ myogenic progenitors arising from the dermyotome between E10.5-E11.5 did not express the transgene and whose myogenic derivatives were not observed in the adult skeletal muscle sections. Together, these studies establish the Myo-*cre* transgenic line as a useful tool for genetic deletional strategies to target the entire skeletal muscle, including myogenic progenitors and their derivatives.

Sin3a expression in skeletal muscle is essential for mouse viability. To determine the functional role of Sin3a in skeletal muscle, I mated Sin3a conditional knockout mice to the Myo-*cre* transgenic line to obtain Sin3a skeletal muscle knockout mice ($Sin3a^{L/L}$:Myo-cre^{+}). Previous studies have demonstrated that the global removal of Sin3a results in massive chromosomal abnormalities and embryonic lethality by E5.5 (Dannenberg *et al*, 2005; Cowley *et al*, 2005). Global haploinsufficiency of Sin3a results in massive apoptosis of T-lymphocyte progenitors and indicates a possible Sin3a dosage requirement (Cowley *et al*, 2005). $Sin3a^{L/L}$:Myo-cre^{+} pups were viable immediately after birth and showed no visible defects in skeletal muscle formation throughout embryonic gestation, virtually indistinguishable from their $Sin3a^{L/L}$ *cre* recombinase negative

littermates. However, approximately four hours following birth, the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups were cyanotic and quickly expired. In over eight separate litters from different breeders, no Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups were viable. However, all Sin3a*L/*+:Myo-*cre*$^{+}$ pups showed no defects and had normal lifespans (data not shown).

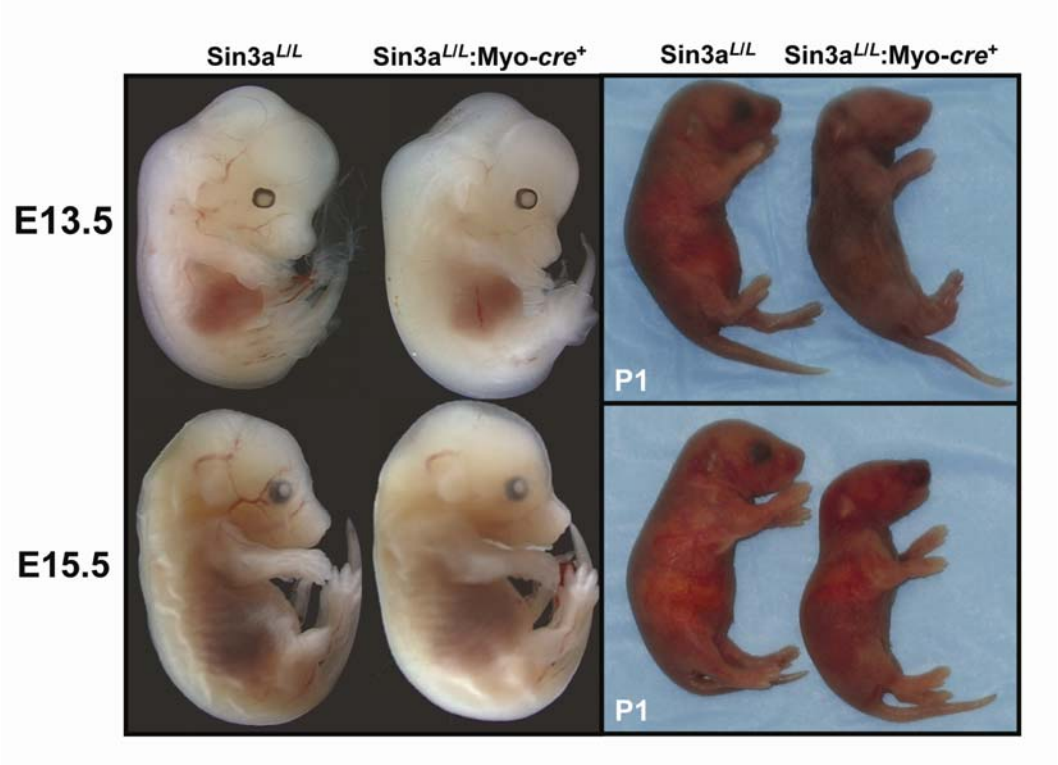


Figure 14. Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups are neonatal lethal. (Left Panels) E13.5 and E15.5 Sin3a$^{L/L}$:Myo-*cre*$^{+}$ and Sin3a$^{L/L}$ embryos reveal no striking abnormalities. (Right Panels) Top panel shows a P1 Sin3a$^{L/L}$ and an expired Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pup 12 hours following birth. Bottom panel shows a P1 Sin3a$^{L/L}$ and a Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pup 2 hours after birth. Note the cynanotic craniofacial regions in the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pup.

Two hours following birth, Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups were ambulatory and had lactate in their stomachs; thereby, excluding the possibility that they starved to death due to an inability to suckle from their mothers (Figure 14). I then examined the histology of the skeletal muscle groups from both expired Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups and pups that were two hours old and still viable. Surprisingly, the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ had intact diaphragms and hindlimb muscles that were histologically indistinguishable from the Sin3a$^{L/L}$ littermates controls (Figure 15A-D) I confirmed the loss of Sin3a in hindlimb muscle by Western blot analysis of the gastrocnemis/plantaris muscle from a two-hour-old litter, which showed no detectable levels of Sin3a (Supplemental Figure 3). I then performed a TUNEL assay, which detects DNA breakage and is a cellular apoptotic indicator, to determine the myogenic cell viability in Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups. The majority of the skeletal muscle of the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups was TUNEL negative; however there was a significant number of TUNEL positive cells and visible myonecrosis around the myotendonous junctions of the rib cage (Figure 15E-H). Thus, while there appeared to be significantly more myonecrosis at the myotendonous junctions, it was unclear whether or not this was the cause of lethality in the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups.

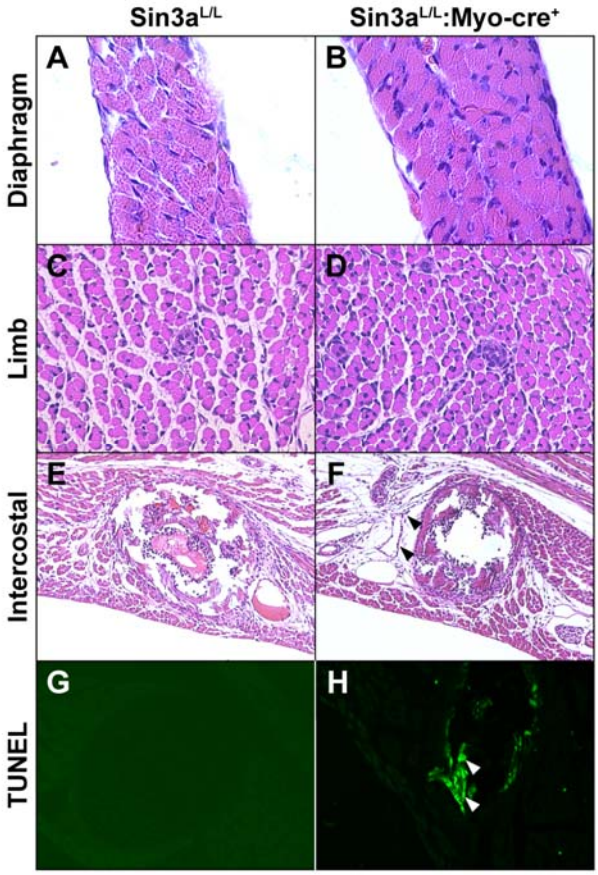


Figure 15. Sin3a-deficient pups have increased necrosis at the myotendonous junctions. (A,C,E,G) (Sin3a$^{L/L}$) and **(B,D,F,H)** (Sin3a$^{L/L}$:Myo-*cre*$^{+}$) histological sections taken from 3-hour-old pups. **A-D** Diaphragm and hindlimb H&E sections reveal that both wild type and Sin3a-muscle knockout mice have histologically unremarkable skeletal muscle. **E** and **F** Intercostal H&E sections of wild type and Sin3a-muscle knockout mice reveal increased myonecrosis at the myotendonous junctions (closed arrowheads). **G and H** TUNEL histochemistry of the same sections reveal increased amounts of TUNEL^{+} nuclei (open arrowheads) at the myotendonous junctions.

Sin3a regulates mitochondrial mass and is essential for myofiber congruity in skeletal muscle. To further examine the cause of lethality in Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups, I utilized transmission electron microscopy (TEM) to examine the ultrastructure of the Sin3a-deficent muscle. Surprisingly, Sin3a skeletal muscle-deficient pups had abnormally large mitochondria throughout their hindlimb and diaphragm muscles (Figure 16A). In both longitudinal and transverse sections, the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ skeletal muscle lacked uniform ultrastructure due to large-sized mitochondria that disrupted myofiber congruity (Figure 16B). Additionally, there was a significant increase in the total number of mitochondria per field in the Sin3a$^{L/L}$:Myo-*cre*$^{+}$ skeletal muscle compared to littermate controls. A previous study analyzing the transcriptome of Sin3a-deficent mouse embryonic fibroblasts revealed a role for Sin3a in regulating various mitochondrial functions including glycolysis/respiration and mitochondrial biogenesis (Dannenberg *et al*, 2005). An additional study involving the siRNA knockdown of *Drosophila* Sin3 (*dSIN3*) in S2 cells also resulted in abnormally large mitochondria (Pile *et al*, 2003). Thus, my studies support the theory that Sin3a is essential for regulating mitochondrial mass and possibly mitochondrial biogenesis as well.

Gene Symbol	Gene	Dysregulated in Sin3a KO muscle?	Dysregulated in Sin3a KO MEFs?
Acsl1	acyl-CoA synthetase 1	Yes	Yes
Ctps2	cytidine 5'-triphosphate synthase 2	Yes	Yes
Fabp5	fatty acid binding protein 5, epidermal	Yes	Yes
Hsd17b4	hydroxysteroid (17-beta) dehydrogenase 4	Yes	Yes
Nr2f2	nuclear receptor subfamily 2, group F, member 2	Yes	Yes
Scarb1	scavenger receptor class B, member 1	Yes	Yes

Table 2. Sin3a is essential for the regulation of several genes involved in mitochondrial mass and synthesis. Comparison of mitochondrial related genes dysregulated in Sin3a-deficient skeletal muscle verse Sin3a-deficient mouse embryonic fibroblasts (MEFs). Sin3a-deficient MEFs previously described in Dannenberg *et al*, 2007.

Sin3a is essential for regulating the mitochondria transcriptome in skeletal muscle. To determine what mitochondrial genes were dysregulated in the Sin3a-deficient skeletal muscle, we harvested two-hour old gastrocnemius/plantaris muscles from Sin3a$^{L/L}$:Myo-*cre*$^{+}$ and Sin3a$^{L/L}$ pups and extracted RNA to make cDNA for oligonucleotide microarrays.

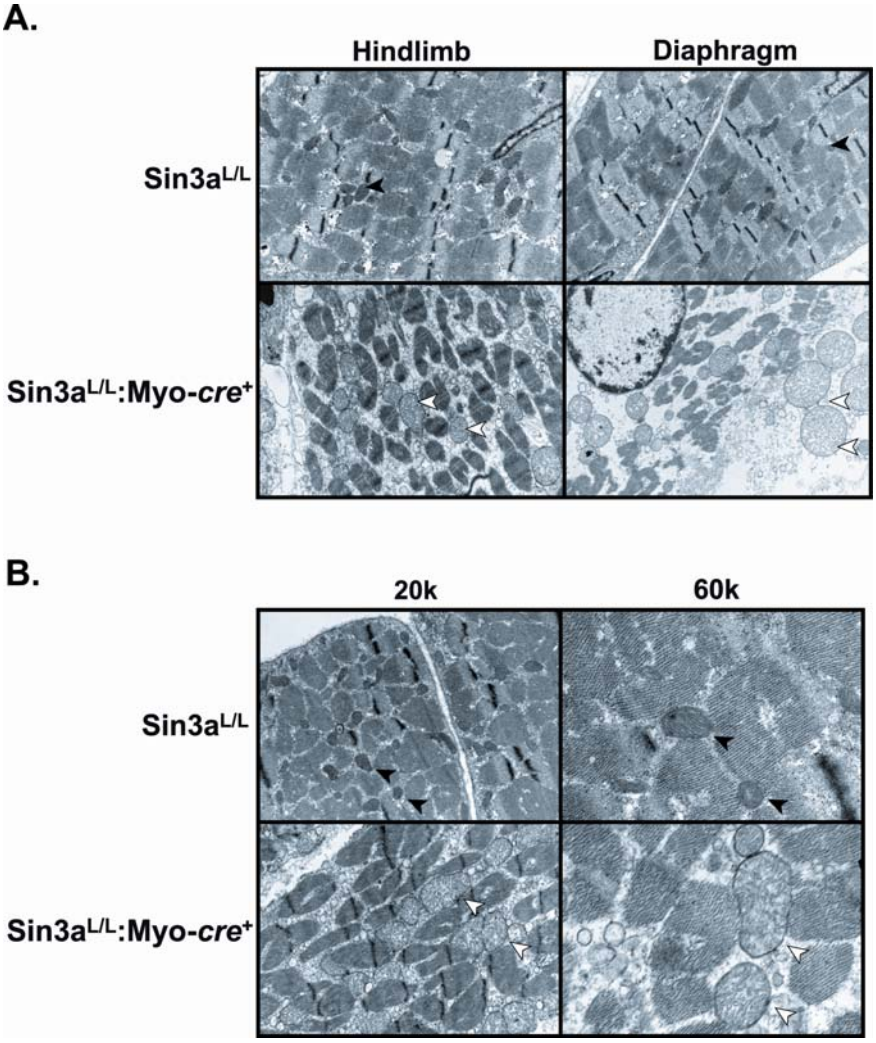


Figure 16. Sin3a is an essential regulator of mitochondrial mass in skeletal muscle. A TEM of Hindlimb and diaphragm muscles taken from a P1 Sin3a$^{L/L}$ and Sin3a$^{L/L}$:Myo-*cre*$^{+}$ 2-hour-old litter. Note the loss of myofiber congruity in the Sin3a-deficient skeletal muscle. 15,000 (15k) magnification. **B** High resolution magnification of Sin3a$^{L/L}$ and Sin3a$^{L/L}$:Myo-*cre*$^{+}$ hindlimb skeletal muscle reveals abnormally large mitochondria (open arrowheads) as compared to normal mitochondria (closed arrowheads).

3423 genes were significantly (greater than 2-fold) upregulated in Sin3a-deficient skeletal muscle; whereas a surprising 3659 genes were downregulated. Although Sin3a functions as a transcriptional repressor, one explanation is that it is likely that the downregulation of over 3600 genes in the Sin3a-deficient skeletal muscle that results in the upregulation of various transcription factors in an indirect manner. A known direct downstream target gene of Sin3a, the cyclin dependent kinase inhibitor Trp53 (also referred to as p53), was downregulated 1.5 fold in the Sin3a-deficient muscle. The mitochondrial metabolism gene *acyl-CoA synthetase 1* (*Acsl1*), an essential regulator of fatty acid synthesis and also upregulated in Sin3a-deficient mouse embryonic fibroblasts, was upregulated 10 fold in Sin3a-deficient skeletal muscle (Dannenberg *et al*, 2005). Other mitochondrial-related genes that were significantly dysregulated in Sin3a-deficient muscle and Sin3a-deficient mouse embryonic fibroblasts include: *Nr2f2* (-9.6 fold), *Ctps2* (-5.4-fold), *Scarb1* (-11.4-fold), *Hsd17b4* (-3.3-fold), and *Fabp5* (-11.9-fold) (Table 2). Given the abnormally large mitochondrial mass and numbers observed in Sin3a-deficient skeletal muscle, it is likely that the severe dysregulation of the mitochondrial transcriptome can account for a significant portion of the phenotype observed.

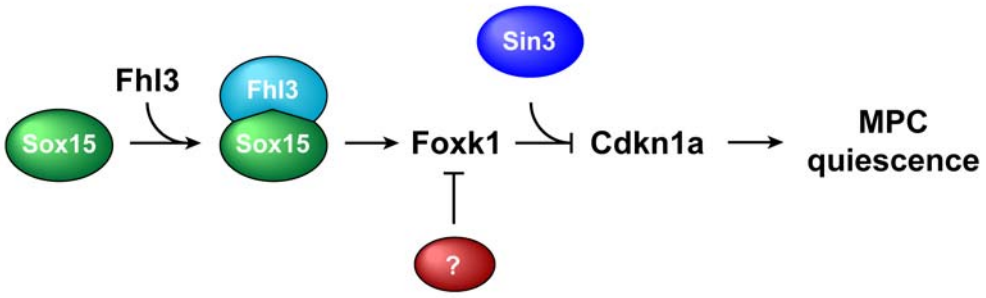


Figure 17. Proposed model for Sin3a function in the regulation of MPC quiescence. Schematic illustrating Foxk1 function in the regulation of Cdkn1a and the cell cycle kinetics of the MPC population. Fhl3 binds to Sox15 to transcriptional activate Foxk1 transcription. Foxk1 then binds to the co-repressor Sin3 (Sin3a/b) to repress *Cdkn1a* (*p21*) transcription. Additional factors (?) may repress Foxk1 transcription in the MPC population. Repression of Cdkn1a levels results in the maintenance of the MPC population in a quiescent state under unperturbed conditions.

Discussion

The loss of Sin3a in skeletal muscle lineages has a deleterious effect on mouse viability as Sin3a-skeletal muscle knockout mice expire approximately four hours after birth. Sin3a-deficent skeletal muscle have abnormally large mitochondria, which may be a result of a severely dysregulated mitochondrial transcriptome. siRNA knockdown of Sin3a in C2C12 myoblasts results in a cell cycle arrest; however, I did not observe any significant changes in the numbers of $Ki67^{+}$ (a marker of proliferating cells) nuclei in the Sin3a-deficient skeletal muscle (data not shown). Sin3a has been shown to repress the transcriptional activation of the p21 gene through the formation of a repression complex with Foxo1, Foxk1, HDAC1/2, and other factors at an evolutionarily conserved *forkhead* binding site located within the p21 5' upstream promoter (Dannenberg *et al*, 2005; unpublished data). Intriguingly, p21 levels in Sin3a-deficient skeletal muscle appear to be only slightly elevated (+1.2-fold). One explanation for this finding may be that Sin3b upregulation may compensate for the loss of Sin3a at specific gene promoters. For example, HDAC1 and HDAC2 cardiac-specific knockout mice have no observable phenotypes; however, the HDAC1/2 double cardiac-specific knockout mice suffer cardiac arrhythmias and expire in the weeks following their birth (Montgomery *et al*, 2007). Indeed, Sin3b is upregulated (+1.7 fold) in the Sin3a-deficient skeletal muscle and may be functionally redundant

with Sin3a in skeletal muscle. However, given the large number of dysregulated genes in the Sin3a-deficent skeletal muscle, it remains likely that the phenotype observed in the Sin3a skeletal muscle knockout mice is independent of a cellular proliferation defect.

In vivo chromatin immunoprecipitation assays followed by oligonucleotide microarrays (ChIP:chip) using endogenous Sin3a antibodies would address the question of what genes does Sin3a transcriptionally regulate under normal growth and regeneration conditions. Additional questions remain as to the exact mechanism of transcriptional repression of Sin3a on gene promoters. Does Sin3a recruit partner proteins to modulate the transcriptional activity of its cofactors or is it an autonomous repressor function for Sin3a? Generation of Sin3b conditional knockout mice and the subsequent Sin3a/b double conditional skeletal knockout mice will enhance our knowledge of the *in vivo* functions of Sin3 proteins during periods of myogenesis and skeletal muscle regeneration (Figure 17).

