2016

Characterization of FAM65B knock-out mice and role of FAM65B in skeletal muscle stem cells differentiation

Colletta, Alessandro

https://hdl.handle.net/2144/16765

Boston University
CHARACTERIZATION OF FAM65B KNOCK-OUT MICE AND ROLE OF FAM65B IN SKELETAL MUSCLE STEM CELLS DIFFERENTIATION

by

ALESSANDRO COLLETTA

B.S., University of Michigan, 2012

Submitted in partial fulfillment of the requirements for the degree of
Master of Science
2016
Approved by

First Reader

Simon Levy, Ph.D.
Associate Professor of Physiology and Biophysics

Second Reader

Emanuela Gussoni, Ph.D.
Associate Professor of Pediatrics
Boston Children’s Hospital
Harvard Medical School
ACKNOWLEDGMENTS

I would like to sincerely thank my mentor, Dr. Emanuela Gussoni, for attentively helping me throughout the writing of this work. I also wish to acknowledge her for the advice and collaboration provided during the research year of my Masters in Medical Sciences; her guidance has helped me become a more knowledgeable and mature scientist. For this reason, I feel grateful to have been given the opportunity to work in her lab.

In addition, I would like to thank Dr. Simon Levy, my BU academic advisor, for his professional contribution to the revision process of this thesis, since his suggestions have allowed me to further improve my work. I also would like to express my appreciation for his advising and support throughout the entire duration of my Masters; his help has been important for me to achieve success in both my academic studies and application to medical school.

Moreover, I want to express my appreciation for the effort and commitment that Dr. Gwynneth Offner, the director of the Masters in Medical Sciences at BU, has devoted to the organization of this academic program. I indeed believe that the structure and curriculum of this program allow students to strongly prepare for their medical school journey and develop the ability to think and reason more critically. Finally, I wish to thank her for helping me connect with Dr. Gussoni and find a fantastic research opportunity for the second year of my Masters.
CHARACTERIZATION OF FAM65B KNOCK-OUT MICE AND ROLE OF FAM65B IN SKELETAL MUSCLE STEM CELLS DIFFERENTIATION

ALESSANDRO COLLETTA

ABSTRACT

The family with sequence similarity 65, member B (Fam65b) protein is thought to facilitate fusion of myocytes and formation of myotubes during the differentiation of human myogenic cells. Fam65b and histone deacetylase 6 (HDAC6) co-immunoprecipitate and together regulate the levels of acetylated tubulin, which might control microtubule stability in myogenic cells. In this thesis, to gain further insight on the role of Fam65b in the differentiation pathway and motility of myogenic cells, we characterized a Fam65b knock-out (KO) mouse model. Genotyping and transcriptional analysis revealed that a thirteen exons-long region of the Fam65b gene has been successfully ablated and the mRNA amplicons within the deleted segment are not transcribed. Nevertheless, mRNA products corresponding to genomic regions downstream of the deleted area are still detected. Furthermore, analysis of skeletal muscle lysates via western blot (WB) does not show a complete loss of Fam65b expression, but only reduced translation of some isoforms. Nevertheless, WBs of myogenic cells that have been directly isolated from Fam65b KO mice and expanded in vitro revealed the absence of a 120 Kd band, which putatively corresponds to the long isoform of Fam65b. Finally, our data show that Fam65b KO mice are significantly heavier than wild type (WT) mice, and that this phenotype is consistently observed across both genders during the first seven months of age. While functional and molecular analyses of the KO mouse
model are still ongoing, future work might include generating a new KO model via the CRISPR-Cas9 technology to ablate all isoforms of Fam65b.
TABLE OF CONTENTS

TITLE..............................................................................................................................i
COPYRIGHT PAGE.........................................................................................................ii
READER APPROVAL PAGE............................................................................................iii
ACKNOWLEDGMENTS ............................................................................................... iv
ABSTRACT....................................................................................................................... v
TABLE OF CONTENTS ............................................................................................... vii
LIST OF TABLES ......................................................................................................... ix
LIST OF FIGURES ....................................................................................................... x
LIST OF ABBREVIATIONS ......................................................................................... xii
INTRODUCTION .......................................................................................................... 1
    Skeletal Myogenesis ............................................................................................... 2
    Myogenic Differentiation in vitro ......................................................................... 5
    The role of Fam65b in skeletal myogenic differentiation .................................... 9
    The Role of Fam65B in other cell types ................................................................. 11
    Aim and Objectives .............................................................................................. 12
METHODS .................................................................................................................. 14
    Genotyping ............................................................................................................. 14
RNA Extraction and RT-PCR.............................................................................................. 15