Chapter 4

Characterization of the Novel Murine Forkhead Foxj3

FoxJ Transcription Factors

The FoxJ subfamily of mammalian *forkhead* transcription factors consists of three separate members: Foxj1 (previously referred to as Hfh-4 and FKHL13), Foxj2 (previously referred to as FHX), and the most recently identified *forkhead* Foxj3. In addition to their *forkhead* DNA-binding domains, FoxJ proteins all share a common glycine-proline rich carboxyl-terminus transactivation domain (also referred to as the "acid blob") that is essential for their transcriptional activity (Pérez-Sánchez *et al*, 2000a). Gal4-DNA binding assays have determined that FoxJ proteins appear to function as transcriptional activators *in vitro*; however, recent *in vivo* studies have expanded their functions to act as transcriptional repressors depending on post-translational modifications (Lin *et al*, 2004). It remains a likely hypothesis that, similar to other *forkhead* proteins, FoxJ proteins undergo changes in their phosphorylation and acetylation status that modifies their functional activity as modulators of gene transcription.

Foxj1

The first *forkhead* of the FoxJ family identified was Foxj1 in a hepatocyte library screen for cDNAs that had homology to the FoxA *forkhead* subfamily (Murphy *et al*, 1997). Foxj1 is ubiquitously expressed; however, there is strong enrichment in the adult kidney, liver, lung cilia, and brain (Whitsett and Tichelaar, 1999). Mice lacking Foxj1 expire immediately after birth due to respiratory failure (Chen *et al*, 1998). Remarkably, Foxj3-deficient newborn pups have *situs invertus* in which their body organs are found on the opposite side of where they normally lie (Brody *et al*, 2000). The respiratory failure phenotype observed in Foxj1-/- mice is a result of impaired ciliogenesis resulting from the absence of the normal 9 + 2 cilia microtubule patterning which is replaced by a 9 + 0 pattern. The subsequent disruption of normal ciliogenesis may subsequently result in the abnormal transcription factor gradient as it has been demonstrated that the “beating” of cilia positions specific cell populations to their appropriate location within the morphogen gradient (Nonaka *et al*, 1998). Ciliogenesis and its regulation by Foxj1, is essential for the normal gradients of transcription factors during gastrulation.

Foxj2

Foxj2 (previously referred to as Fhx) was initially identified in a screen for novel *forkhead* transcription factors highly expressed in the liver (Pérez-Sanchez *et al*, 2000b). Foxj2 is ubiquitously expressed and is enriched in adult skeletal muscle, ovaries, testis, B- and T-lymphocytes, and in the brain (Pérez-Sanchez *et al*, 2000a). Foxj2 is highly expressed as early as the 8 cell embryo, and throughout the development of the blastocyst and inner cell mass (ICM) (Granadino *et al*, 2000). A recent paper from Wijchers and colleagues characterized strong expression of Foxj2 in spatially-restricted regions of the adult brain, which include the hippocampus, dentate gyrus, cerebellum, and the granular cell layer (Wijchers *et al*, 2006). More recently it has been reported that Foxj2 is strongly expressed in the nuclei of cardiomyocytes and is dysregulated during human heart failure, making it a potential target for cardiac hypertrophy therapeutic treatments (Hannenhalli *et al*, 2006).

Foxj3

Foxj3 is the most recent family member of the FoxJ forkhead subfamily identified in a genomic database screen for uncharacterized *forkhead* genes (Landgren and Carlsson, 2004). *In situ* hybridization revealed strong expression of Foxj3 in

the neural crest, neuroectoderm, limb buds, somites, and somatic derivatives. Orthologs of the Foxj3 gene are found in zebrafish, which have a gene duplication for Foxj3 (Hubbard *et al*, 2007). Given the recent evolution of the Foxj3 gene, it is likely that it functions to regulate a specific cellular population at a specific developmental time point.

Rationale and Aim

The final aim of my dissertation involved the generation and subsequent characterization of Foxj3-deficient mice, along with the characterization of Foxj3's role as a modulator of gene transcription specifically within the confines of myogenic-derived lineages. Foxj3's high expression in the adult heart and enriched expression in the developing limb buds as opposed to other novel *forkhead* factors which were enriched in other tissues. Foxj3 had previously been enriched in $MyoD^{+}$ somites in a cDNA microarray data experiment that our lab had previously conducted (Goetsch *et al*, 2003). The subsequent characterization of Foxj3 and its uncertain functional role in myogenesis, made it an ideal candidate for further study.

Materials and Methods

Transgenic Mice. Foxj3 mutant mice were generated from the BayGenomics™ Foxj3-trapped ES-cell line (XL913) of the129P2OlaHsd strain (Stryke *et al*, 2003). After ES cell expansion and sequence confirmation, the Foxj3-trapped ES cells were injected into E3.5 blastocysts, and chimeric mice were born to foster mothers. Offspring mice that were greater than 90% chimeric were then mated to both C57/B6J (Jackson Laboratory) and the 129P2OlaHsd (Harlan) to ensure germline transmission. The subsequent F2 generation of Foxj3+/- mice were then backcrossed for six generations to ensure >95% isogenicity and eliminate any strain variances. All experiments were conducted in the C57/B6J background. Unless specified, wild type littermates from Foxj3 heterozygote crosses were used for all experiments (See supplemental materials and methods for genotyping primers).

Mice were kept on a 4% fat irradiated chow (Harlan) diet, and exposed to standard 12-hour light/dark cycles. Mouse weights were recorded on a Mettler PE 200 scale. Mice were housed in a sterile, pathogen-free animal facility. All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center at Dallas.

Wholemount preparation of embryos. Mouse embryos were staged, harvested, and immersion fixed in cold 4% paraformaldehyde (30 min to 60 min based on gestational age) as previously described (Meeson *et al*, 2007). Staging of the embryos was performed by counting the presence of the vaginal plug as day 0.5 after conception, the number of somites and the presence of developmental anatomical features. Embryos were obtained from Foxj3+/- by wild type matings. Following fixation, embryos were washed in PBS and stained overnight at 37^0C for β-galactosidase expression using a standard lacZ staining protocol. Embryos were then washed, post-fixed overnight at 4^0C in 4% paraformaldehyde and photographed utilizing an Olympus dissection microscope and Optronics VI-470 CCD camera. Images were captured with Scion imaging software and image processing was completed using Adobe Photoshop CS2 prior to paraffin embedding and histological analysis.

Immunohistochemistry of Histological Sections. All mouse tissues were perfusion fixed in 4% paraformaldehyde overnight at 4^0C, and washed in 1xPBS before sectioning. Sections were stained with hemotoxylin and eosin, nuclear fast red, and other antiseras as previously described (Meeson *et al,* 2007).

TUNEL assays were done using the Dead End Fluorometric TUNEL System (Promega), as per the manufacturer's instructions. TUNEL sections were costained with propidium iodide (Molecular Probes) and other molecular markers.

Skeletal muscle tissues for fiber type analysis was performed following cryoembedding of tissues in OCT (Tissue Tek) and Gum Tragacanth (Sigma) mix as previously described (Meeson *et al,* 2007). The protocol for metachromatic ATPase fibertyping has been previously described (Garry *et al,* 1996).

Primers. All primers used in this study were from Operon, and RT-PCR primer sequences can be found in Supplemental Table 3.

Plasmids. The murine Foxj3 gene (NM_172699; NCBI Gene Bank) consisting of an 1869 base pair open reading frame was cloned into the pCDNA3.1-NT-GFP (Invitrogen), pM1 (Gal4 vector) and the pCDNA3.1-N-myc plasmids using standard techniques. A portion of Foxj3 that contained the amino terminus and the DNA-binding domain ($Foxj3_{1-176}$) was cloned in frame in the same expression plasmids. The Gal4-UAS-luciferase system has been previously described (McKinsey *et al,* 2006). More detailed information about the plasmids used in this manuscript is available upon request.

The Mef2c skeletal muscle-specific promoter (-1.1 kb/+77 bp) containing the *forkhead* binding site has been previously described (Wang *et al*, 2001). The promoter region was amplified from genomic DNA and cloned into the pGL3-basic promoter with a minimal TATA promoter (pGL3-TATA). Mutagenesis of the *forkhead* binding site in the Mef2c-*luciferase* promoter construct was performed using the QuikChange II Site-Directed Mutagenesis Kit (Strategene). The Mef2c FBS was mutated using the mutant primer 5' TAATAGGAAC**AGGTGGGCGG**CTACAAAGC 3' and its reverse complement.

Cell culture and Transfection Assays. Unless otherwise mentioned, all transfections were done using C2C12 myoblasts grown in 20% fetal calf serum/DMEM (Gibco). Transfections of 10^5 C2C12 myoblasts were performed in 6-well plates using a Lipofectamine (Invitrogen) kit, and total protein was harvested 48 hours post transfection. Up to 2 µg of plasmid DNA was transfected in serum-free Optimem media (Gibco). Luciferase assays were performed using a Luciferase Assay System (Promega) following the manufacturer's instructions. All fold changes were normalized to a lacZ loading control, as previously described (Meeson *et al,* 2007).

Myotube differentiation was promoted by exposing 80% confluent myoblast cultures to differentiation medium (DMEM supplemented with 2% heat

inactivated horse serum, antibiotics, insulin and transferrin) as previously described (Meeson *et al,* 2007).

shRNA Experiments. Four separate sets of small interfering RNA (shRNA; HuSH-29 set, Origene) directed against murine Foxj3 were cloned into the pRS plasmid under the U6 promoter and used for transfection assays in C2C12 myoblasts. The empty vector (pRS) and a nonsense (pRS-GFP) plasmid were used as negative controls. Effectiveness of the Foxj3 knockdown was tested in C2C12 myoblasts using both semi-quantitative RT-PCR for endogenous Foxj3. 0.5 μg of shFoxj3 or vector was transfected into C2C12 myoblasts using lipofectamine (Invitrogen) in serum free Optimem (Gibco) media for four hours. After four hours, the transfection media was replaced with growth media containing 20% FBS in DMEM (Gibco). Cells were harvested 48 hours post transfection for RNA, protein, and cellular content in a manner previously described (Meeson *et al*, 2007).

Antisera. The following antibodies were used in this paper. Anti-β-*galactosidase* (rabbit polyclonal serum; Rockland Immunologicals, 1:1000). Anti-β-*galactosidase* (mouse monoclonal serum; Iowa Hybridoma Bank; 1:200 dilution). Anti-c-myc serum (Santa Cruz; 1:1000 dilution). Anti-Ki67 serum (mouse monoclonal; Nova Castra; 1:1000 dilution). Anti-GFP serum (rabbit

polyclonal; Molecular Probes; 1:1000 dilution). Anti-Mef2c serum (goat polyclonal serum; Santa Cruz; 1:200 dilution).

Secondary antisera included a FITC-conjugated goat anti-mouse serum (1:50 dilution; Jackson ImmunoResearch) and a rhodamine-conjugated goat anti-rabbit serum (1:50 dilution; Jackson ImmunoResearch).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed using the ChIP Kit-M for mammalian cells (Progeneron, Inc). C2C12 myoblasts (2×10^6) were transfected with 4 µg if Foxj3-*gfp*, and harvested for nuclear protein 48 hours post-transfected. Nuclear lysates were sonicated using a 550 Sonic Dismembrator (Fisher Scientific) sonicator, and immunoprecipitated with 6 µg of anti-GFP antibody (Molecular Probes) overnight at 4^0C. Selected samples were incubated with IgG (Sigma) as an internal control. Lysates were then bound on UltralLink Immobilized Protein A/G beads (Pierce), and chromatin was then purified using Tripure (Roche) before elution in sterile water. 6 µL of five-fold diluted input and antibody-bound chromatin were used in the PCR reaction with primers specific for the region in the Mef2c skeletal muscle enhancer containing the *forkhead* binding site.

Primary MPC isolation, culture and immunohistochemical analyses. As previously described (Meeson *et al,* 2007), primary MPC cultures were established following the isolation of MPCs from hindlimb skeletal muscle of 2-day-old neonatal mice. Cells were preplated and cultured in F-10 growth medium (Gibco/Invitrogen) supplemented with 25 ng/ml fibroblast growth factor (Invitrogen). WT and Foxj3-/- MPCs were isolated from postnatal day 2 littermate pups generated from Foxj3 heterozygote breeders. Asynchronously dividing WT and Foxj3-/- MPCs were fixed for 10 min with 4% paraformaldehyde and immunostained as previously described (Meeson *et al,* 2007).

Electron microscopy and satellite cell quantitation. As previously described (Meeson *et al,* 2007), tibialis anterior muscles from 3-4 month old male adult WT, and Foxj3-/- mice were harvested, perfusion fixed with 3% glutaraldehyde and postfixed with buffered 1% osmium tetroxide. Samples were then dehydreated with ethanol, embedded in Spurr resin, polymerized, sectioned and placed on copper grids. Sections were examined using a JEOL 1200 EXII TEM. MPCs were quantified using criteria as outlined previously (Garry *et al,* 1997).

RT-PCR. Total RNA was extracted from cells and homogenized tissues using Tripure (Roche). 1 µg of total RNA was reverse transcribed using the SuperScript First Strand Synthesis kit (Invitrogen). cDNA was then diluted ten-

fold in water, and PCR amplified using Go Taq Master Mix (Promega), and visualized on 1% agarose gels stained with ethidium bromide.

Quantitative RT-PCR. Quantitative (real time) RT-PCR was performed using an AbiPrism 7000 light thermocycler (Applied Biosystems). cDNA was diluted ten-fold, and amplified with SYBER GREEN PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. All samples were run in triplicate, and all samples were run in three separate reactions (n = 9). Data were analyzed using the manufacture's software, and fold changes were calculated by taking the inverse log of the $2^{-\Delta\Delta Ct}$, normalized to a Gapdh loading control as previously described (Meeson *et al*, 2007; Livak and Schmittgen, 2001).

cDNA Microarray Data. Total RNA was harvested from wild type and Foxj3-/- myoblasts, and labeled for Affymetrix arrays as previously described (Goetsch *et al*, 2003).

Cardiotoxin-induced muscle regeneration. 100 µl of 10 µM cardiotoxin (Calbiochem) was delivered intramuscularly into the gastrocnemius muscle of adult transgenic mice as previously described (Meeson *et al*, 2007).

Hypoxia experiments. Hypoxia experiments and the hypoxia chamber have been previously described (Mammen *et al*, 2002). 10^5 C2C12 myoblasts were transfected with either Mef2c-Flag or an empty vector control, and 36 hours later exposed to either normoxia (20% oxygen) or anoxia (0% oxygen) for approximately 16 hours. Cells were then collected and stained with Trypan blue (Gibco/Invitrogen), and counted (500 cells per well; n = 3).

Statistical analysis. All p values were calculated using Student's t-test analysis (two-tailed).

Results

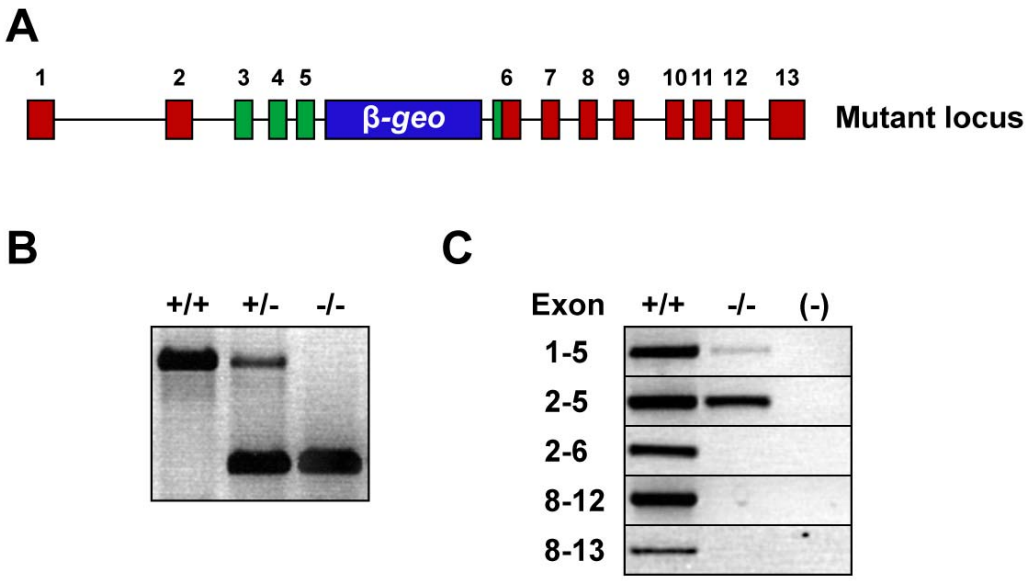


Figure 18. Generation of Foxj3 gene trapped mice. A Foxj3 gene "trapped" mutant locus. Note that a portion of the *forkhead* DNA-binding domain (last 6 amino acids) of Foxj3 is disrupted by the β-*geo* insertion cassette. Splice acceptors flank both ends of the the β-*geo* insertion cassette to ensure that the lacZ reporter is properly spliced in frame. **B** PCR genotyping of tail clippings from a Foxj3 heterozygote mating. The WT band (upper) is approximately 800 base pairs, while the mutant band (lower) is approximately 300 base pairs. **C** RT-PCR from gastrocnemis/plantarius skeletal muscle from WT and Foxj3-/- mice. Note that no transcript is detected beyond exon 6, confirming that the Foxj3 mutant transcript is effectively transcribed. (-) denotes without reverse transcriptase.

Generation of Foxj3-/- transgenic mice. Previous studies of unperturbed skeletal muscle have revealed the dynamic expression of Fox factors at discreet phases during regeneration. To further elucidate the function of Foxj3, I generated Foxj3 mutant mice (hereby referred to as Foxj3-/-) transgenic mouse line from a BayGenomics© ES cell "trapped" line (Stryke *et al*, 2003). The Foxj3 trapped ES cell line contained an insertion of a β-*galactosidase/neomyocin* (β-*geo*) cassette with two separate splice acceptors (Figure 18A). The resulting mRNA transcript encodes exons 1 through 5, and contains all but the last six residues of the *forkhead* DNA-binding domain, and no detectable transcript following exon 6 (Figure 18C). Previous studies from Pérez-Sánchez and colleages demonstrated through Gal4 transcriptional assays that the carboxy-terminus is the activation domain of FoxJ proteins and is essential for the ability of the Foxj2 *forkhead*'s activity (Pérez-Sánchez *et al*, 2000a). Thus, the predicted fusion protein encoded from Foxj3-/- mice is predicted to result in a non-functional, transcriptionally inactive, fusion protein. Additionally, the subsequent Foxj3-β-*gal* fusion protein would be predicted to recapitulate the endogenous expression of Foxj3 and be transcriptionally regulated by its endogenous promoter elements.

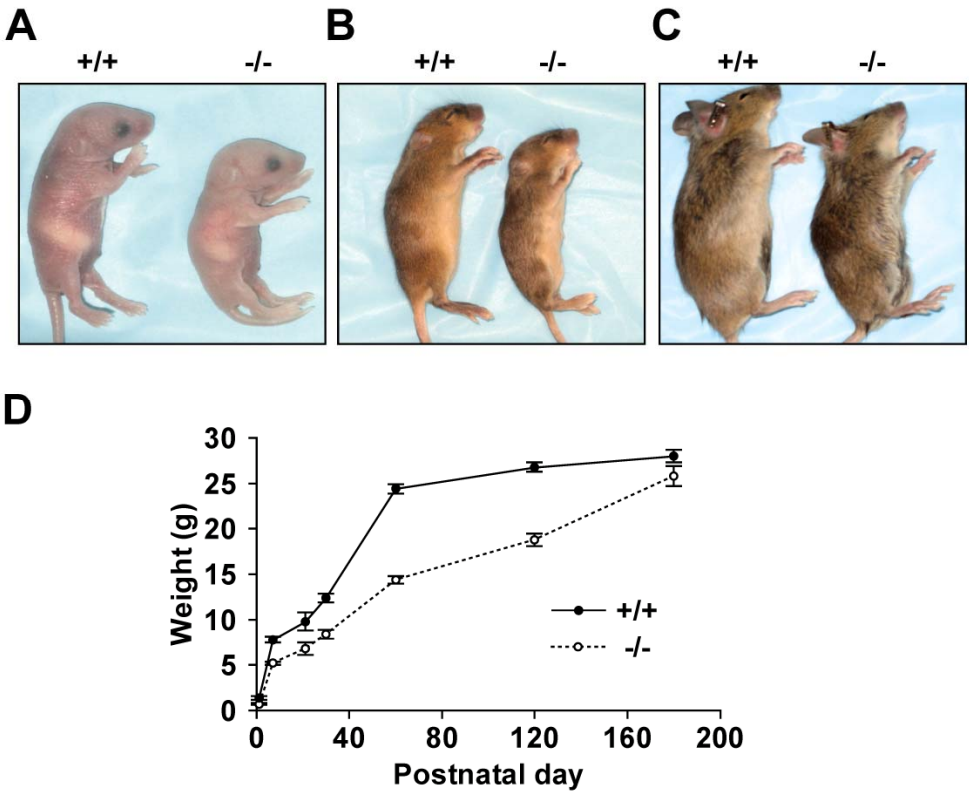


Figure 19. Foxj3 is essential for normal growth and development. **A-C** Postnatal day 1 (P1), 14 (P14), and 30 (P30) littermate pups born from Foxj3 heterozygote matings. **D** Foxj3-/- mice weigh less compared to their wild type littermates. Weights were taken from eight separate Foxj3 heterozygote crosses (n = 8 litters, n = 4 mice evaluated for each litter). All mice were subjected to the same 4% fat irradiated chow diet and normal 12 hour light/dark cycles under the same pathogen-free cages. Not shown, Foxj3-/- females also weigh less than their wild type littermates.

Foxj3 is essential for normal growth and female fertility. Mice homozygous for the Foxj3-β-*geo* targeted allele were generated from combinatorial matings of heterozygotes (Figure 18B). Initially, my studies utilized the C57/B6J:129OlaP2Hsd mixed strain; however, I have since backcrossed the original chimeric mice with inbred C57/B6J and 129OlaP2Hsd strains separately and over six generations to ensure isogenicity. No strain variances have been observed. Mice homozygous with the Foxj3 mutant alleles, generated from heterozygote matings, were viable and born at normal Mendelian ratios (Supplemental Figure 4A). However, the Foxj3-/- mice were noticeably reduced in weight and size, as compared to their wild type and heterozygote littermates. As Foxj3-/- mice develop, they remain growth retarded throughout their longevity; although their weights approach that of the wild type controls by six-months of age. The decreased mass phenotype in Foxj3-/- mice was observed in both male and female mice (data not shown). Female Foxj3-/- mice had poor fertility and produced no more than two litters consisting of no more than three pups when mated to wild type males. Consistent with the GeneAtlas© microarray data, we observed high expression of Foxj3 in the uterine horn following X-gal staining of Foxj3-/- female mice (Supplemental Figure 4B). The high expression of Foxj3 in the female reproductive system shifted our focus to the study of male Foxj3 mutant mice to rule out any estrogen-related effects. Similar to the phenotype

observed in Foxk1-deficient mice, Foxj3 appears to be essential for normal growth during development (Garry *et al*, 2000).

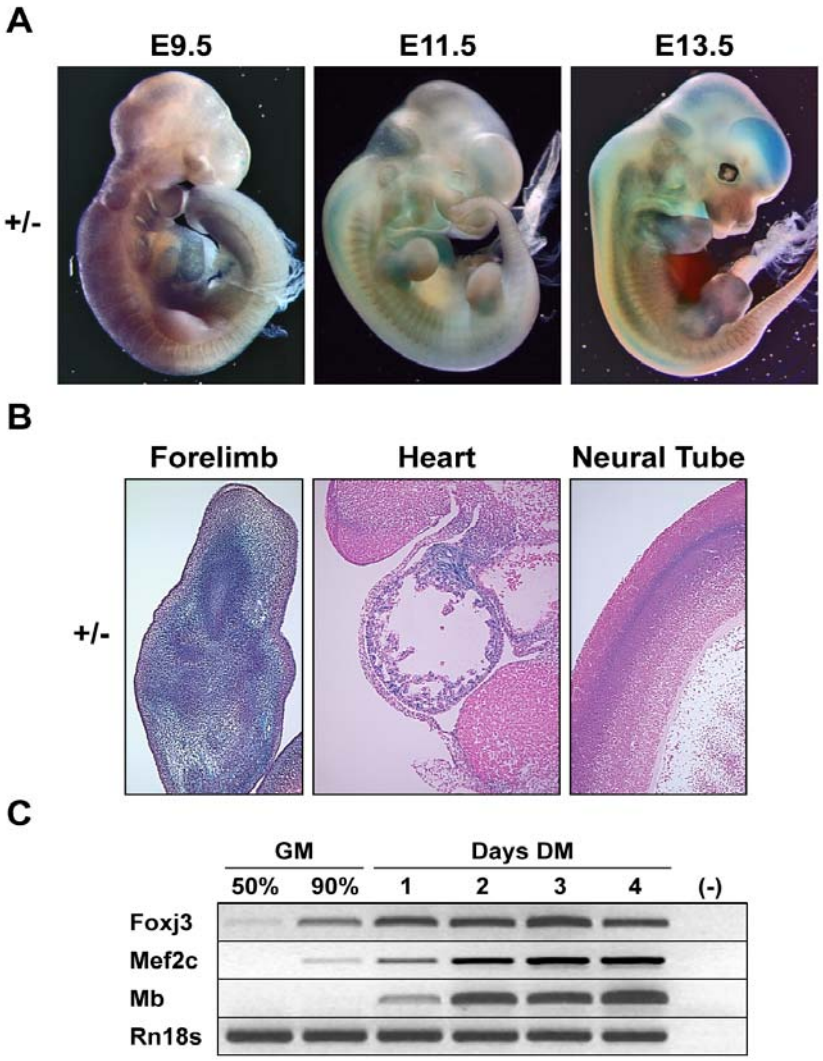


Figure 20. Foxj3 is expressed in myogenic lineages during early embryogenesis. **A** (Left-Right) X-gal histochemistry of Foxj3 heterozygote embryos harvested at E9.5, E11.5, and E13.5. Note the expression of the Foxj3-lacZ fusion protein in the developing heart, somites, and neural tube. **B** Foxj3 expression is strongly enriched in the developing limb buds, heart, and neural tube at E11.5. **C** C2C12 myoblast differentiation reveals the upregulation of Foxj3 transcript upon exposure to differentiation media. Myoblasts were initially placed in growth media at 50% confluency, and switched to differentiation media at 90% confluency. Note the subsequent upregulation of Mef2c transcript upon differentiation. Mb, myoglobin (marker of C2C12 differentation) control. Rn18s, 18s ribosomal RNA loading control. GM, growth media (20%FBS/DMEM). DM, differentiation media (2%FBS/DMEM).

Foxj3 is upregulated during myoblast differentiation. I examined the spatial expression pattern of Foxj3 in adult organs using primers specific for murine Foxj3. Semi-quantitative and real time PCR revealed that Foxj3 is broadly expressed, and is highly expressed in the adult brain, heart, and spleen. Moderate expression was observed in isolated gastrocenmius/plantaris skeletal muscle and liver, while Foxj3 was barely detectable in adult testis. These findings are consistent with the adult mouse and human cDNA microarrays in the

public database GenAtlas (http://symatlas.gnf.org/SymAtlas/). Interestingly, the *forkhead*/winged-helix DNA binding domain of Foxj3 has a greater similarity with the *forkhead* Foxk1, a regulator of myogenic progenitor cells, than with the other two *forkhead* genes in the FoxJ subfamily. To further examine the role of Foxj3 in myogenic lineages, I examined the expression of Foxj3 in differentiating C2C12 myoblasts, a cell culture model for myogenic differentiation (Figure 20C). Foxj3 transcription was significantly upregulated during C2C12 differentiation and was at its highest levels four days post-differentiation. The studies support the hypothesis that Foxj3 has an important role in post-embryonic myogenesis.

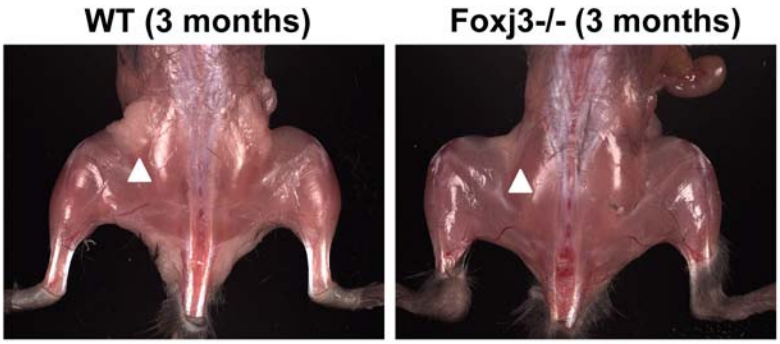


Figure 21. Foxj3-/- mice have decreased amounts of fat and lean skeletal muscle. (Left Panel) WT three month old male mouse, deskinned to display skeletal muscles and fat pads. (Right Panel) Foxj3-/- 3 month old male littermate, also deskinned to display fat pads and skeletal muscle architecture. (Open Arrowhead) demarcates the rear abdominal fat pad. Note the reduced size of the abdominal fat pad and the overall "redness" of the hindlimb muscles of the Foxj3-/- mouse.

Foxj3-/- mice have abnormal skeletal muscles. I first observed that Foxj3-/- mice had an abnormal walking stride after they were weaned from their mothers at twenty-one days postnatal. Upon closer examination of their hindlimb skeletal muscle, I noticed that the Foxj3-/- mice had reduced fat pads and abnormally rigid hindlimb muscles (Figure 21). This abnormal architecture of the hindlimb muscles of the Foxj3 mutant mice may account for their unusual gait. However, due to the high expression of Foxj3 in the adult brain, I cannot rule out the possibility that this phenotype observed was due neurological deficits.

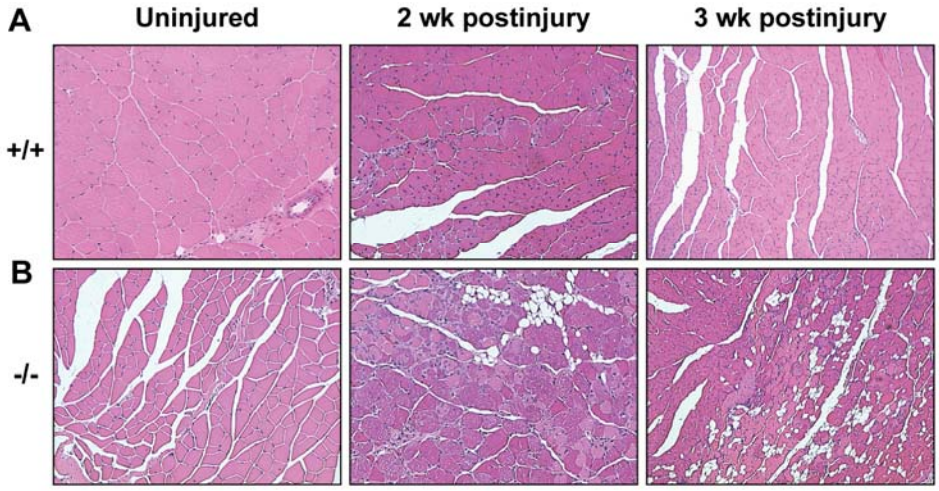


Figure 22. Foxj3-/- mice have perturbed skeletal muscle regeneration following cardiotoxin-induced injury. A Wild type adult mouse gastrocnemius/plantarius (G/P) skeletal muscle uninjured, 2 weeks post-injury, and 3 weeks post-injury. **B** Foxj3-/- adult mouse G/P uninjured, 2 weeks post-injury, and 3 weeks post-injury. (Open arrowheads) demarcate areas of adipogenesis. (Black arrowheads) demarcate regions of necrosis. H&E stained sections taken at 10x magnification.

Foxj3-/- mice have impaired skeletal muscle regeneration following injury. I tested the ability of Foxj3-/- mice to regenerate their skeletal muscle following a cardiotoxin-injury to their gastrocnemius muscle. Whereas, mice normally regenerate their skeletal muscle within two weeks post-injury, Foxj3-/- mice had areas of necrosis and adipocytes (Figure 22). I next examined the Foxj3-/- mice for the presence of muscle satellite cells using transmission electron microscopy. Satellite cells were present in the tibialis anterior muscle of Foxj3-/- mice and consisted of 3% out of 1000 total nuclei counted, slightly higher than the 2% counted in their wild type littermates, although not statistically significant. I then counted the total number of dividing cells present in uninjured and injured skeletal muscle using the proliferation marker Ki67. I noted that Foxj3-/- mice had twice the number of $Ki67^+$ cells as their wild type littermates post-injury; although uninjured skeletal muscle had the same amount of $Ki67^+$ cells as wild type controls. This supported the hypothesis that Foxj3 might play a role in either the maintenance of myogenic progenitor cells or in the proliferative capacity. I then performed TUNEL assays on uninjured and injured wild type and Foxj3-/- skeletal muscle to determine if Foxj3 was essential for the maintenance of myogenic progenitor cells. However, there were no changes in the number of $TUNEL^+$ cells in wild type and Foxj3-/- skeletal muscle before and after injury. These findings indicate that Foxj3 is not essential for the maintenance of quiescent or activated myogenic progenitor cells.

Foxj3 is expressed in myogenic lineages during embryogenesis. X-gal histochemistry of Foxj3+/- embryos revealed strong Foxj3 expression in the developing heart and later in the somites, brain, neural tube, and limb buds from through mid-gestation (Figure 20A and B). The lacZ reporter expression from the Foxj3+/- embryos is consistant with that of the *in situ* hybridizations of Foxj3 expression performed by Landgren and Carlsson (Landgren and Carlsson, 2004). Expression of Foxj3 appears to be more ubiquitous after E13.5; although, I detected strong enrichment of Foxj3 in the adult brain, spleen, and heart.

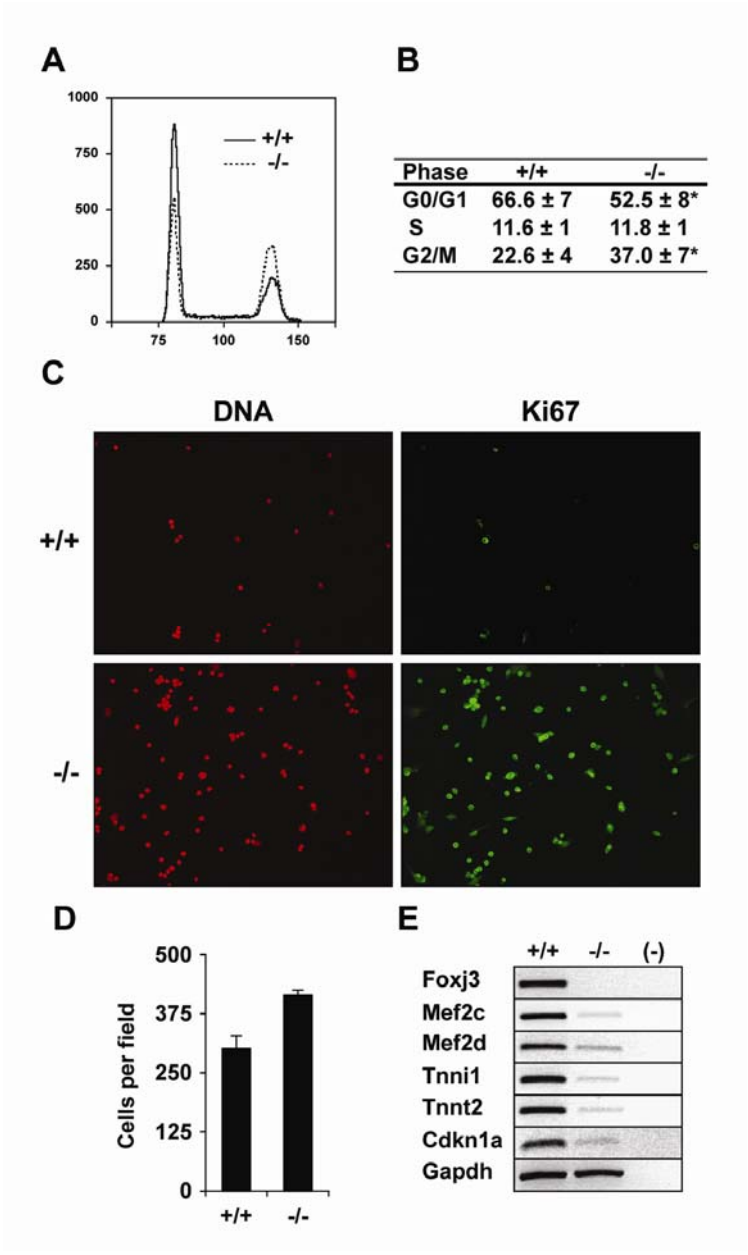
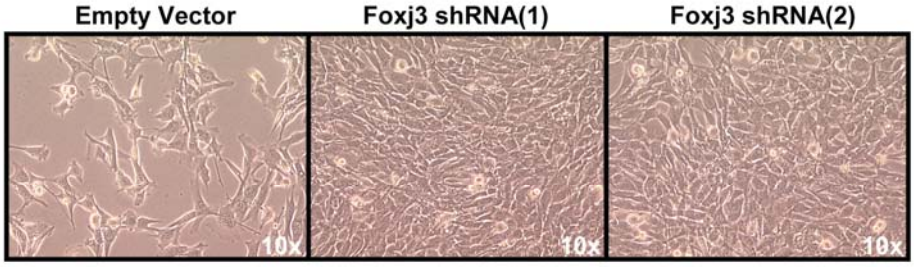

A
1000
750
500
250
0
75
100
150
+/+
-/-
B
Phase +/+ -/-
G0/G1 66.6 ± 7 52.5 ± 8*
S 11.6 ± 1 11.8 ± 1
G2/M 22.6 ± 4 37.0 ± 7*
C
DNA
Ki67
+/+
-/-
D
Cells per field
500
375
250
125
0
+/+
-/-
E
+/+
-/-
(-)
Foxj3
Mef2c
Mef2d
Tnni1
Tnnt2
Cdkn1a
Gapdh

Figure 23. Foxj3-/- myoblasts have increased proliferation and a dysregulated myogenic transcriptome. **A** FACS histogram of wild type and Foxj3-/- primary myoblasts. Upper peak indicates the cells in the G0/G1 phase of the cell cycle. Bottom valley indicates cells in the S phase of the cell cycle. Smaller peak indicates the G2/M phase of the cell cycle. **B** Summary table of FACS profiles of wild type and Foxj3-/- primary myoblasts cell cycle phases (n = 3 separate pups; 3 separate heterzyogote mating litters). Note the increase in cells found in the G2/M phase. * p value < 0.005. **C** Foxj3-/- skeletal muscle (G/P) has increased amounts of $Ki67^+$ (green) cells 2 weeks following cardiotoxin-injury. Cellular DNA is labeled with the nuclear stain propidium iodide (red). (Arrowheads) demarcate $Ki67^+$ cells. **D** Quantitation of the increased amounts of $Ki67^+$ cells observed in Foxj3-/- skeletal muscle following injury. **E** Semi-quantitative RT-PCR of wild type and Foxj3-/- myoblasts. (-), without reverse transcriptase.