Primary human and murine muscle cell isolation and FACS analysis of MCAM+ cells. ........................................................................................................................................... 17

Cell culture of myogenic cells ......................................................................................... 17

Animal Husbandry ........................................................................................................... 19

RESULTS ............................................................................................................................ 21

Description of Fam65B Deletion Cassette....................................................................... 22

Genomic analysis of Fam65b-/ mice.................................................................................. 23

RNA isolation and RT-PCR analysis ................................................................................ 27

Analysis of Fam65B protein expression ......................................................................... 30

Fam65B and its potential role in adiposity ....................................................................... 35

DISCUSSION ...................................................................................................................... 37

REFERENCES .................................................................................................................. 49

CURRICULUM VITAE ....................................................................................................... 62
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR conditions for genotyping of WT and Fam65b-/- mice</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>RT-PCR reaction conditions for transcriptional analysis of WT and Fam65b-/- mice</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>Promoter locations downstream of Fam65B deletion cassette</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Summary of western blots experimental condition</td>
<td>31</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Representation of skeletal muscle myofibers and satellite stem cells</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Schematic of adult myogenesis</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Murine myogenic cells in proliferation and differentiation medium</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Fam65B protein coding transcripts in Mus musculus</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Deletion strategy for Fam65B -/- ECS clone 15686A-B6</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Genotyping strategy for Fam65b-/- and WT mice</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>PCR reaction 1</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>PCR reaction 2</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>PCR Reaction 3 and 4</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>PCR Reaction 4</td>
<td>26</td>
</tr>
<tr>
<td>11</td>
<td>RT-PCR Reactions of WT and KO with DMSO</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>RT-PCR Reactions of WT and KO without DMSO</td>
<td>29</td>
</tr>
<tr>
<td>13</td>
<td>Fam65b expression analysis in murine skeletal muscle tissue lysates with MO1 Ab</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>Fam65b expression analysis in murine skeletal muscle tissue lysates with custom made Ab</td>
<td>33</td>
</tr>
<tr>
<td>Page</td>
<td>Section Description</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>Fam65b expression analysis in lysates of murine myoblast with MO1 Ab</td>
<td>34</td>
</tr>
<tr>
<td>16</td>
<td>Fam65−/− and WT mice weight chart</td>
<td>36</td>
</tr>
<tr>
<td>17</td>
<td>Model of CRISPR Cas9 editing strategy</td>
<td>42</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BHLH</td>
<td>Basic helix–loop–helix</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CTX</td>
<td>Cardiotoxin</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno-precipitation</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Cluster of Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>DM</td>
<td>Dermomyotome</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Stranded Break</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>Fam65B</td>
<td>Family with Sequence Similarity 65b</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucorticoid Receptor</td>
</tr>
</tbody>
</table>
HDAC ................................................................. Histone Deacetylase
HDR ................................................................. Homologous Directed Repair
H&E .................................................................. Hematoxalin and Eosin
Heat Shock Protein 90 ........................................ HSP90
Immunoglobulin G .............................................. IgG
ITGA7 ................................................................... α7 Integrin
KO .................................................................... Knock-Out
KOMP ............................................................... Knock-Out Mouse Project
MAPK ................................................................. Mitogen-Activated Protein Kinase
MEF .................................................................. Myocyte Enhancer Factor
MHC .................................................................. Myosin Heavy Chain
MRF .................................................................. Myogenic Regulatory Factor
mTOR ............................................................... Mammalian Target of Rapamycin
MyoD ................................................................. Myoblast Determination Protein
Myf .................................................................. Myogenic Factor
NCAM .............................................................. Neuronal Cell Adhesion Molecule
NFDM ............................................................... Non-fat Dry Milk
NHEJ ................................................................. Non Homologous End Joining
Pax ................................................................... Paired Box
PCR ................................................................. Polymerase Chain Reaction
PK .................................................................. Protein Kinase
PLA ................................................................ Proximity Ligation Assay
pMLC .......................................................... Phosphorylated Myosin Light Chain
Rac1 .......................................................... Ras-related C3 botulinum toxin substrate 1
RhoA .......................................................... Ras Homolog Gene Family Member A
RT ................................................................. Reverse Transcriptase
SEM ............................................................. Scanning Electron Microscope
sgRNA ......................................................... Single Guide RNA
siRNA ......................................................... Small Interfering RNA
SMP ............................................................. Skeletal Myogenic Progenitor
TA ............................................................... Tibialis Anterior
TEM ............................................................. Transmission Electron Microscope
WB .............................................................. Western Blot
WT .............................................................. Wild Type
INTRODUCTION