A.



B.

Phase	Empty Vector	Foxj3 shRNA (1)	Foxj3 shRNA (2)
%G0/G1	69.4 ± 1	62.1 ± 1*	60.1 ± 1**
%S	12.5 ± 2	13.8 ± 1	12.6 ± 1
%G2/M	17.8 ± 1	24.1 ± 3	27.5 ± 1**

* $p < 0.03$
** $p < 0.003$

C.

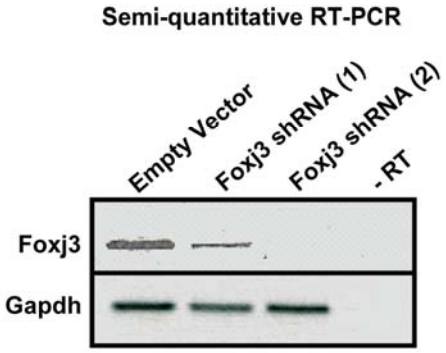


Figure 24. shRNA knockdown of Foxj3 in C2C12 myoblasts results in perturbed cell cycle kinetics. **A** Phase microscopy of C2C12 myoblasts transfected with either empty vector or two shRNAs directed against mouse Foxj3. Note the increased amount of proliferation of the cells transfected with the Foxj3 shRNAs. **B** Summary of a FACS histogram outlining the increased amount of cells in the proliferative G2/M phase as compared to empty vector. **C** Semi-quantitative RT-PCR demonstrating the effectiveness of knockdown of endogenous Foxj3. – RT, without reverse transcriptase (negative control).

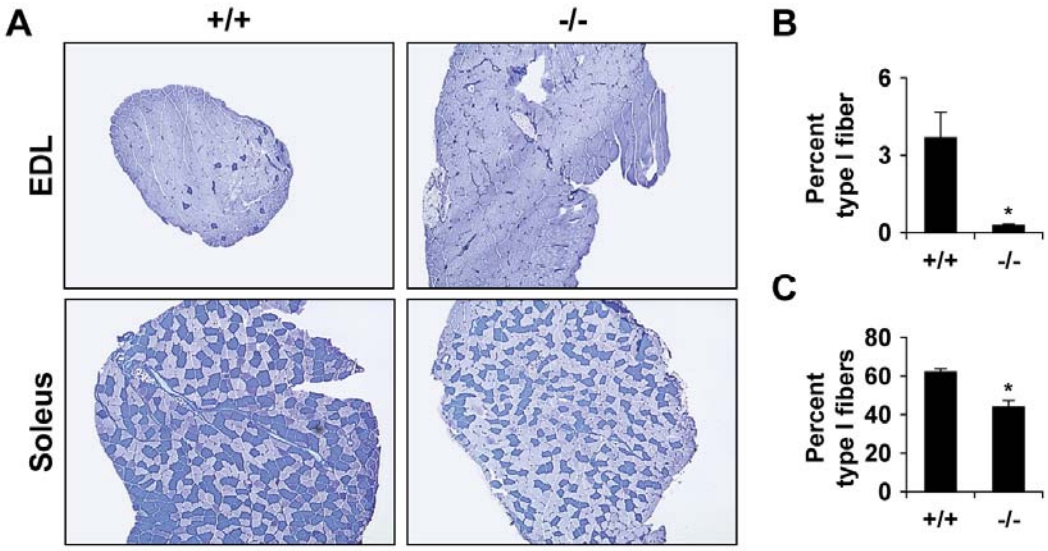


Figure 25. Foxj3 is essential for determining adult skeletal muscle myofiber identity. A Metachromatic ATPase staining of wild type and Foxj3-/- EDL and soleus muscles taken from 4-month-old male littermates. Note the reduction in type I (dark blue; oxidative) fibers in the Foxj3-/- soleus muscle. Non-oxidative, Type IIa/b fibers are stained turquoise/light blue. 10x magnification. **B and C** Total percentages of over 500 counted type I myofibers taken from EDL and soleus muscles 3 separate adult mice. * p value < 0.05; n = 3 mice per genotype.

Foxj3-/- mice have decreased Type I fibers. I next examined the fiber type diversity of the two mixed muscle groups, the soleus and EDL in Foxj3-/- mice using metachromatic ATPase fiber type analysis. Both the soleus and EDL

muscles of the Foxj3-/- mice had decreased numbers of oxidative Type I fibers and significantly higher amounts of Type IIa/b fibers (Figure 25A.-C.; p value < 0.05; n = 3 mice per genotype). The TA muscles from the Foxj3-/- mice showed no significant differences in percentages of fiber types.

Foxj3-/- myoblasts proliferate at a faster rate. Having ruled out the possibility that Foxj3 is necessary for myogenic progenitor cell maintenance, I then hypothesized that Foxj3 may play a role in cell cycle kinetics. I then isolated primary myoblasts from neonatal hindlimb muscle, which contains 30-50% myogenic progenitor cells. Surprisingly, Foxj3-/- myoblasts proliferate at a faster rate, with over 50% in the S- or G2/M-phase, as compared to their wild type littermates (Figure 23; n = 3 separate experiments, n = 9 total pups). I confirmed this proliferation defect using immunohistochemistry for the proliferation marker Ki67, and counting the total number of $Ki67^+$ cells. Foxj3-/- myoblasts had a significantly higher amount of $Ki67^+$ cells out of 500 nuclei counted and average number counted per field, as compared to wild type controls (Figure 23B). Together, these studies establish Foxj3 as an essential regulator of myoblast cell cycle kinetics.

shRNA knockdown of endogenous Foxj3 results in increased myoblast proliferation. To validate the *in vivo* increased myoblast proliferation phenotype observed in the Foxj3-/- mice, I undertook shRNA knockdown of endogenous Foxj3 in C2C12 myoblasts. Using four separate shRNA oligos and an empty vector control, I observed partial and severe knockdown of Foxj3 transcript in two out of the four shRNA constructs examined (Figure 24C). Knockdown of endogenous Foxj3 in C2C12 myoblasts resulted in hyperproliferation of Foxj3 myoblasts and an induction of cells in the proliferative phase of the cell cycle (Figure 24A and B). No significant amounts of cellular death were observed in any of the samples (n = 3 replicates, performed in three separate experiments).

Foxj3 functions as a transcriptional activator in myoblasts. I next undertook a series of transcriptional assays to evaluate Foxj3's functional role as a modulator of gene transcription. I utilized an *in vitro* Gal4-UAS-*luciferase* reporter cell-based assay in C2C12 myoblasts (Chang *et al*, 2005). The Foxj3 full length open reading frame and the $Foxj3_{1\text{-}176\ aa}$ mutant transcript were fused to the Gal4-DNA binding domain and transfected along with a UAS-*luciferase* reporter into C2C12 myoblasts. Overexpression of the full-length Gal4-Foxj3 transcript was capable of activating the reporter 12-fold in a dose-dependant fashion. Whereas, the $Foxj3_{1\text{-}176aa}$ mutant transcript had no transcriptional activity using the same UAS-*luc* reporter in transfected C2C12 myoblasts.

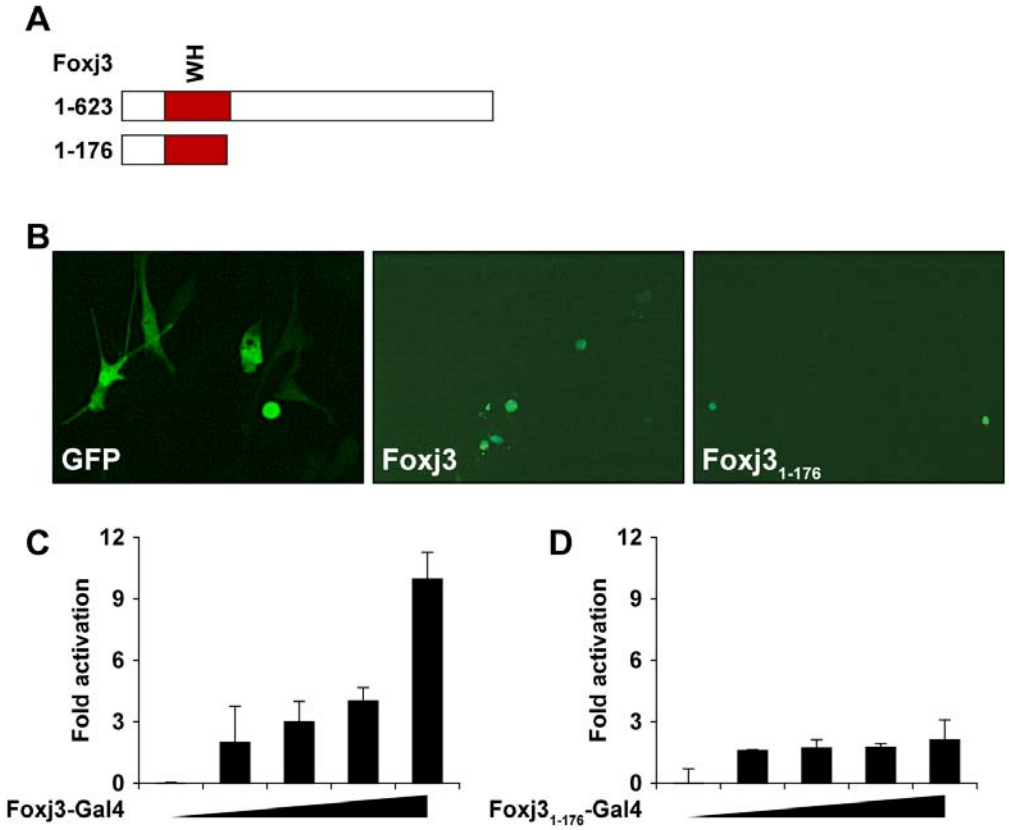


Figure 26. Foxj3 is a transcriptional activator and requires its carboxyl-terminus for full activity. **A** Schematic displaying both full length Foxj3 (1-623aa) and the truncated transcript produced in the Foxj3-/- mice (1-176aa). Note that only a small region (six amino acids) of the *forkhead* DNA-binding domain (also referred to as the "winged helix", WH) does not get fully transcribed in the Foxj3-/- mice. **B** Foxj3-GFP is predominantly nuclear when overexpressed in C2C12 myoblasts. While the GFP vector alone is ubiquitously expressed, both full length Foxj3 and the Foxj3 truncated mutant are expressed

exclusively in the nucleus of the cells. C2C12 cells are at approximately 90% confluency. **C** Gal4/UAS-luc transcription assay demonstrats that only full length Foxj3 is capable of functioning as a transcriptional activator in C2C12 myoblasts. A greater than 9-fold induction of the UAS-*luc* reporter is observed in the full length Gal4-Foxj3 fusion construct; whereas, the truncated transcript has minimal transcriptional activity.

Mef2c is a direct downstream target gene of Foxj3 in myoblasts. To identify potential downstream target genes of Foxj3, I utilized oligonucleotide microarray technologies to examine dysregulated genes in Foxj3-/- myoblasts. Over 900 genes were significantly dysregulated ($p < 0.005$) greater than two-fold and I validated several of the most dysregulated transcripts by semi-quantitative RT-PCR (Table 3). Myocyte enhancer factor 2C (Mef2c) was downregulated greater than 10-fold in the Foxj3 mutant myoblasts compared to the wild type controls. Upon examination of the 5' upstream promoter of Mef2c, I identified a highly conserved *forkhead* binding within the skeletal muscle enhancer region (-1.1 kb/+77 bp, Wang *et al*, 2001). Based on evolutionary conservation, I then fused the -1.1 kb/+77 bp (subsequently referred to as 1.2 kb Mef2c) to a *luciferase* reporter and transfected C2C12 myoblasts with increasing amounts of Foxj3 (Figure 27A). I noted that Foxj3 trancriptionally activated the 1.2 kb Mef2c-*luc* up to 8-fold in a dose dependant fashion (Figure 27C). The $Foxj3_{1\text{-}176\ aa}$ mutant

transcript that was produced in the Foxj3-/- mice had virtually no transcriptional activity (Figure 27B). Using site-directed mutagenesis, I mutated the *forkhead* binding site (FBS) in the 1.2 kb Mef2c-*luc* reporter, and noted that overexpression of Foxj3 in C2C12 myoblasts was significantly reduced (Figure 27E). Together these experiments confirmed the hypothesis that *Mef2c* was a direct downstream target gene of Foxj3 *in vitro*.

MEF2-dependent Dysregulated Transcripts in Foxj3-/- Myoblast Microarray

Gene Symbol	Gene	Fold Change
Cdkn1a	cyclin-dependent kinase inhibitor 1a (p21)	-1.74
Mef2d	myocyte enhancer factor 2D	-2.41
Myog	myogenin	-3.60
Cryab	crystalline, alpha B	-4.13
Mylpf	myosin light chain, phosphorylatable	-4.49
Tnnt2	troponin T2	-5.28
Tnnt1	troponin T1	-5.41
Tnni1	troponin I1	-5.97
Myom2	myomesin 2	-8.76
Myl4	myosin, light polypeptide 4	-9.14
Tnnc1	troponin C1	-9.39
Mef2c	myocyte enhancer factor 2C	-9.55
Myh3	myosin, heavy polypeptide 3	-9.89
Tnnt3	troponin T3	-10.43

Table 3. Foxj3-/- myoblasts have reduced transcription of MEF2-dependent signaling pathway. Oligonucleotide microarray results taken from a comparison of wild type and Foxj3-/- primary myoblasts. Dysregulated transcripts were validated by semi-quantitative and quantitative PCR.

To confirm my hypothesis that Foxj3 was a direct downstream target of Mef2c *in vivo*, I performed a chromatin immunoprecipitation (ChIP) assay and PCR with primers specific for the FBS located in the Mef2c 5' upstream skeletal enhancer region. When the Foxj3-GFP fusion construct is overexpressed in C2C12 myoblasts the bound chromatin is purified, the segment containing the FBS in the 1.2 kb *Mef2c* upstream skeletal muscle enhancer was specifically amplified (Figure 27B).

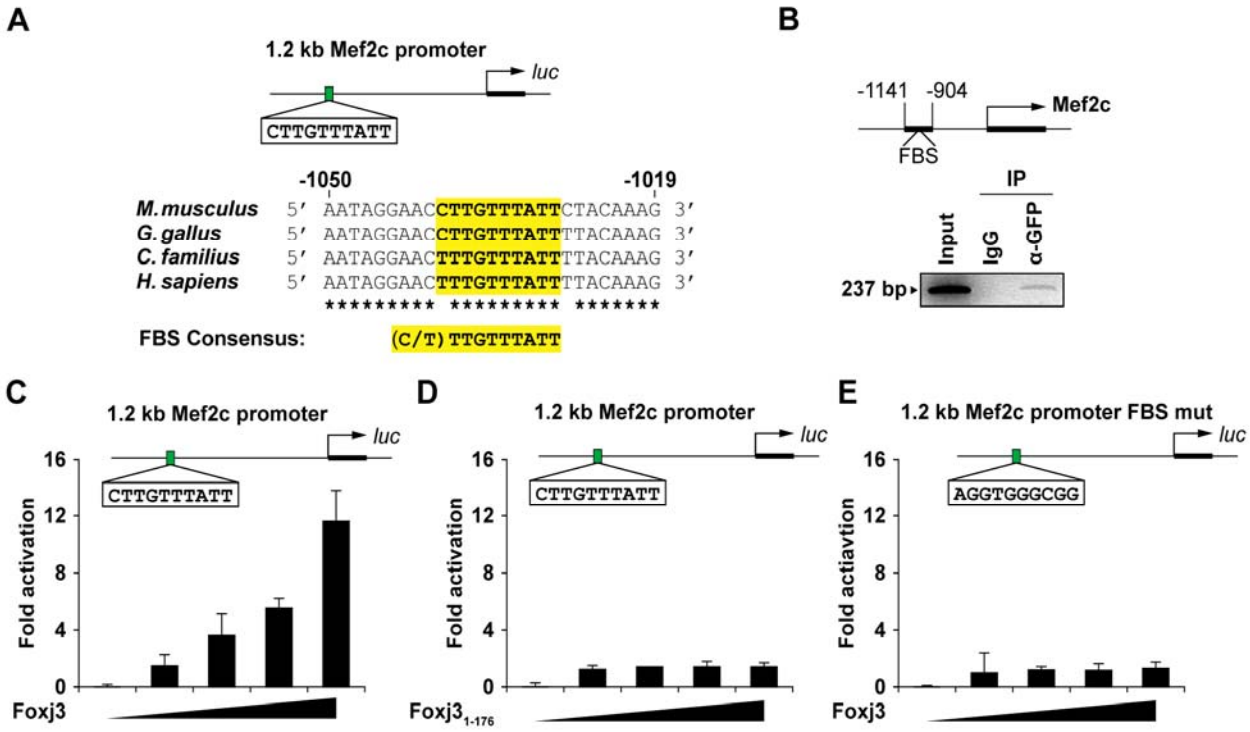


Figure 27. Mef2c is a direct downstream target gene of Foxj3. A Schematic illustrating the evolutionarily conserved *forkhead* binding site (FBS) located

withing the 1.2 kb Mef2c 5' upstream skeletal muscle specific enhancer. **B** ChIP of C2C12 myoblasts transfected with a Foxj3-GFP fusion construct. 10% total input and IgG controls shown. **C** Foxj3 transcriptional activates the 1.2 kb Mef2c-*luc* reporter in a dose-dependant fashion in C2C12 myoblasts. **D** The truncated $Foxj3_{1\text{-}176aa}$ transcript cannot activate the 1.2 kb Mef2c-*luc* reporter. **E** Mutagenesis of the FBS results in ablation of Foxj3 activation of the 1.2 kb Mef2c reporter.

Foxj3-/- mice have skeletal defects. In addition to the skeletal muscle phenotype observed in Foxj3-/- mice, I also noticed that beginning at one month of age Foxj3 mutant mice had an abnormal gait. I stained wild type and Foxj3-/- mice front paws with red ink and their back paws with blue ink, and measured their gait diameters after placing them in cardboard tubes lined with Whatman paper. I observed that Foxj3-/- had a significantly wider gait diameter, possibly resulting from either a neurological and/or a skeletal defects (Figure 29). It has been recently shown that Mef2c plays an important role in the regulation of chondrocyte hypertrophy in development (Arnold *et al*, 2007). Mice in which Mef2c has been conditionally deleted in the *twist2* and *collagen-IIa* lineages, have abnormally ossified bones and are growth retarded, which is strikingly similar to the Foxj3-/- mouse phenotype we observed. To confirm the presence of a skeletal phenotype in Foxj3-/- mice, I harvested postnatal day 1 (P1) femurs

from wild type and Foxj3-/- pups, and stained them for markers of bone development. Foxj3-/- femurs had overall decreased Von Kassa staining compared to their wild type littermates (Figure 28A and C).

Having initially identified severe deficits in Foxj3-/- neonatal bone, I next examined the femurs of adult Foxj3 mutant mice. Foxj3-/- mice have severely reduced growth plates, and a significant decrease in overall size and organization compared to their wild type littermates (Figure 28B and D). This loss of growth plate structure has also been observed in the Mef2c bone knockout mice (Arnold *et al*, 2007). The femoral growth plate is an essential site for chondrocyte hypertrophy, and its subsequent disruption in the Foxj3-/- mice is indicative of an osteoblast/osteoclast progenitor malfunction.

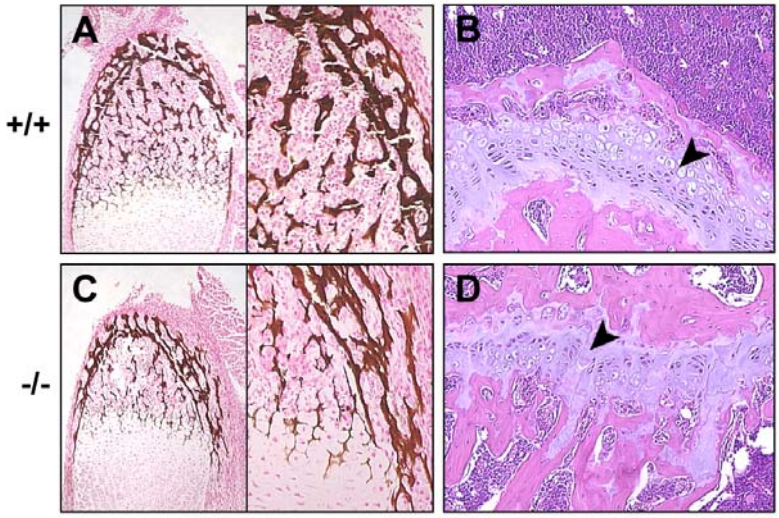


Figure 28. Foxj3 is essential for normal bone growth plate formation.

A and C Von Kassa staining (light brown) of P1 wild type (+/+) and Foxj3 mutant (-/-) femors. Note the reduction in total amount of Von Kassa positive regions in the Foxj3-/- mice. 4x magnification. **B and D** H&E sections of the femoral growth plates of adult wild type and Foxj3-/- mice. Note the decreased amounts of chondrocyte hypertrophic cells (arrowhead) in the Foxj3-/- mice.

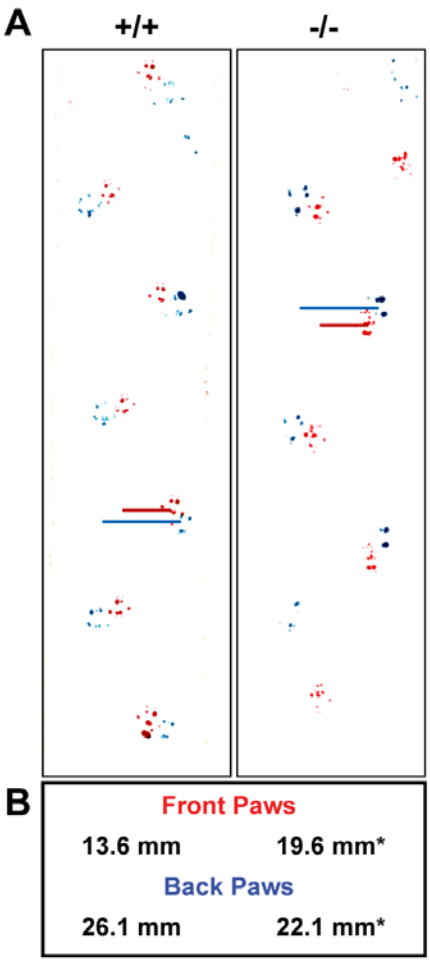


Figure 29. Foxj3-/- have an abnormal gait. **A** Wild type (+/+) and the Foxj3 mutant (-/-) mice abnormal gaits observed by ink blotting of their front (red ink) and back paws (blue inks). Lines indicate average diameters measured from the center-to-center of the paw prints. **B** Summary of the measured gaits in wild type and Foxj3-/- mice highlights the abnormally large hindlimb stance in the mutant mice. Ten separate measurements recorded from five separate adult male mice per strain.

Discussion

Foxj3 plays an important role in normal growth and skeletal muscle development.

The overall decrease in size of Foxj3 mutant mice was unexpected given the induction of proliferation in Foxj3-/- myoblasts. However, given the fact that Foxj3 appears to be induced during early development of important organs (heart, limbs, brain), Foxj3 may play a role in regulating the expression of key genes involved in cellular expansion. Additionally, given the high percentage of similarity between Foxj3 and Foxj2, it is possible that Foxj3 may play some redundant roles with Foxj2 in certain lineages. It has been suggested that Foxj3 may play explicit redundant functional roles with another *forkhead* transcription factor, Foxs1 (Heglind *et al*, 2005). However, we did not detect any differences in the levels of Foxs1 in Foxj3-/- muscle, and given the mild phenotype of the Foxs1 knockout mice, we conclude that Foxj3 most likely plays a role independent of Foxs1.

Foxj3 has its highest degree of homology *forkhead* domain evolutionary homology with the *Drosophila sloppy-paired-1* (*slp1*) genes, the *C.elegans hfh-8* gene, and the *S. cerevisiae Hcm1* gene. Slp1 is essential for embryonic

patterning of the fly through its direct interaction with RNA polymerase II at several critical developmental gene promoters (Wang *et al*, 2007). On a similar note, yeast lacking the *Hcm1* gene undergo rapid proliferation and massive genomic instability (Pramila *et al*, 2006). Thus, it is likely that Foxj3 has a specialized function in the regulation of cellular proliferation of specific cell populations at key developmental time points.

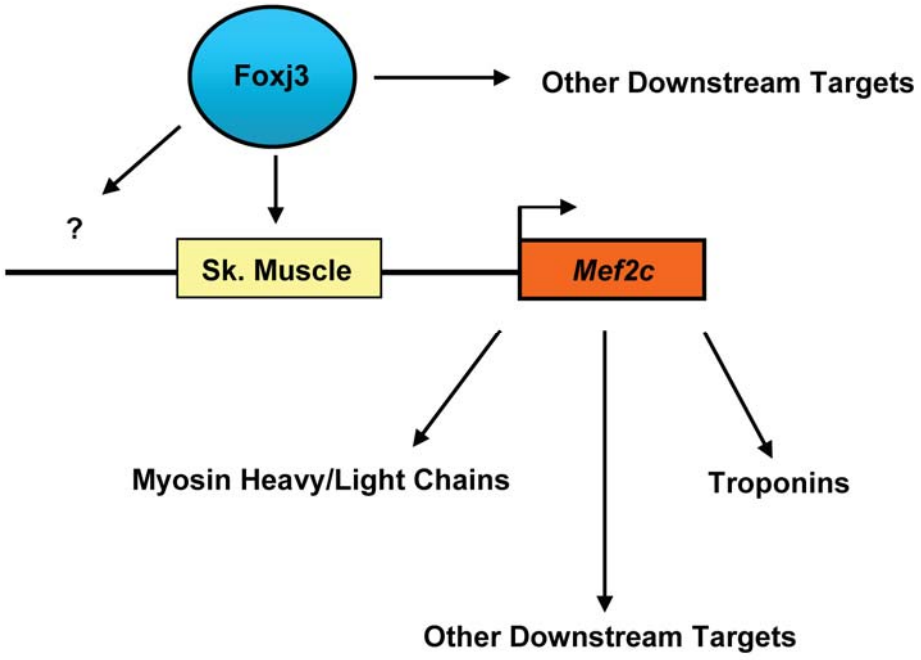


Figure 30. Schematic illustrating Foxj3 function as a transcriptional activator of Mef2c. Foxj3 (turquoise) transcriptionally activates *Mef2c* (red) at the evolutionarily conserved FBS site located withing the upstream skeletal muscle enhancer region (yellow). Mef2c then transcriptionally activates members of the troponin and myosin heavy and light chains. Foxj3 additionally might transcriptionally activate *Mef2c* at another location in the Mef2c 5' upstream region (?).

The role of *forkheads* in human disease states.

Several *forkhead* genes have been implicated in human disease states; although, given many of their important roles in the regulation of embryogenesis, it is likely that many go undetected do to non-viability of the embryo. Mutations of the *forkhead* transcription factor Foxp2 result in language and speech disorders that are transmitted familially (MacDermot *et al*, 2005). Given the fact that many *forkheads* appear to regulate cellular proliferation, many of them play important roles as potential cancer targets. The formation of fusion proteins involving Foxo1 and Pax3/Pax7, result in alveolar rhabdomyosarcoma (ARMS) (Mercado and Barr, 2007).

Mef2c as a determinant of skeletal muscle fiber type identity.

The regulation of Mef2c transcription by Foxj3 further elucidates a role for Mef2c and other MEFs as regulators of muscle fiber type. Mice lacking Mef2c are embryonic lethal at E9.5 due to massive defects in the looping of the heart tube (Lin *et al*, 1997). While the role of Mef2c in the heart has been elucidated in the past few years, the exact role of Mef2c in myogenesis is still not completely understood (reviewed by Berkes and Tapscott, 2005). A recent study utilizing a temperature-sensitive *Drosophila* MEF2 orthologue (*dMEF2*), surprisingly

revealed that dMef2 was not essential for adult myogenesis (Baker *et al*, 2005). While flies lacking dMEF2 had severe neurological deficits and lacked the ability to fly, they had normal patterning of skeletal muscle.

More recently, work done by Hughes and colleagues demonstrated that simultaneous morpholino knockdown of zebrafish Mef2c and Mef2d resulted in a loss of thick filament proteins and sarcomeric structure breakdown (Hinits and Hughes, 2007). It has been conclusively demonstrated that Mef2c is an essential upstream transcriptional activator of troponins in skeletal muscle (Di Lisi *et al*, 1998; Blais *et al*, 2005) and myofiber identity (Wu *et al*, 2000). Conditional transgenic technologies have revealed a broader role for Mef2c in cellular maintenance in various lineages. Conditional deletion of Mef2c in skeletal muscle lineages by a MCK-*cre* transgenic line, resulted in a severe decrease of Type 1 fibers, a striking similar of that of the Foxj3-/- mice (Potthoff *et al*, 2007). Together these results establish Foxj3 as a determinant of myofiber identity through a MEF2-dependent pathway. Recent studies performed by members of the Accili lab, demonstrated that the *forkhead* factor, Foxo1, is also a determinant of myofiber identity (Kitamura *et al*, 2007).

It remains to be seen as to whether or not Foxj3 is a regulator of cell cycle proliferation directly through Mef2c. While there are some studies of Mef2c

transcriptionally regulating cardiac cellular proliferation through *cdkn1a* (*p21*) by the RNA helicase CHAMP (Mov10l1) (Liu and Olson, 2002), it is unclear whether or not Mef2c regulates the cell cycle in skeletal muscle through this pathway.

Foxj3 as a modulator of gene transcription.

Forkhead proteins often require partner cofactors to modulate the transcriptional activity of a gene. The *forkhead* transcription factor Foxk1 directly interacts with the transcriptional repressor Sin3b to form a large repression complex (Yang *et al*, 2000). The *forkhead* transcription factor Foxp3, an essential regulator of T-lymphocytes, histone actylases and class II histone deactylases to modulate *interleukin-2* levels (Li *et al*, 2007). *In vivo* chromatin immunoprecipitation assays followed by hybridization to DNA microarrays (ChIP:chip) have revealed that forkhead transcription factors can act as transcriptional activators and repressors of distinct genes even within the same cellular population. ChIP:chip involving the purification of GFP^+ T-lymphocytes from a Foxp3-GFP knock-in mouse, identified several direct downstream target genes of Foxp3 that were both activated and repressed in the same population (Zheng *et al*, 2007). Additionally, the phosphorylation status of *forkhead* proteins also appears to regulate their transcriptional function in addition to the cellular localization. Phosphorylation of

the forkhead factor Foxo1 inhibits its interaction with the histone deactylase Sir2 (Sirt1), and attenuates Foxo1's transcriptional activity (Matsuzaki *et al*, 2005).

Foxj3 has several predicted phosphorylation sites, which may play a role in its nuclear localization and transcriptional activity. In summary, it is probable that interactions with other transcriptional cofactors regulate Foxj3's transcriptional function and activity of a gene's transcription at the promoter level.

Chapter 5

Concluding Statements

Sox15 Transcriptionally Activates Foxk1 in Myogenic Progenitor Cells

The molecular mechanisms for the transcriptional regulation of *Foxk1* transcription in myogenic progenitor cells is unknown. Based on evolutionary conservation I have identified a Sox transcription factor site located in a 4.6 kb enhancer region of the 5' *Foxk1* upstream promoter. I have fused both the 4.6 kb *Foxk1* enhancer to a β-*galactosidase* reporter and one with a mutation in the SBE resulting in substantial ablation of *Foxk1* expression based on X-gal histochemistry. When I overexpressed the same 4.6 kb Foxk1 promoter fused to a *luciferase* reporter and transfected C2C12 myoblasts with two myogenic progenitor cell markers, Sox8 and Sox15, only Sox15 was capable of potently activating the 4.6 kb Foxk1-*luc* reporter in a dose-dependant fashion. Mutagenesis of the SBE resulted in an over 90% ablation of transcriptional activity of the 4.6 kb Foxk1-*luc* reporter. Electrophoretic mobility shift assays of *in vitro* with recombinant Sox15 demonstrated that it was capable of binding to the radiolabeled SBE in the mouse and human *Foxk1* promoters and that the binding could be competed with cold competitor oligo and the protein-DNA complex supershifted with antibody against the myc-tagged Sox15.

RNA interference of Sox15 in C2C12 myoblasts resulted in a significant arrest of cells in the G0/G1 state of the cell cycle, and a dysregulation of the cyclin-dependent kinase inhibitor p21. Sox15 mutant mice fail to fully regenerate their skeletal muscle following a cardiotoxin-induced injury. Myoblasts isolated from Sox15 mutant mice, have a dysregulated myogenic transcriptome and perturbed cellular kinetics. Sox15 mutant mice have overall decreased numbers of total myogenic progenitor cells in the muscles based on electron microscopy analysis of their TA muscles. Together, these studies establish Sox15 as a potent transcriptional activator of Foxk1 in myogenic progenitor cells, and an essential regulator of normal MPC cell cycle maintenance and kinetics.

Sin3a Expression in Skeletal Muscle is Essential For Mouse Viability and Regulation of Mitochondrial Mass

Sin3a is a repressor of gene transcription and is essential for maintenance of chromosomal stability and normal gastrulation. I have characterized a Myo-*cre* transgenic mouse line that expresses *cre* recombinase in all myogenic-derived lineages, including myogenic progenitor cells. To further elucidate the *in vivo* function of Sin3a, I have combinatorially mated the Sin3a conditional knockout mouse line to the Myo-*cre* transgenic line, to generate Sin3a-muscle knockout mice. Mice lacking Sin3a in their skeletal muscle are perinatal lethal, and expire

within six hours after birth. Sin3a-deficient diaphragms, hindlimbs, and other myogenic-progenitor derived muscle appear histologically normal compared to their wild type littermates. Using transmission electron microscopy, I have observed that Sin3a muscle-deficient mice have abnormally large mitochondria resulting in a disruption of the muscle ultrastructure. Sin3a-deficient muscle also has increased apoptosis and myonecrosis around the myotendous junctions of the rib cage. Transcriptome analysis of Sin3a muscle-deficient gastrocnemises revealed a significant number of genes essential for the regulation of mitochondrial respiration and fatty acid synthesis. Together these studies identify a critical role for Sin3a in the regulation of myoblast mitochondrial function and mouse viability.

Foxj3 Transcriptionally Activates Mef2c, and is Essential for Muscle Fiber Type Identity

Foxj3 is a relatively uncharacterized *forkhead* transcription factor that is upregulated during C2C12 myoblast differentiation. Previous studies have demonstrated that Foxj3 is enriched in the developing heart, limb buds, and neural tube during embryogenesis. To further characterize the function of Foxj3, I have generated Foxj3-/- mice from a BayGenomics™ "gene-trapped" ES cell line.

Mice homozygous for the targeted allele are viable, but are growth retarded compared to their wild type littermates. X-gal histochemistry revealed confirmed that Foxj3 is expressed in the developing myotome, heart, and neural tube until E12.5, when its expression becomes ubiquitous. The skeletal muscle of Foxj3-/- mice is redder with reduced fat pads. Additionally, Foxj3 mutant mice have severely reduced numbers of Type I myofibers and have a noticeably abnormal gait. Foxj3-/- mice fail to fully regenerate their skeletal muscle following a cardiotoxin-induced injury. Myoblasts isolated from Foxj3-/- mice proliferate at a faster rate, and have dysregulated MEF2-dependant transcriptome.

Sequence analysis of the *Mef2c* skeletal muscle enhancer revealed an evolutionarily conserved *forkhead* binding site. Transfection of the Mef2c skeletal muscle enhancer fused to a *luciferase* reporter in C2C12 myoblasts along with Foxj3 resulted in an eight fold activation of the reporter in a dose-dependent manner. Mutagenesis of the *forkhead* binding site severely ablated Foxj3's ability to activate the Mef2c skeletal muscle reporter. Correspondingly to the Mef2c bone conditional knockout mice, mice lacking Foxj3 have an abnormally calcified skeleton and develop chondrocyte hypertrophy as they develop into adulthood. Together these studies establish Foxj3 as a transcriptional activator of *Mef2c*, and an essential regulator of muscle fiber type identity and normal bone development.

Chapter 6

Future Directions and Experiments

Chapter 2: Identification of novel regulators of Foxk1 transcription

Sox15's recruitment to the SBE located in the 5' upstream region of the Foxk1 promoter is essential for Foxk1's transcriptional activation. However, Sox proteins have been previously shown to interact with partner proteins to modulate gene transcription (see Figure 2). An important question to address is what partner proteins interact with Sox15 to regulate Foxk1 transcription in MPCs? In data not shown, a yeast-two-hybrid with Sox15 as bait identified Fhl3 as being a direct interacting partner with Sox15. Later experiments using GST-pull downs and transfection assays in C2C12 myoblasts would identify the specificity and functional role for this interaction. Fhl3 was shown to synergistically activate Foxk1 transcription through its interaction with Sox15 at the SBE (Meeson *et al*, 2007). An important experiment to conduct would involve the overexpression of Sox15 in a myoblast cell line, and the useage of mass spectrometry to identify novel interacting partners (both direct and indirect) of Sox15 in a more relevant cell population (cultured myoblasts as opposed to the yeast-two-hybrid screen).

However, Fhl3 and Sox15 are not found in all of the same cellular populations in which Foxk1 has sustained expression (Garry *et al*, 1997). Foxk1 has sustained expression in dopaminergic neurons of adult mammals of which Sox15 and Fhl3 are not detected (Wijchers *et al*, 2006). The question remains, what Sox factors might regulate Foxk1 transcription in this neuronal population? Of the Sox factors that have sustained expression in the brain (in particularly the dopaminergic regions), Sox1, Sox10, and Sox21 are all strongly enriched in these regions and are the strongest candidate genes. Sox8, while expressed in the same regions as Foxk1 in the brain, did not activate the Foxk1-*luciferase* reporter in myoblasts, fibroblasts, and kidney cells (see Figure 6). Sox1, Sox10, and Sox21 have all been implicated as regulators of neuronal progenitor maintenance through loss of function and RNAi knockdown studies (Sock *et al*, 2001; Sandberg *et al*, 2005). A simple experiment would be to perform immunohistochemistry using a β-*galactosidase* (Foxk1) and various Sox antibodies on the brain sections from the 4.6 kb Foxk1-*lacZ* transgenic mouse line.