Skeletal muscle is a striated tissue principally composed of multinucleated syncytial cells named myofibers. As it accounts for about 35 to 40% of human body weight, it represents the most abundant tissue in our body. Skeletal muscles allow for the accomplishment of vital functions such as locomotion, physical support, breathing and thermogenesis. Unlike smooth and cardiac muscle tissues, skeletal muscle cells are directly innervated by the somatic nervous system, which allows for voluntary contraction (Boron & Boulpaep, 2012). One distinguishing feature of skeletal muscle lies in its ability to adapt to the environment, which can be observed under a multitude of physiological and disease-like states. Indeed, while endurance training may lead to muscle hypertrophy and enhanced strength, other conditions, like cancer, chronic obstructive pulmonary disease and denervation, can induce atrophy and loss of function. In addition to this remarkable adaptive potential, skeletal muscle exhibits great regenerative capacity. In fact, few weeks after a major traumatic event, skeletal muscle structure and function can be restored. This property is due to the activity of skeletal muscle stem cells, named satellite cells (Mauro, 1961). The capacity of this cell population to re-enter the cell cycle, differentiate and fuse to form regenerating myofibers allows for the maintenance of homeostasis of skeletal muscle tissue (Dumont et al., 2015).
Skeletal Myogenesis

The generation of muscle tissue - myogenesis - can be mainly organized into embryonic and adult phases. During embryonic development, muscle founder stem cells, which are located in the dorsal somites of the paraxial mesoderm, give rise to the dermomyotome (DM), the first site where commitment to myogenesis occurs (Buckingham et al., 2003; Sambasivan & Tajbakhsh, 2007; Endo, 2015). These stem cells are marked by the expression of the transcription factors paired box 3 (Pax3) and paired box 7 (Pax7) and low expression of the basic helix-loop-helix (BHLH) transcription factor myogenic factor 5 (Myf5) (Jostes, Walther, & Gruss, 1990; Goulding et al., 1991; Kiefer & Hauschka, 2001). At this developmental stage, the progenitor cells of the DM, undergo an epithelial to mesenchymal transition, migrate underneath the DM
and differentiate into mononucleated myocytes to form the myotome. These mononuclear precursor cells, known as myoblasts, express high levels of Myf5 and myoblast differentiation protein (MyoD) and are marked for terminal differentiation to the muscle lineage. Myf5 and MyoD are two myogenic regulatory factors (MRFs) and both belong to the basic helix loop helix (bHLH) transcription factor family (Pownall, Gustafsson, & Emerson, 2002; Francetic & Li, 2011). Myoblasts first fuse together in a process referred to as “primary fusion” and give rise to nascent myotubes, which are very long, plurinucleated syncytia. During a second phase, additional myoblasts unite with nascent myotubes, a step known as “secondary fusion” (Buckingham, 2001). This process results in the growth of myotubes, myonuclear accretion and the subsequent formation of myofibers, which are the multinucleated contractile cells typical of adult skeletal muscle (Hindi, Tajrishi, & Kumar, 2013; Yin, Price, & Rudnicki, 2013).