A more conclusive experiment to address the question of what additional Sox factors regulate Foxk1 transcription in non-myogenic populations would be to generate a Foxk1-*gfp* knockin transgenic mouse. Specific regions from the brains of these Foxk1-*gfp* heterozygote mice could be isolated, and the cells can

be sorted for GFP (Foxk1) expression and later microarray analysis in an *in vivo* context. Additional studies in other cell populations would prove fruitful in identifying novel regulators of Foxk1 transcription in both myogenic and non-myogenic populations.

Chapter 3: Determination of the extent of Sin3b's functional redundancy with Sin3a in myogenic lineages

As mentioned earlier, it has been previously demonstrated that Sin3b plays a functional redundancy with Sin3a in the regulation of specific gene promoters. However, it is also been proven that this functional redundancy is not complete, and that Sin3b has unique functions in the repression of specific gene transcription during various developmental time points.

To address what areas Sin3b and Sin3a have a functional overlap and what are unique to Sin3a, I would propose to generate two inducible knockin transgenic mouse lines that would each turn on Sin3a or Sin3b using the other's endogenous gene promoter. Intramuscular injections of the inducing agent (such as doxcycline or polyinosine-polycytidylic acid) would specifically activate the knockin Sin3 gene, and allow for study of Sin3 functional redundancy using adult muscle injury models. The Sin3a/b knockin inducible mouse lines would serve

as an important model for understanding what in vivo tissues and cellular populations Sin3a or Sin3b play a redundant role, and what functions are unique to each gene.

Chapter 4: Characterizing a functional role for Foxj3 in the adult heart

Foxj3 is significantly expressed in both the developing and the adult heart (Landgren and Carlsson, 2004; Alexander *et al*, 2007, *in preparation*). As I have mentioned previously, I have generated a Foxj3 trangenic mouse line that has a functional β-*galactosidase* gene fused to Foxj3 to utilize as a marker of its expression. X-gal histochemistry and RTPCR revealed that Foxj3 is expressed in the myonuclei of the endocardium, aorta, and a few cells in the epicardium of the adult mammalian heart (data not shown). Additionally, it has been reported by Hannenhalli and colleagues that Foxj3 is significantly upregulated along with other forkhead factors, in human heart failure microarray data (Hannenhalli *et al*, 2006). Together, these studies ask the question of what functional role does Foxj3 play in the adult heart, particularly under the condition of cardiac hypertrophy.

To better understand the role of Foxj3 in cardiac hypertrophy, I would propose using the Foxj3 mutant mouse model. Foxj3-/- hearts have no gross defects and

have normal heart/body weight ratios (data not shown). I would propose using thorasic aortic banding (TAB) both wild type and Foxj3 mutant mice and evaluating their hypertrophic response (i.e. survival, heart/body weight ratio, gross histology, etc.) and performing microarray analyses on the hearts. Similar studies have been done by Ni and colleagues have proven fruitful in identifying Foxo3 as being an essential regulator of the Akt pathway during heart failure (Ni *et al*, 2006). A similar study using the Foxj3 mutant hearts might identify the pathway activated by increased levels of Foxj3 during heart failure.

It is important to note that Foxj3 is an activator of *Mef2c* transcription through a skeletal muscle-specific enhancer; however, it is unclear if it activates *Mef2c* transcriptions in other tissues. A quick survey of the Mef2c cardiac-specific enhancer (a 6 kb enhancer described in Wang *et al*, 2001) reveals two potential *forkhead* binding sites. Transfections with the 6 kb enhancer fused to a luciferase reporter and Foxj3 in either primary cardiomyocytes or an cardiomyocyte-derived line (such as H9C2 myocytes), might validate *Mef2c* as a downstream target of Foxj3. One problem might be that the cardiomyocyte transfection conditions might have to mimic that of heart failure, which can be done using cell culture hypertrophic mimics such as phenylephrine or isoproterenol. However, the best way to determine the functional role of Foxj3 during heart failure might be to undertake a Chip-on-chip approach on Foxj3

mutant and wild type mice. This experiment would no doubt reveal potential downstream target genes of Foxj3 during heart failure, as well as identify the consequences of a lack of Foxj3 in the heart.

In conclusion, the studies that I have presented provide a molecular mechanism for the regulation of the MPC population. Whereas, there is a great deal of information in regards to the physiological processes that affect MPC regulation, little is known about the molecular pathways that govern their function. Through the characterization of the molecular pathways that regulate Foxk1 and Foxj3, I have identified a novel level of transcriptional control that is essential for MPC cell cycle kinetics and myogenesis at the transcriptional level.

Supplemental Data

Chapter 2:

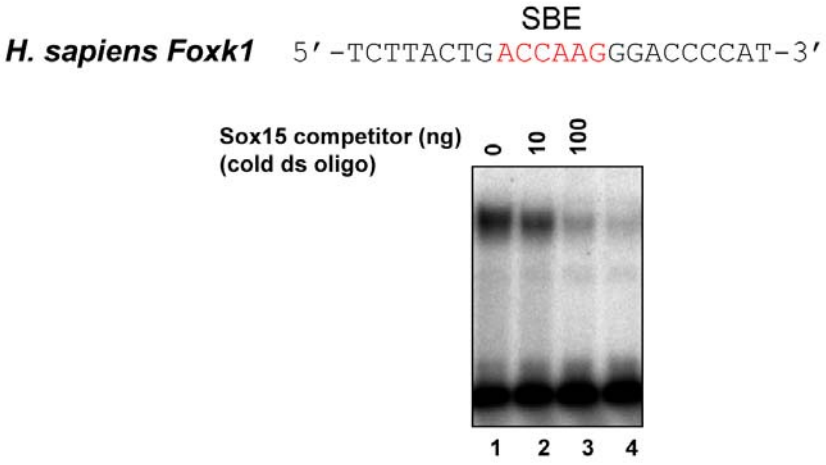
Supplemental Table 1.

Foxk1 reporter expression during embryogenisis

				MPC	
Reporter fragment	**E9.5.**	**E11.0**	**E13.5**	**Adult**	***mdx***
4.6 kb - lacZ	-	+	+++	+++	++
1.6 kb - lacZ	-	+	++	-	N/A
0.6 kb - lacZ	-	-	++	-	N/A

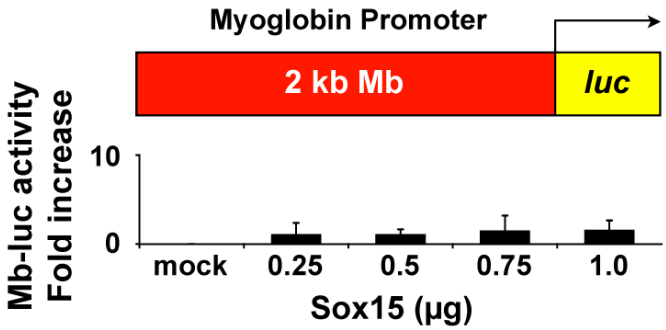
Supplemental Table 1. Foxk1 reporter expression observed during embryogenesis. Table summarizing the X-gal reporter expression observed in 3 separate Foxk1-lacZ promoter transgenic mouse lines. Only the 4.6 kb Foxk1-lacZ transgenic line showed reporter expression during early embryogenesis and in both unperturbed and myopathic (*mdx*) mouse models. N/A not applicable or examined.

Supplemental Figure 1.



Supplemental Figure 1. EMSA of mouse Sox15 binds to the human Foxk1 SBE. Recombinant mouse Sox15 is capable of binding to the human Foxk1 radiolabeled probe. Lane 1, recombinant murine Sox15 protein forms a protein-DNA complex with the human Foxk1 SBE. Lanes 2 and 3, increasing amounts of unlabled "cold" ds oligo can compete out the Sox15-Foxk1 SBE protein-DNA complex. Lane 4, endogous protein control.

Supplemental Figure 2.



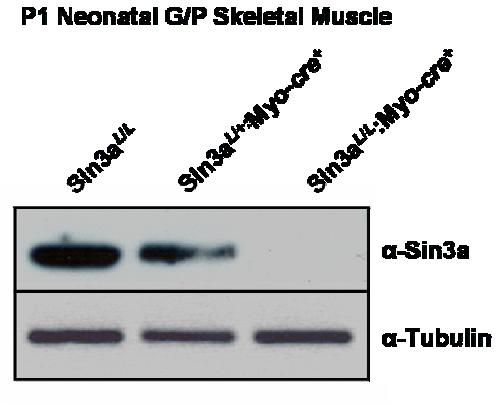
Supplemental Figure 2. Sox15 does not activate the human *myoglobin* promoter. Overexpression of Sox15 cannot activate the well-characterized human *myoglobin* reporter (2 kb Mb) in transfected C2C12 myoblasts. 10^5 C2C12 myoblasts transfected with 0.5 µg and increasing amounts of Sox15 and harvested 48 hours post transfection.

Supplemental Table 2.

RT-PCR primers (5'-3')	
Foxk1 F	GCAGGAAGGTGGAGTGAAAC
Foxk1 R	CCATGGGAGGACAGGAGATA
Sox15 F	CGGCGTAAGAGCAAAAACTC
Sox15 R	TGGATCACTCTGAGGGAAG
Rn18s F	CTCAACACGGGAAACCTCAC
Rn18s R	TGCCAGAGTCTCGTTCGTTAT
Cdc2a F	GGTCCGTCGTAACCTGTTGA
Cdc2a R	CTCCTTCTTCCTCGCTTTCA
Cdkn1a F	TTGCACTCTGGTGTCTGAGC
Cdkn1a R	CTGCGCTTGGAGTGATAGAA
Cdkn1b F	TTGGGTCTCAGGCAAACTCT
Cdkn1b R	TCTGTTCTGTTGGCCCTTTT
Trp53 F	AAGTCCTTTGCCCTGAACTG
Trp53 R	CTGTAGCATGGGCATCCTTT
Ccnb1 F	AACCTGAGCCTGAACCTGAA
Ccnb1 R	GCGTCTACGTCACTCACTGC
Ccnd1 F	CGGATGAGAACAAGCAGACC
Ccnd1 R	GCAGGAGAGGAAGTTGTTGG
Fhl3 F	TGAGTGTTACTGCACAGCCTTC
Fhl3 R	GCTGATCACGATATGTCACTCC
Mb F	CAAGATCCCGGTCAAGTACC
Mb R	AAGTCCCCGGAATGTCTCTT
Gapdh F	GTGGCAAAGTGGAGATTGTTGCC
Gapdh R	GATGATGACCCGTTTGGCTCC

Supplemental Table 2. Quantiative and semiquantitative RT-PCR primers used in these experiments. All primers diluted to 10 μM concentrations in sterile water.

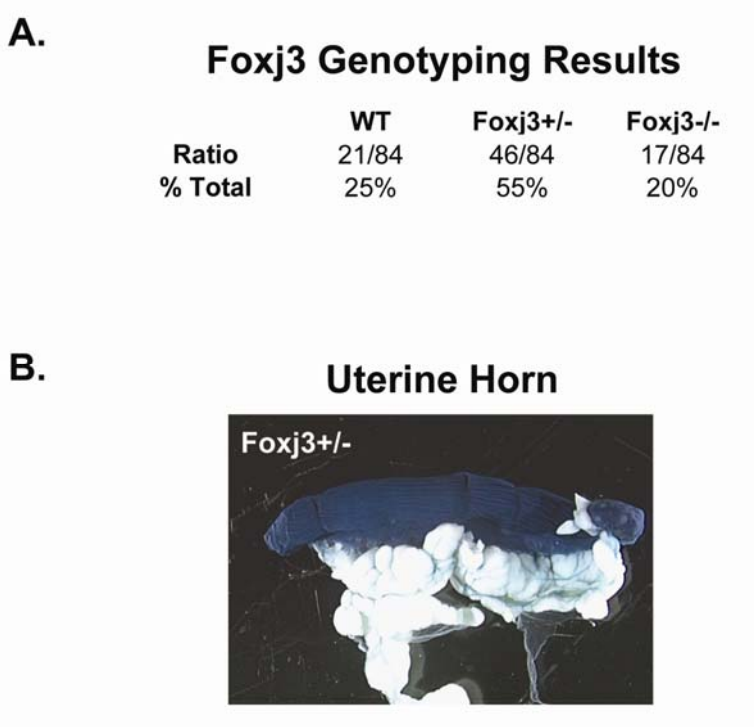
Chapter 3:



Supplemental Figure 3. Sin3a is not expressed in Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pup skeletal muscle. Western blot of G/P skeletal muscle homogenates taken from 2-hour-old Sin3a$^{L/L}$, Sin3a$^{L/+}$:Myo-*cre*$^{+}$, Sin3a$^{L/L}$:Myo-*cre*$^{+}$ pups. 20 µg of total protein run on a 7.5% polyacrylamide gel. Anti-tubulin used as a total input of protein loading control.

Chapter 4:

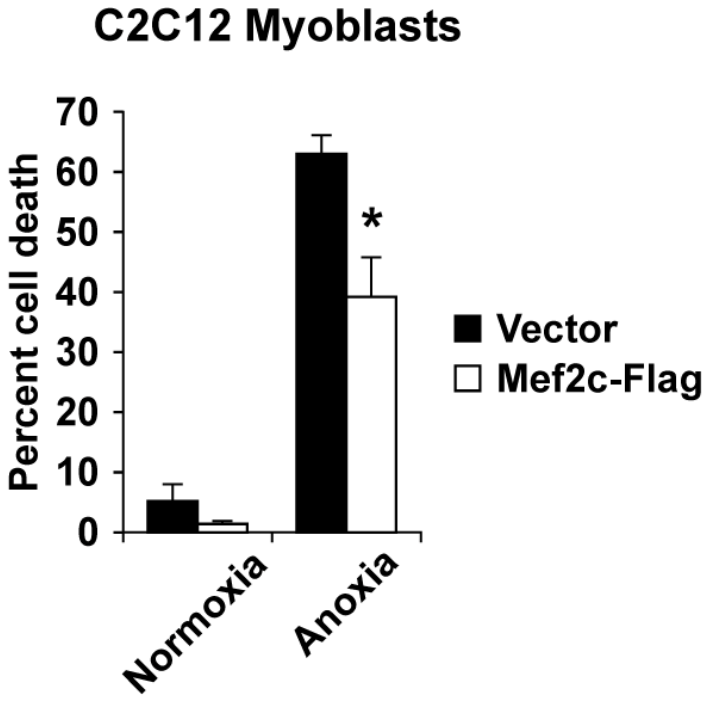
Supplemental Figure 4.



Supplemental Figure 4. Foxj3 is essential for female fertility but not viability. A Table summarizing the genotyping results from 8 separate Foxj3 heterozygote genotyping crosses. Foxj3 heterozygote crosses result in live births at Medelian ratios. **B** X-gal histochemistry of a Foxj3+/- 4-month-old

female reveals strong staining (and subsequently Foxj3 expression) in the uterine horn.

Supplemental Figure 5.



Supplemental Figure 5. Overexpression of Mef2c protects C2C12 myoblasts from anoxia-induced apoptosis. Transfection of Mef2c-Flag in C2C12 myoblasts results in a significant (63% in vector alone versus 39%) decrease in cell dealth in C2C12 myoblasts exposed to anoxia (0% oxygen) for 16 hours. Normoxic conditions consist of cells grown in 20% oxygen. Cell death is measured as the total number of trypan blue positive cells out of 500 total cells (n = 3 replicates). * p value < 0.05.

Supplemental Table 3

RT-PCR Primers

Primer	Sequence
Foxj3_F	5’ GCTTGTCCATCTGTAACTTC 3’
Foxj3_R	5’ CTATGCTATATGGAGTTGAGGC 3’
Mef2c_F	5’ GTCTCACCTGGTAACCTGAACAAG 3’
Mef2c_R	5’ GCAGATGGCGGCATGTTATGTAGG 3’
Mef2d_F	5’ CCACACGAGAGCCGCACCAATGC 3’
Mef2d_F	5’ GGCAAAGTTGGGGGCCGGAACAG 3’
Tnni1_F	5’ GAAATCCAAGATCACTGCCTCCC 3’
Tnni1 _R	5’ CCACCACCTCTACCTTGGCATG 3’
Tnnt2 _F	5’ GCCACCCAAGATCCCCGATGGAG 3’
Tnnt2 _R	5’ CCTCCTCCTCACGCCGGGCCCTC 3’
Cdkn1a_F	5’ TTGCACTCTGGTGTCTGAGC 3’
Cdkn1a_R	5’ CTGCGCTTGGAGTGATAGAA 3’
Gapdh_F	5’ GTGGCAAAGTGGAGATTGTTGCC 3’
Gapdh_R	5’ GATGATGACCCGTTTGGCTCC 3’
18sRb_F	5' CTCAACACGGGAAACCTCAC 3'
18sRb_R	5' TGCCAGAGTCTCGTTCGTTAT 3'

Supplemental Table 3. RT-PCR primers used in Foxj3-related experiments.

Primers were used for both semi-quantiative and quantitative RT-PCR.

Bibliography

1. Ang S.L. and Rossant, J. HNF-3 beta is essential for node and notochord formation in mouse development. *Cell*. 1994. 78(4):561-574.

2. Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., Olson, E.N. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev. Cell*. 2007. 12(3):377-389.

3. Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L, Vivian, N., and Lovell-Badge, R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes. Dev*. 2003. 17(1):126-140.

4. Ayer, D.E., Lawrence, Q.A., and Eisenman, R.N. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell*. 1995. 80(5):767-777.

5. Bailey, P., Downes, M., Lau, P., Harris, J., Chen, S.L., Hamamori, Y., Sartorelli, V., and Muscat, G.E. The nuclear receptor corepressor N-

CoR regulates differentiation: N-CoR directly interacts with MyoD. *Mol. Endocrinol*. 1999. 13(7):1155-1168.

6. Baker, P.W., Tanaka, K.K., Klitgord, N., Cripps, R.M. Adult myogenesis in Drosophila melanogaster can proceed independently of myocyte enhancer factor-2. *Genetics*. 2005. 170(4):1747-1759.

7. Bassel-Duby, R., Hernandez, M.D., Yang, Q., Rochelle, J.M., Seldin, M.F., and Williams, R.S. Myocyte nuclear factor, a novel winged-helix transcription factor under both developmental and neural regulation in striated myocytes. *Mol. Cell. Biol*. 1994. 14(7):4596-4605.

8. Ben-Yair, R. and Kalcheim, C. Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates. *Development*. 2005. 132(4):689-701.

9. Béranger, F., Méjean, C., Moniot, B., Berta, P., and Vandromme, M. Muscle differentiation is antagonized by SOX15, a new member of the SOX protein family. *J. Biol. Chem*. 2000. 275(21):16103-16109.

10. Bergsland, M., Werme, M., Malewicz, M. Perlmann, T., and Muhr, J. The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev*. 2006. 20(24):3475-3486.

11. Berkes, C.A., Tapscott, S.J. MyoD and the transcriptional control of myogenesis. *Semin. Cell Dev. Biol*. 2005. 16(4-5):585-595.

12. Blais, A., Tsikitis, M., Acosta-Avear, D., Sharan, R., Kluger, Y., Dynlacht, B.D. An initial blueprint for myogenic differentiation. *Genes Dev*. 2005. 19(5):553-569.

13. Blomqvist, S.R., Vidarsson, H., Söder, O., and Enerbäch, S. Epididymal expression of the forkhead transcription factor Foxi1 is required for male fertility. *EMBO. J*. 2006. 25(17):4131-4141.

14. Bois, P.R. and Grosveld, G.C. FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. *EMBO. J*. 2003. 22(5):1147-1157.

15. Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G.,

Gifford, D.K., Melton, D.A., Jaenisch, R., and Young, R.A. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*. 2005. 122(6):947-956.

16. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., Arnold, H.H. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J*. 1989. 8(3):701-719.

17. Braun, T., Rudnicki, M.A., Arnold, H.H., and Jaenisch, R. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell*. 1992. 71(3):369-382.

18. Brennan, K., Baylies, M., and Arias, A.M. Repression by notch is required before Wingless signaling during muscle progenitor cell development in Drosophila. *Curr. Biol*. 1999. 9(13):707-710.

19. Bridgewater, L.C., Walker, M.D., Miller, G.C., Ellison, T.A., Holsinger, L.D., Potter, J.L., Jackson, T.L., Chen, R.K, Winkel, V.L, Zhang, Z., McKinney, S., and de Crumbrugghe, B. 2003. Adjacent DNA sequences modulate Sox9 transcriptional activation at paired Sox sites in

three chondrocyte-specific enhancer elements. *Nucleic Acids Res*. 2003. 31(5):1541-1553.

20. Brody, S.L., Yan, X.H., Wuerffel, M.K., Song, S.K., and Shapiro, S.D. Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. *Am. J. Respir. Cell Mol. Biol*. 2000. 23(1):45-51.

21. Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y., Hu, L.S., Cheng, H.L., Jedrychowski, M.P., Gygi, S.P., Sinclair, D.A., Alt, F.W., and Greenberg, M.E. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science*. 2004. 303(5666):2011-2015.

22. Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. The formation of skeletal muscle: from somite to limb. *J. Anat*. 2003. 202(1):59-68.

23. Castrillion, D.H., Miao, L., Kollipara, R., Horner, J.W., and DePinho, R.A. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*. 2003. 301(5630)215-218.

24. Catena, R., Tiveron, C., Ronchi, A., Porta, S., Ferri, A., Tatangelo, L., Cavallaro, M., Favaro, R., Ottolenghi, S., Reinbold, R., Schöler, H., and Nicolis, S.K. Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells. *J. Biol. Chem.* 2004. 279(40):41846-41857.

25. Chang, S., Bezprozvannaya, S., Li, S., and Olson, E.N. An expression screen reveals modulators of class II histone deacetylase phosphorylation. *Proc. Natl. Acad. Sci*. 2005. 102(23):8120-8125.

26. Chen, J., Knowles, H.J., Herbert, J.L., and Hackett, B.P. Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. *J. Clin. Invest*. 1998. 102(6):1077-1082.

27. Chen, J.C., Mortimer, J., Marley, J., and Goldhamer, D.J. MyoD-cre transgenic mice: a model for conditional mutagenesis and lineage tracing of skeletal muscle. *Genesis*. 2005. 41(3):116-121.

28. Cheng, T.C., Wallace, M.C., Merlie, J.P., and Olson, E.N. Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science*. 1993. 261(5118):215-218.

29. Chew, J.L., Loh, Y.H., Zhang, W., Chen, X., Tam, W.L., Yeap, L.S., Li, P., Ang, Y.S., Lim, B., Robson, P., and Ng, H.H. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol. Cell. Biol*. 2005. 25(14):6031-6046.

30. Conboy, I.M. and Rando, T.A. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev. Cell*. 2002. 3(3):397-409.

31. Cowley, S.M., Iritani, B.M., Mendrysa, S.M., Xu, T., Cheng, P.F., Yada, J., Liggett, H.D., and Eisenmann, R.N. The mSin3A chromatin-modifying complex is essential for embryogenesis and T-cell development. *Mol. Cell. Biol*. 2005. 25(16):6990-7004.

32. Chen, J.F. Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L., and Wang, D.Z. The role of microRNA-1

and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genetics*. 2006. 38(2):228-233.

33. Davis, R.L., Weintraub, H., and Lasser, A.B. Expression of a single cDNA converts fibroblasts to myoblasts. *Cell*. 1987. 51(6):987-1000.

34. Dannenberg, J.H., David, G., Zhong, S., van der Torre, J., Wong, W.H., and DePinho, R.A. mSin3A corepressor regulates diverse transcriptional networks governing normal and neoplastic growth and survival. *Genes. Dev*. 2005. 19(13):1581-1595.

35. De Felice, M., Ovitt, C., Biffali, E., Rodriguez-Mallon, A., Arra, C., Anastassiadis, K., Macchia, P.E., Mattei, M.G., Mariano, A., Schöler, H., Macchia, V., and Di Lauro, R. A mouse model for hereditary thyroid dysgenesis and cleft palate. *Nat. Genet*. 1998. 19(4):395-398.

36. Denny, P., Swift, S., Brand, N., Dabhade, N., Barton, P., and Ashworth, A. A conserved family of genes related to the testis determining gene, SRY. *Nucleic Acids Res*. 1992. 20(11):2887.

37. Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W.,

Koenderman, L, and Coffer, P.J. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol. Cell. Biol*. 2000. 20(24):9138-9148.

38. Di Lisi, R., Millino, C., Calabria, E., Altruda, F., Schiaffino, S., Ausoni, S. Combinatorial cis-acting elements control tissue-specific activation of the cardiac troponin I gene in vitro and in vivo. *J. Biol. Chem*. 1998. 273(39):25371-25380.

39. Duncan, S.A., Navas, M.A., Dufort, D., Rossant, J., and Stoffel, M. Regulation of a transcription factor network required for differentiation and metabolism. *Science*. 1998. 281(5377):692-695.

40. Durocher, D. and Jackson, S.P. The FHA domain. *FEBS. Lett*. 2002. 513(1):58-66.

41. Ehebauer, M., Hayward, P. and Martinez-Arias, A. Notch signaling pathway. *Science STKE*. 2006. 364:cm7(1-4).

42. Flanagan, S.P. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet. Res*. 1966. 8(3):295-309.

43. Fleischer, T.C., Yun, U.J., and Ayer, D.E. Identification and characterization of three new components of the mSin3A corepressor complex. *Mol. Cell. Biol*. 2003. 23(10):3456-3467.

44. Foglietti, C., Filocamo, G., Cundari, E., De Rinaldis, E., Lahm, A., Cortese, R., and Steinkühler, C. Dissecting the biological functions of Drosophila histone deacetylases by RNA interference and transcriptional profiling. *J. Biol. Chem*. 2006. 281(26):17968-17976.

45. Freddie, C.T., Ji, Z., Marais, A., and Sharrocks, A.D. Functional interactions between the Forkhead transcription factor FOXK1 and the MADS-box protein SRF. *Nucleic Acids. Res*. 2007. 35(15):5203-5212.

46. Garry, D.J., Bassel-Duby, R.S., Richardson, J.A., Grayson, J., Neufer, P.D., Williams, R.S. Postnatal development and plasticity of specialized muscle fiber characteristics in the hindlimb. *Dev. Genet*. 1996. 19(2):146-156.

47. Garry, D.J., Yang, Q., Bassel-Duby, R., and Williams, R.S. Persistent expression of MNF identifies myogenic stem cells in postnatal muscles. *Dev. Biol*. 1997. 188(2):280-294.

48. Garry, D.J., Meeson, A., Elterman, J., Zhao, Y., Yang, P., Bassel-Duby, R., and Williams, R.S. Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. *Proc. Natl. Acad. Sci*. 2000. 97(10):5416-5421.

49. Giebel, B. The notch signaling pathway is required to specify myogenic progenitor cells in Drosophila. *Mech. Dev*. 1999. 86(1-2):137-145.

50. Goetsch, S.C., Hawke, T.J., Gallardo, T.D., Richardson, J.A., and Garry, D.J. Transcriptional profiling and regulation of the extracellular matrix during muscle regeneration. *Physiol. Genomics*. 2003. 14(3):261-271.

51. Granadino, B., Arias-de-la-Fuente, C., Pérez-Sánchez, C., Párraga M., López-Fernández, L.A., del Mazo, J., and Rey-Campos, J. Fhx (Foxj2) expression is activated during spermatogenesis and very early in embryonic development. *Mech. Dev*. 2000. 97(1-2):157-160.

52. Guo, Y., Costa, R., Ramsey, H., Starnes, T., Vance, G., Robertson, K., Kelley, M., Reinbold, R., Schöler, H., and Hromas, R. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proc. Natl. Acad. Sci*. 2002. 99(6):3663-3667.

53. Hanna, L.A., Foreman, R.K., Tarasenko, I.A., Kessler, D.S., and Labosky, P.A. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. *Genes. Dev*. 2002. 16(20):2650-2661.

54. Hannenhalli, S., Putt, M.E., Gilmore, J.M., Wang, J., Parmacek, M.S., Epstein, J.A., Morrisey, E.E., Margulies, K.B., and Cappola, T.P. Transcriptional genomics associates FOX transcription factors with human heart failure. *Circ. Res*. 2006. 114(12):1269-1276.

55. Hatini, V., Huh, S.O., Herzlinger, D., Soares, V.C., and Lai, E. Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. *Genes. Dev*. 1996. 10(12):1467-1478.

56. Hawke, T.J. and Garry, D.J. Myogenic satellite cells: physiology to molecular biology. *J. Appl. Physiol*. 2001. 91:531-551.

57. Hawke, T.J., Jiang, N., and Garry, D.J. Absence of $p21^{CIP}$ rescues myogenic progenitor cell proliferative and regenerative capacity in Foxk1 null mice. *J. Biol. Chem*. 2003. 278(6):4015-4020.

58. Heglind, M., Cederberg, A., Aquino, J., Lucas, G., Ernfors, P., and Enerbäck, S. Lack of the central nervous system- and neural crest-expressed forkhead gene Foxs1 affects motor function and body weight. *Mol. Cell. Biol*. 2005. 25(13):5616-5625.

59. Heidt, A.B. and Black, B.L. Transgenic mice that express Cre recombinase under control of a skeletal muscle-specific promoter from mef2c. *Genesis*. 2005. 42(1):28-32.

60. Heinzel, T., Lavinsky, R.M., Mullen, T.M., Söderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., Seto, E., Eisenmann, R.N., Rose, D.W., Glass, C.K., and Rosenfeld, M.G. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature*. 1997. 387(6628):43-48.

61. Heyer, W.D., Thuriaux, P., Kohli, J., Ebert, P., Kersten, H., Gehrke, C., Kuo, K.C., and Agris, P.F. An antisuppressor mutation of *Schizosaccharomyces pombe* affects the post-transcriptional modification of the "wobble" base in the anticodon of tRNAs. *J. Biol. Chem*. 1984. 259(5):2856-2862.

62. Hiraoka, Y., Ogawa, M., Sakai, Y., Taniguichi, K., Fujii, T., Umezawa, A., Hata, J., and Aiso, S. Isolation and expression of a human SRY-related cDNA hSOX20. *Biochem. Biophys. Acta*. 1998. 1396(2):132-137.

63. Hoodless, P.A., Pye, M., Chazaud, C., Labbé, E., Attisano, L., Rossant, J., and Wrana, J.L. FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. *Genes. Dev*. 2001. 15(10):1257-1271.

64. Hosaka, T., Biggs, W.H., Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., and Arden, K.C. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc. Natl. Acad. Sci*. 2004. 101(9):2975-2980.

65. Hribal, M.L., Nakae, J., Kitamura, T., Shutter, J.R., and Accili, D. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J. Cell. Biol*. 2003. 162(4):535-541.

66. Huang, J.T. and Lee, V. Identification and characterization of a novel human FOXK1 gene in silico. *Int. J. Oncol.* 2004. 25(3):751-757.

67. Hubbard, T.J., Aken, B.L., Beal, K., Ballester, B., Caccamo, M., Chen, Y., Clarke, L., Coates, G., Cunningham, F., Cutts, T., Down, T., Dyer, S.C., Fitzgerald, S., Fernandez-Banet, J., Graf, S., Haider, S., Hammond, M., Herrero, J., Holland, R., Howe, K., Howe, K., Johnson, N., Kahari, A., Keefe, D., Kokocinski, F., Kulesha, E., Lawson, D., Longden, I., Melsopp, C., Megy, K., Meidl, P., Ouverdin, B., Parker, A., Prlic, A., Rice, S., Rios, D., Schuster, M., Sealy, I., Severin, J., Slater, G., Smedley, D., Spudich, G., Trevanion, S., Vilella, A., Vogel, J., White, S., Wood, M., Cox, T., Curwen, V., Durbin, R., Fernandez-Suarez, X.M., Flicek, P., Kasprzyk, A., Proctor, G., Searle, S., Smith, J., Ureta-Vidal, A., and Birney, E. Ensembl 2007. *Nucleic Acids Res*. 2007. 35:D610-617.

68. Huh, M.S., Parker, M.H., Scimè, A., Parks, R., and Rudnicki, M.A. Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J. Cell. Biol*. 2004. 166(6):865-876.

69. Hulander, M., Wurst, W., Carlsson, P., and Enerbäck, S. The winged helix transcription factor Fkh10 is required for normal development of the inner ear. *Nat. Genet*. 1998. 20(4):374-376.

70. Iida, K., Koseki, H., Kakinuma, H., Kato, N., Mizutani-Koseki, Y., Ohuchi, H., Yoshioka, H., Noji, S., Kawamura, K., Kataoka, Y., Ueno, F., Taniguchi, M., Yoshida, N., Sugiyama, T., and Miura, N. Essential roles of the winged helix transcription factor MFH-1 in aortic arch patterning and skeletogenesis. *Development*. 1997. 124(22):4627-4638.

71. Kaestner, K.H., Silberg, D.G., Traber, P.G., and Schütz, G. The mesenchymal winged helix transcription factor Fkh6 is required for the control of gastrointestinal proliferation and differentiation. *Genes. Dev*. 1997. 11(12):1583-1595.

72. Kaestner, K.H., Hiemisch, H., and Schütz, G. Targeted disruption of the gene encoding hepatocyte nuclear factor 3γ results in reduced transcription of hepatocyte-specific genes. *Mol. Cell. Biol*. 1998. 18(7):4245-4251.

73. Kaestner, K.H., Knochel, W., and Martinez, D.E. Unified nomenclature for the winged helix/*forkhead* transcription factors. *Genes. Dev*. 2000. 14(2):142-146.

74. Kasser-Duchossoy, L, Gayroud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. Mrf4 determines skeletal muscle identity in MyoD-Myf5 double-mutant mice. *Nature*. 2004. 431(7007):466-471.

75. Kasten, M.M., Ayer, D.E. and Stillman, D.J. SIN3-dependent transcriptional repression by interaction with the Mad1 DNA-binding protein. *Mol. Cell. Biol*. 1996. 16(8):4215-4221.

76. Kellerer, S., Schreiner, S., Stolt, C.C., Scholz, S., Bösl, M.R., and Wegner, M. Replacement of the Sox10 transcription factor by Sox8

reveals incomplete functional equivalence. *Development*. 2006. 133(15):2875-2886.

77. Kim, J., Lo, L., Dormand, E., and Anderson, D.J. SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron*. 2003. 38(1):17-31.

78. Kitamura, T., Kitamura, Y.I., Funahashi, Y., Shawber, C.J., Castrillon, D.H., Kollipara, R., Depinho, R.A., Kitajewski, J., Accili, D. A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. *J. Clin. Invest*. 2007. *Epub*. Aug. 24.

79. Korver, W., Schilham, M.W., Moerer, P., van den Hoff, M.J., Dam, K., Lamers, W.H., Medema, R.H., and Clevers, H. Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. *Curr. Biol*. 1998. 8(24):1327-1330.

80. Kouzarides, T. Chromatin modifications and their function. *Cell*. 2007. 128(4):693-705.

81. Kume, T., Deng, K.Y., Winfrey, V., Gould, D.B., Walter, M.A., and Hogan B.L. The forkhead/winged helix gene Mf1 is disrupted in the

pleiotropic mouse mutation congenital hydrocephalus. *Cell*. 1998. 93(6):985-996.

82. Kume, T., Deng, K., and Hogan, B.L. Minimal phenotype of mice homozygous for a null mutation in the forkhead/winged helix gene, Mf2. *Mol. Cell. Biol*. 2000. 20(4):1419-1425.

83. Landgren, H. and Carlsson, P. FoxJ3, a novel mammalian forkhead gene expressed in neuroectoderm, neural crest, and myotome. *Dev. Dyn*. 2004. 231(2):396-401.

84. Laudet, V., Stehelin, D., and Clevers, H. Ancestry and diversity of the HMG box superfamily. *Nucleic Acids Res*. 1993. 21(10):2493-2501.

85. Lee, H.J., Göring, W., Ochs, M., Mühlfeld, C., Steding, G., Paprotta, I., Engel, W., and Adham, I.M. Sox15 is required for skeletal muscle regeneration. *Mol. Cell. Biol*. 2004. 24(19):8428-8436.

86. Lensch, M.W. and Daley, G.Q. Origins of mammalian hematopoiesis: in vivo paradigms and in vitro models. *Curr. Top. Dev. Biol*. 2004. 60:127-196.

87. Li, B., Samanta, A., Song, X., Iacono, K.T., Bembas, K., Tao, R., Basu, S., Riley, J.L., Hancock, W.W., Shen, Y., Saouaf, S.J., and Greene, M.I. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc. Natl. Acad. Sci*. 2007. 104(11):4571-4576.

88. Li, S., Mo, Z., Yang, X., Price, S.M., Shen, M.M., and Xiang, M. Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. *Neuron*. 2004. 43(6):795-807.

89. Li, S., Zhou, D., Lu, M.M., and Morrisey, E.E. Advanced cardiac morphogenesis does not require heart tube fusion. *Science*. 2004. 305(5690):1619-1622.

90. Li, S., Czutbryt, M.P., McAnally, J., Bassel-Duby, R., Richardson, J.A., Wiebel, F.F., Nordheim, A., and Olson, E.N. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. *Proc. Natl. Acad. Sci*. 2005. 102(4):1082-1087.

91. Lin, L., Spoor, M.S., Gerth, A.J., Brody, S.L., and Peng, S.L. Modulation of Th1 activation and inflammation by the NF-kappaB repressor Foxj1. *Science*. 2004. 303(5660):1017-1020.

92. Lin, Q., Schwarz, J., Bucana, C., Olson, E.N. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science*. 1997. 276(5317):1404-1407.

93. Liu, L., Rajareddy, S., Reddy, P., Du, C., Jagarlamundi, K., Shen, Y., Gunnarsson, D., Selstam, G., Boman, K., and Liu, K. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. *Development*. 2007. 134(1):199-209.

94. Liu, Z.P. and Olson, E.N. Suppression of proliferation and cardiomyocyte hypertrophy by CHAMP, a cardiac-specific RNA helicase. *Proc. Natl. Acad. Sci.* 2002. 99(4):2043-2048.

95. Livak, K.J. and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001. 25(4):402-408.

96. Lyon, M.F., Peters, J., Glenister, P.H., Ball, S., and Wright, E. The scurfy mouse mutant has previously unrecognized hematological abnormalities and resembles Wiskott-Aldrich syndrome. *Proc. Natl. Acad. Sci*. 1990. 87(7):2433-2437.

97. Ma, K., Chan, J.K., Zhu, G., and Wu, Z. Myocyte enhancer factor 2 acetylation by p300 enhances its DNA binding activity, transcriptional activity, and myogenic differentiation. *Mol. Cell. Biol*. 2005. 25(9):3575-3582.

98. MacDermot, K.D., Bonora, E., Sykes, N., Coupe, A.M., Lai, C.S., Vernes, S.C., Vargha-Khadem, F., McKenzie, F., Smith, R.L., Monaco, A.P., Fisher, S.E. Identification of FOXP2 truncation as a novel cause of developmental speech and language deficits. *Am. J. Hum. Genet*. 2005. 76(6):1074-1080.

99. Machida, S., Spangenburg, E.E., and Booth, F.W. Forkhead transcription factor FoxO1 transduces insulin-like growth factor's signal to p27Kip1 in primary skeletal muscle satellite cells. *J. Cell. Physiol*. 2003. 196(3):523-531.

100. Mahlapuu, M., Ormestad, M., Enerbäck, S., and Carlsson, P. The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. *Development*. 2001. 128(2):155-166.

101. Major, M.H. Satin. sa. *Mouse News Letter*. 1955. 12(47).

102. Mammen, P.P., Shelton, J.M., Goetsch, S.C., Williams, S.C., Richardson, J.A., Garry, M.G., and Garry, D.J. Neuroglobin, a novel member of the globin family, is expressed in focal regions of the brain. 2002. *J. Histochem. Cytochem.* 50(12):1591-1598.

103. Martin, J.F., Schwarz, J.J., and Olson, E.N. Myocyte enhancer factor (MEF) 2C: a tissue-restricted member of the MEF-2 family of transcription factors. *Proc. Natl. Acad. Sci*. 1993. 90(11):5282-5286.

104. Maruyama, M., Ichisaka, T., Nakagawa, M., and Yamanaka, S. Differential roles for Sox15 and Sox2 in transcriptional control in mouse embryonic stem cells. *J. Biol. Chem*. 2005. 280(26):24371-24379.

105. Masino, A.M., Gallardo, T.D., Wilcox, C.A., Olson, E.N., Williams, R.S., and Garry, D.J. Transcriptional regulation of cardiac progenitor cell populations. *Circ. Res*. 2004. 95(4):389-397.

106. Matsuzaki, H., Daitoku, H., Hatta, M., Aoyama, H., Yoshimochi, Y., and Fukamizu, A. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proc. Natl. Acad. Sci*. 2005. 102(32):11278-11283.

107. Mauro, A. Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 1961. 9:493-498.

108. McKinsey, T.A., Kuwahara, K., Bezprozvannaya, S., Olson, E.N. Class II histone deacetylases confer signal responsiveness to the ankyrin-repeat proteins ANKRA2 and RFXANK. *Mol. Cell. Biol*. 2006. 17(1):438-447.

109. Medina-Martinez, O., Brownell, I., Amaya-Manzanares, F., Hu, Q., Behringer, R.R., and Jamrich, M. Severe defects in proliferation and differentiation of lens cells in Foxe3 null mice. *Mol. Cell. Biol*. 2005. 25(20):8854-8863.

110. Meeson, A.P., Hawke, T.J., Graham, S., Jiang, N., Hutcheson, K., Dimaio, J., Gallardo, T.D., and Garry, D.J. Cellular and molecular regulation of skeletal muscle side population cells. *Stem Cells*. 2004. 22(7):1305-1320.

111. Meeson, A.P., Shi, X., Alexander, M.S., Williams, R.S., Allen, R.E., Jiang, N., Adham, I.M., Goetsch, S.C., Hammer, R.E., and Garry, D.J. Sox15 and Fhl3 transcriptionally coactivate Foxk1 and regulate myogenic progenitor cells. *EMBO. J*. 2007. 26(7):1902-1912.

112. Mercado, G.E. and Barr, F.G. Fusions involving PAX and FOX genes in the molecular pathogenesis of alveolar rhabdomyosarcoma: recent advances. *Curr. Mol. Med*. 2007. 7(1):47-61.

113. Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., Chen, C., Hosokawa, K., Nakauchi, H., Nakayama, K., Nakayama, K.I., Harada, M., Motoyama, N., Suda, T., and Hirao, A. Foxo3a is essential for the maintenance of the hematopoetic stem cell pool. *Cell Stem Cell*. 1:101-112.

114. Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. *Mol. Cell. Biol*. 1996. 16(6):2627-2636.

115. Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., Partridge, T., and Buckingham, M. Direct isolation of satellite cells for skeletal muscle regeneration. *Science*. 2005. 309(5743):2064-2067.

116. Montgomery, R.L., Davis, C.A., Potthoff, M.J., Haberland, M., Fielitz, J., Qi, X., Hill, J.A., Richardson, J.A., and Olson, E.N. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. *Genes. Dev*. 2007. 21(14):1790-1802.

117. Muir, A.R., Kanji A.H., and Allbrook D. The structure of the satellite cells in skeletal muscle. *J. Anat.* 1965. 99:435-444.

118. Murphy, D.B., Seemann, S., Wiese, S., Kirschner, R., Grzeschik, K.H., and Thies, U. The human hepatocyte nuclear factor 3/fork head gene FKHL13: genomic structure and pattern of expression. *Genomics*. 1997. 40(3):462-469.

119. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W.H., Arden, K.C., and Accili, D. The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev. Cell*. 2003. 4(1):119-129.

120. Ni, Y.G., Berenji, K., Wang, N., Oh, M., Sachan, N., Dey, A., Cheng, J., Lu, G., Morris, D.J., Castrillon, D.H., Gerard, R.D., Rothermel, B.A., Hill, J.A. Foxo transcription factors blunt cardiac hypertrophy by inhibiting calcineurin signaling. *Circulation*. 2006. 114(11):1159-1168.

121. Nicole, S., Desforges, B., Millet, G., Lesbordes, J., Cifuentes-Diaz, C., Vertes, D., Cao, M.L., De Backer, F., Languille, L., Roblot, N., Joshi, V., Gillis, J.M., and Melki, J. Intact satellite cells lead to remarkable protection against Smn gene defect in differentiated skeletal muscle. *J. Cell. Biol*. 2003. 161(3):571-582.

122. Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A, Kanai, Y, Kido, M, and Hirokawa, M. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell.* 1998. 95(6):829-837.

123. Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B.R., Miura, N., and Carlsson, P. Foxf1 and Foxf2 control murine gut

development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development*. 2006. 133(5):833-843.

124. Oustanina, S., Hause, G., and Braun, T. Pax7 directs postnatal renewal and propagation of muscle satellite cells but not their specification. *EMBO J*. 2004. 23(16):3430-3439.

125. Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., Jiang, S., Gilliland, D.G., Chin, L., Wong, W.H., Castrillion, D.H., and DePinho, R.A. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell*. 2007. 128(2):309-323.

126. Pennetta, G. and Pauli, D. The Drosophila Sin3 gene encodes a widely distributed transcription factor essential for embryonic viability. *Dev. Genes. Evol*. 1998. 208(9):531-536.

127. Pérez-Sánchez, C., Arias-de-la-Fuente, C., Gómez-Ferrería, M.A., Granadino, B., Rey-Campos, J. FHX.L and FHX.S, two isoforms of the human fork-head factor FHX (FOXJ2) with differential activity. *J. Mol. Biol*. 2000a. 301(4):795-806.

128. Pérez-Sánchez, C., Gómez-Ferrería, M.A., de La Fuente, C.A., Granadino, B., Velasco, G., Esteban-Gamboa, A., and Rey-Campos, J. FHX, a novel fork head factor with a dual DNA binding specificity. *J. Biol. Chem*. 2000b. 275(17):12909-12916.

129. Pile, L.A., Spellman, P.T., Katzenberger, R.J. and Wassarman, D.A. The SIN3 deacetylase complex represses genes encoding mitochondrial proteins: implications for the regulation of energy metabolism. *J. Biol. Chem*. 2003. 278(39):37840-37848.

130. Potthoff, M.J., Wu, H., Arnold, M.A., Shelton, J.M., Backs, J., McAnally, J., Qi, X., Bassel-Duby, R., and Olson, E.N. Histone deacetylase degradation andMEF2 activation promote the formation of slow-twitch myofibers. *J. Clin. Invest*. 2007. 117(9):2459-2467.

131. Pramila, T., Wu, W., Miles, S., Noble, W.S., Breeden, L.L. The Forkhead transcription factor Hcm1 regulates chromosome segregation genes and fills the S-phase gap in the transcriptional circuitry of the cell cycle. *Genes. Dev*. 2006. 20(16):2266-2278.

132. Relaix, F., Rocancourt, D., Mansouri, A., Buckingham, M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev*. 2004. 18(9):1088-1105.

133. Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature*. 2005. 435(7044):948-954.

134. Relaix, F., Montarras D., Zaffran, S., Gayraud-Morel, B., Rocancourt, D., Tajbakhsh, S., Mansouri, A., Cumano, A, and Buckingham, M. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J. Cell Biol*. 2006. 172(1):91-102.

135. Rhodes, S.J., and Konieczny, S.F. Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev*. 1989. 12(B):2050-2061.

136. Rodda, D.J., Chew, J.L., Lim, L.H., Loh, Y.H., Wang, B., Ng, H.H., and Robson, P. Transcriptional regulation of nanog by OCT4 and SOX2. *J. Biol. Chem*. 2005. 280(26):24731-24737.

137. Rosenberg, M.I., Georges, S.A., Asawachaicharn, A., Analau, E., Tapscott, S.J. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miRNA-206. *J. Cell Biol*. 2006. 175(1):77-85.

138. Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. Inactivation of MyoD in mice leads to upregulation of the HLH-gene Myf5 and results in apparently normal muscle development. *Cell*. 1992. 71(3):383-390.

139. Sampaolesi, M., Blot, S., D'Antona, G., Granger, N., Tonlorenzi, R., Innocenzi, A., Mognol, P., Thibaud, J.L., Galvez, B.G., Barthélémy, I., Perani, L., Mantero, S., Guttinger, M., Pansarasa, O., Rinaldi, C., Cusella De Angelis, M.G., Torrente, Y., Bordignon, C., Bottinelli, R., and Cossu, G. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature*. 2006. 444(7119):574-579.

140. Sandberg, M., Källström, M., and Muhr, J. Sox21 promotes the progression of vertebrate neurogenesis. *Nat. Neurosci*. 2005. 8(8):995-1001.

141. Savare, J., Bonneaud, N., and Girard, F. SUMO represses transcriptional activity of the Drosophila SoxNeuro and human Sox3 central nervous system-specific transcription factors. *Mol. Biol. Cell*. 2005. 16(6):2660-2669.

142. Seale, P., Sabourin, L.A., Girgis-Garbardo, A., Mansouri, A., Gruss, P., and Rudnicki, M.A. Pax7 is required for the specification of muscle satellite cells. *Cell*. 2000. 102(6):777-786.

143. Schepers, G.E., Teasdale, R.D., Koopman, P. Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. *Dev. Cell*. 2002. 3(2):167-170.

144. Schilham, M.W., Oosterwegel, M.A., Moerer, P., Ya, J., de Boer, P.A., van de Wetering, M., Verbeek, S., Lamer, W.H., Kruisbeek, A.M., Cumano, A., and Clevers, H. Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. *Nature*. 1996. 380(6576):711-714.

145. Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.C., and Treier, M. The murine winged-helix transcription factor

Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development*. 2004. 131(4):933-942.

146. Schmidt, K., Glaser, G., Wernig, A., Wegner, M., and Rosorius, O. Sox8 is a specific marker for muscle satellite cells and inhibits myogenesis. *J. Biol. Chem*. 2003. 278(32):29769-29775.

147. Sheeba, C.J., Palmeirim, I., and Andrade, R.P. Chick Hairy1 protein interacts with Sap18, a component of the Sin3/HDAC transcriptional repressor complex. *BMC Dev. Biol*. 2007. 7(83):1-8.

148. Shimamura, M., Garcia, J.M., Prough, D.S., Dewitt, D.S., Uchida, T., Shah, S.A., Avila, M.A., and Hellmich, H.L. Analysis of long-term gene expression in neurons of the hippocampal subfields following traumatic brain injury in rats. *Neuroscience*. 2005. 131(1):87-97.

149. Shinin, V., Gayraud-Morel, B., Gomes, D. and Tajbakhsh, S. Asymmetric division and the cosegregation of template DNA strands in muscle satellite cells. *Nat. Cell Biol.* 2006. 8(7):677-687.

150. Shu, W., Cho, J.Y., Jiang, Y., Zhang, M., Weisz, D., Elder, G.A., Schmeidler, J., De Gasperi, R., Sosa, M.A., Rabidou, D., Santucci, A.C., Perl, D., Morrisey, E., Buxbaum, J.D. Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. *Proc. Natl. Acad. Sci*. 2005. 102(27):9643-9648.

151. Sock, E., Schmidt, K., Hermanns-Borgmeyer, I., Bösl, M.R., and Wegner, M. Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. *Mol. Cell. Biol*. 2001. 21(20):6951-6959.

152. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet*. 1999. 21(1):70-71.

153. Sternberg, P.W., Stern, M.J., Clark, I., and Herskowitz, I. Activation of the yeast HO gene by release from multiple negative controls. *Cell*. 1987. 48(4):567-577.

154. Stryke, D., Kawamoto, M., Huang, C.C., Johns, S.J., King, L.A., Harper, C.A., Meng, E.C., Lee, R.E., Yee, A., L'Italien, L., Chuang, P.T., Young, S.G., Skarnes, W.C., Babbitt, P.C, Ferrin, T.E. BayGenomics: a

resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res*. 2003. 31(1):278-281.

155. Sutton, J., Costa, R., Klug, M., Field, L., Xu, D., Largaespada, D.A., Fletcher, C.F., Jenkins, N.A., Copeland, N.G., Klemsz, M., and Hromas, R. Genesis, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. *J. Biol. Chem*. 1996. 271(38):23126-23133.

156. Tallquist, M.D., Weismann, K.E., Hellström, M., and Soriano, P. Early myotome specification regulates PDGFA expression and axial skeleton development. *Development*. 2000. 127(23). 5059-5070.

157. Taylor, K.M. and Labonne, C. SoxE factors function equivalently during neural crest and inner ear development and their activity is regulated by SUMOylation. *Dev. Cell*. 2005. 9(5):593-603.

158. Tompers, D.M., Foreman, R.K., Wang, Q., Kumanova, M., and Labosky, P.A. Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo. *Dev. Biol*. 2005. 285(1):126-137.

159. Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillion, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., Armstrong, S.A., Passagué, E., DePinho, R.A., and Gilliland, D.G. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell*. 2007. 128(2):325-339.

160. Vasyutina, E., Lenhard, D.C., Wende, H., Erdmann, B., Epstein, J.A. and Birchmeier, C. RBP-J (Rbpsuh) is essential to maintain muscle progenitor cells and generate satellite cells. *Proc. Natl. Acad. Sci*. 2007. 104(11):4443-4448.

161. Wang, B., Weidenfeld, J., Lu, M.M., Maika, S., Kuziel, W.A., Morrisey, E.E., and Tucker, P.W. Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. *Development*. 2004. 131(18):4477-4487.

162. Wang, D.Z., Valdez, M.R., McAnally, J., Richardson, J., Olson, E.N. The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. *Development*. 2001. 128(22):4623-4633.

163. Wang, I.C., Chen, Y.J., Hughes, D., Petrovic, V., Major, M.L., Park, H.J., Tan, Y., Ackerson, T., and Costa, R.H. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol. Cell. Biol*. 2005. 25(24):10875-10894.

164. Wang, T., Tamakoshi, T., Uezato, T., Shu, F., Kanzaki-Kato, N., Fu, Y., Koseki, H., Yoshida, N., Sugiyama, T., and Miura, N. Forkhead transcription factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. *Dev. Biol*. 2003. 259(1):83-94.

165. Wang, X., Kiyokawa, H., Dennewitz, M.B., and Costa, R.H. The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. *Proc. Natl. Acad. Sci*. 2002. 99(26):16881-16886.

166. Wang, X., Lee, C., Gilmour, D.S., Gergen, J.P. Transcription elongation controls cell fate specification in the Drosophila embryo. *Genes. Dev*. 2007. 21(9):1031-1036.

167. Wehr, R., Mansouri, A., de Maeyer, T., and Gruss, P. Fkh5-deficient mice show dysgenesis in the caudal midbrain and hypothalamic mammillary body. *Development*. 1997. 124(22):4447-4456.

168. White, J. and Dalton, S. Cell cycle control of embryonic stem cells. *Stem Cell. Rev*. 2005. 1(2):131-138.

169. Whitsett, J.A. and Tichelaar, J.W. Forkhead transcription factor HFH-4 and respiratory epithelial cell differentiation. *Am. J. Respir. Cell Mol. Biol*. 1999. 21(2):153-154.

170. Wijchers, P.J., Hoekmann, M.F., Burbach, J.P., and Smidt, M.P. Identification of forkhead transcription factors in cortical and dopaminergic areas of the adult murine brain. *Brain Res*. 2006. 1068(1):23-33.

171. Wilson, M. and Koopman, P. Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. *Curr. Opin. Genet. Dev*. 2002. 12(4):441-446.

172. Wright, W.E., Induction of muscle genes in neural cells. *J. Cell. Biol*. 1984. 98(2):427-435.

173. Wright, W.E., Sasson, D.A., and Lin, V.K. Myogenin, a factor related to myogenesis, has a domain homologous to MyoD. *Cell*. 1989. 56(4):607-617.

174. Wu, H., Naya, F.J., McKinsey, T.A., Mercer, B., Shelton, J.M., Chin, E.R., Simard, A.R., Michel, R.N., Bassel-Duby, R., Olson, E.N., Williams, R.S. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *EMBO. J*. 2000. 19(9):1963-1973.

175. Xuan, S., Baptista, C.A., Balas, G., Tao, W., Soares, V.C., and Lai, E. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron*. 1995. 14(6):1141-1152.

176. Yamamoto, M., Meno, C., Sakai, Y., Shiratori, H., Mochida, K., Ikawa, Y., Saijoh, Y., and Hamada, H. The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse. *Genes. Dev*. 2001. 15(10):1242-1256.

177. Yang, Q., Kong, Y., Rothermel, B., Garry, D.J., Bassel-Duby, R., and Williams, R.S. The winged-helix/forkhead protein myocyte nuclear

factor beta (MNF-beta) forms a co-repressor complex with mammalian sin3B. *Biochem. J.* 2000. 345(2):335-343.

178. Yang, Y., Hou, H., Haller, E.M., Nicosia, S.V., and Bai, W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. *EMBO. J*. 2005. 24(5):1021-1032.

179. Zhao, Y., Samal, E., and Srivastava, D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. 2005. *Nature*. 436(7048):214-220.

180. Zheng, Y., Josefowicz, S.Z., Kas, A., Chu, T.T., Gavin, M.A., and Rudensky, A.Y. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007. 445(7130):936-940.

Vitae

Matthew Scott Alexander was born in Manhassett, New York on February 2, 1982, the only child of Elizabeth M. Royer and Michael A. Alexander. After graduating in the top of his class at East Mecklenburg Senior High School in Charlotte, North Carolina, he entered the University of North Carolina at Wilmington. While earning a Bachelor of Science in Biology with a minor in Chemistry, he worked as an undergraduate intern in the lab of Thomas Shafer, Ph.D. where he studied the molecular regulation of osmoregulation of the blue crab, *Callinectes sapidus*. He entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas in August 2003 to pursue the degree of Doctor of Philosophy in Genetics and Development. He joined the lab of Daniel J. Garry M.D., Ph.D. in June 2004. His doctoral studies involved characterizing the molecular regulation of *forkhead* transcription factors in stem cell populations, myogenesis, cardiogenesis, and during muscle regeneration. Following the departure of Dr. Garry in July 2007, Matthew entered the lab of Dr. Joseph A. Garcia M.D., Ph.D., where he currently works on characterizing of Hif2α and its downstream target genes.

Permanent Address:
2301 Knickerbocker Drive
Charlotte, NC 28212