Myofibers are post mitotic, arrested in the G0 phase of the cell cycle and unable to proliferate. They have a longitudinal, cylindrical shape and are internally constituted of myofibrils, which contain myosin and actin filaments that allow for muscular contractions. These myofibers are compounded into bundles by a complex and structured network of extracellular matrix (ECM), specifically, the endomysium and the basement membrane. The endomysium represents the most external membrane, made primarily of collagen fibers and interlaced with a network of nerve fibers and blood vessels. The basement membrane, located between the endomysium and the myofiber membrane (plasmalemma), can be further subdivided into the basal lamina and the reticular lamina (Dumont et al., 2015; Boron & Boulpaep, 2012).
Unlike the *de novo* embryonic generation of muscle tissue described above, adult myogenesis is dependent upon the retention of the ECM scaffold, where a specific population of quiescent stem cells resides (Le Grand & Rudnicki, 2007). These progenitor cells, which originate from the central portion of the dermomyotome early in embryogenesis, and later, during fetal development, position themselves between the plasmalemma and the basal lamina and are marked by the constitutive expression of the transcription factor Pax7 (Seale et al., 2000; Gros et al., 2005). Following activation, which may be induced by either stress, trauma, or microenvironment-secreted growth factors, satellite cells move outside of the basal lamina, re-enter the cell cycle and co-express the transcription factors Pax7, Myf5, MyoD. Then, these activated satellite cells, also known as cycling myoblasts, differentiate into myocytes by downregulating Pax7 expression while maintaining elevated levels of MyoD and by upregulating two other MRFs, MRF4 and myogenin. Finally, downregulation of MyoD and prolonged expression of both MRF4 and myogenin induce myocytes to either fuse homotypically to form nascent multinucleated myotubes or to fuse with regenerating myofiber end-fragments (Dumont et al., 2015).

It should be noted that a stochastic mode of symmetric cell division into a more committed cell type would result in depletion of the satellite stem cell pool aforementioned. Instead, satellite cells utilize asymmetric division to self-renew and generate another cell that is identical to the original stem cell in addition to a committed daughter cell that will commit to myogenic differentiation. This process allows for self-
maintenance of the satellite cell pool and, at the same time, creates a more committed myogenic progeny (Shinin et al., 2006; Kuang et al., 2007).

Figure 2. Schematic of adult myogenesis. The process of adult myogenesis and the temporal expression of critical transcription factors are illustrated. Adapted from Le Grand & Rudnicki, 2007.

Myogenic Differentiation in vitro

The maintenance of murine and human myoblasts in vitro is an essential technique for studying the differentiation pathways of myogenic progenitors, the characterization of muscle disorders and the potential development of cell-based therapies. In vitro culturing of C2C12 cells, an immortalized murine myoblast cell line that was originally isolated by Yaffe D. in 1977, is often utilized to study mammalian
skeletal myogenesis due to its ease of culture, differentiation potential and accessibility (Yaffe & Saxel, 1977; Nag & Foster, 1981; Burattini et al., 2004). Conversely, pure cultures of primary human myoblasts are more difficult to obtain because of the presence of non-myogenic mononuclear cells in human skeletal muscle, like adipocytes, fibroblasts and lymphocytes (Malatesta et al., 2013). In order to specifically enrich for myoblasts within a dissociated human muscle sample, fluorescence-activated cell sorting (FACS) is used to select cells expressing human neural cell adhesion molecule (NCAM), also known as cluster of differentiation (CD) 56 (Webster et al., 1988); indeed, this cell surface molecule was discovered to be highly expressed on myogenic cells (Walsh & Ritter, 1981). Myogenic cells derived from human skeletal muscle can be purified by positive selection for CD56 expression and negative selection of CD34 (CD56+ CD34-cells), which select for myogenic but non-adipogenic precursors in human skeletal muscle (Pisani et al., 2010). Recently, melanoma cell adhesion molecule (MCAM), an antigen expressed in mononuclear proliferating myoblasts, but significantly downregulated during fusion, has been utilized to selectively purify myogenic from dissociated human fetal skeletal muscle (Cerletti et al., 2006; Lapan & Gussoni, 2012). MCAM, however, does not select for adult human myoblasts. Primary cultures of murine myoblasts can be obtained from dissociated mouse muscle tissue following purification of satellite cells by FACS. In 2001, cells isolated from hindlimb muscles of neonatal mice containing a mixture of myoblasts, fibroblasts and other cell types were enriched for a purified myoblast population by selecting for a7 integrin (ITGA7) expression (Blanco-Bose et al., 2001). Optimization of murine skeletal myogenic progenitors (SMPs) was
subsequently achieved via FACS purification using CD34 and ITGA7 as positive selection markers, while CD31, CD45 and CD11b were utilized as negative selection markers (Sacco et al., 2008; Bareja et al., 2014).

Several factors affect the switch from the proliferation to the differentiation state of both murine and human skeletal myoblasts; this process is characterized by cell cycle exit and the formation of multinucleated myotubes. *In vitro*, myoblasts can be expanded in the presence of high serum concentrations (10 to 20% fetal calf serum) and by supplementing mitogenic growth factors, such as basic fibroblast growth factor (bFGF) (Sheehan & Allen, 1999). Undifferentiated cells are about 20-80 µm in size, look flat, not confluent, and are closely adherent to their substrate; they appear star-shaped or fusiform, possess one central nucleus with numerous nucleoli, and are characterized by the presence mitotic figures. Differentiation and fusion of neighboring myonuclear cells is triggered by switching the culture to low serum medium (1-4% fetal calf or horse serum) and the withdrawal of mitogens. As myoblasts begin their process of differentiation, they acquire a mainly elongated shape, and intercellular spaces progressively recede. Myotubes become evident after 36 to 48 hours in differentiation medium, and progressively continue to grow in size. At this time point, they are multinucleated and frequently elongated in three to four directions. Transmission electron microscope (TEM) analysis reveals that, within the cytoplasm, thin and thick filaments both organize to form sarcomeres and myofibrils, and these structures appear to be similar to those present in adult skeletal muscle (Burattini et al., 2004). However, mature myotubes only appear 5 days post addition of differentiation medium (Curci et al., 2008).
Distinct signaling pathways regulate myogenic progression and subsequent differentiation \textit{in vitro}. Downregulation of Pax7 transcription via the action of specific microRNAs and increased expression of MyoD due to mitogen-activated protein kinase (MAPK) signaling protein p38α play a pivotal role in the transition from myoblast proliferation to differentiation (Lluís et al., 2005). MyoD initiates differentiation by inducing cell cycle inhibitors like p21, while upregulation of myogenin and MRF4 has a critical role in late differentiation (Halevy et al., 1995; Rawls et al., 1998). Indeed, MyoD/- myoblasts fail to upregulate MRF4 and myogenin, thus displaying differentiation defects and impaired muscle regeneration (Cornelison et al., 2000).

Following induction of the differentiation program, myoblasts undergo cell fusion which results in the formation of myotubes. Numerous proteins involved in cell-cell adhesion and actin dynamics have been implicated in syncytial fusion (Charrasse, 2002; Charrasse et al., 2007). Myomarker is the only muscle specific membrane protein
identified so far that is both necessary and sufficient to induce the plasma membrane of myoblasts to fuse \textit{in vivo} and \textit{in vitro}. It should be noted that while ablation of myomarker prevents the formation of multinucleated myotubes and myofibers, it does not affect MyoD and myogenin expression levels (Millay et al., 2013), implying that expression of myomarker is downstream of MyoD and myogenin.

\textit{The role of Fam65b in skeletal myogenic differentiation}

With the scope of discovering new genes involved in the differentiation and fusion of human skeletal muscle cells, microarray analyses of primary fetal myoblasts were conducted to identify differentially expressed genes during myogenic differentiation. Among others, the transcription levels of Family with sequence similarity (Fam65b), also known as \textit{C6orf32} and \textit{pl48}, was highly elevated during early myogenic differentiation, specifically when myotubes had up to 5 nuclei. Fam65b expression levels were up to 16 times greater than in mononuclear cells until myotubes reached a size up to 15 nuclei (Cerletti et al., 2006). Expression of Fam65b protein was confirmed during early fusion of human fetal myoblasts and its polar localization within the cells (Yoon et al., 2007). It appeared that Fam65B was mainly located within the cell cytoplasm, in proximity of the plasma membrane. This protein seemed to be associated with the cell cytoskeleton and localized to one side of the cell, leading us to hypothesize that Fam65b may have a function in cell polarity (Yoon et al., 2007). mRNA knock-down of Fam65b using small interfering RNAs (siRNAs) using oligonucleotides in C2C12 myoblasts showed that cell fusion and expression of myosin heavy chain (MHC), a marker of
medium-late myogenic differentiation, were significantly compromised. While the expression of MyoD, the master regulator of skeletal myogenic differentiation in proliferating myoblast, was not altered, the expression of myogenin, a differentiation marker downstream of MyoD, was significantly reduced (Yoon et al., 2007). These findings indicated a role for Fam65b during myogenic differentiation at a stage between MyoD and myogenin expression. Overexpression of Fam65B in C2C12 cells leads to the formation of significantly longer filopodia-like structures relative to control cells. Furthermore, in-frame deletion constructs of Fam65B identify that the N terminal region (55aa-113aa) is important for protrusion formation, suggesting a role in cytoskeletal rearrangement and possible cell motility.

Fam65B putatively encodes for 12 alternatively spliced polypeptides and WB analysis of lysates of human fetal myoblast found two major isoform of Fam65B: one of 105Kd (1018 aa) and one of 70Kd (591 aa). Both these isoforms contain an extremely conserved protein domain in the N-terminus of the protein (aa 79-116) that has similarities to a putative HDAC-binding site. To address if Fam65b is a an HDAC-binding protein, immunoprecipitation experiments were conducted and Fam65B was found to bind histone deacetylase 6 (HDAC6) (Balasubramanian et al., 2014). As the literature reports, HDAC6 is a cytoplasmic HDAC that regulates microtubule dynamics via deacetylation of α-tubulin (Y. Zhang et al., 2003; X. Zhang et al., 2007). Increased expression of Fam65B at 1 day following differentiation resulted in a decrease in HDAC6 de-acetylating activity, which was followed by an increase in the levels of acetylated tubulin and myogenin expression (Balasubramanian et al., 2014). These results
suggest that Fam65B, by binding HDAC6, prevents this protein from deacetylating tubulin. As increased levels of acetylated tubulin lead to increased microtubule stability and increased expression of myogenin, it may further explain why overexpression of Fam65B leads to a significantly greater number of cell protrusions (Yoon et al., 2007; Conacci-Sorrell, Ngouenet, & Eisenman, 2010). Silencing of HDAC6 RNA expression in human primary myogenic cells resulted in tubulin hyperacetylation (Balasubramanian et al., 2014). As high levels of acetylated tubulin were detected, the cells responded via a mechanism of negative feedback and downregulated expression levels of Fam65B and myogenin, which consequently led to impaired myogenic differentiation. This result was further supported by the fact that prolonged treatment of human myoblasts with HDAC6 inhibitors, as also indicated by other studies, prevented rather than favored skeletal muscle differentiation (Nebbioso et al., 2009; Balasubramanian et al., 2014).

The Role of Fam65B in other cell types

Recently, the function of Fam65b has also been investigated in non-myogenic cells and results showed that its role may be pivotal to both hearing and immune competence (Pablo Rougerie et al., 2013; Diaz-Horta et al., 2014; Gao et al., 2015). Studies have indeed shown that Fam65B associates with the plasma membrane of hair cells and lack or reduced expression of this protein can impair the mechanotransduction processes in the inner ear. Indeed, mutations leading to exon skipping in humans as well as Fam65b mRNA silencing in zebrafish are responsible for hearing loss (Diaz-Horta et al., 2014). Instead, experiments on neutrophils and Jurkat cells, an immortalized line of
human T lymphocyte cells, showed that Fam65B binds to Ras Homolog Gene Family Member A (RhoA) and plays a role in the regulation of cell migration and polarization (P. Rougerie et al., 2013; Gao et al., 2015). Although both studies showed that Fam65B negatively regulates the activity of RhoA by inhibiting the loading of GTP onto RhoA, different conclusions were drawn in regard to the role of Fam65B in relation to cell polarity and migration. Indeed, Rougerie et al. (2013) showed that Fam65B negatively regulates cell migration and polarity in T cells. Yet, Gao et al. (2015) demonstrated that the same protein polarizes at the leading edge of neutrophils, and that contributes to localize RhoA and phosphorylated myosin light chain (pMLC) to the back of the cells; furthermore, this study reported that neutrophils of Fam65B KO mice showed impaired directionality during chemotaxis and impaired migration toward areas where inflammation had been induced. Consequently, it remains important to elucidate which role Fam65B may play in cell motility, polarity and regulation of RhoA activity within differentiating skeletal myoblasts. As our lab has generated a Fam65B knock-out mouse model, comparison of aforementioned parameters between wild type (WT) and knock-out tissues will improve the validity of our analysis.

Aim and Objectives

In this thesis, we generated a mouse KO model to allow us to better analyze the function of Fam65b in the differentiation of myogenic cells in vivo. Fam65B knock-out mice were characterized at the gene, transcript and protein levels. We conducted polymerase chain reaction (PCR), Reverse Transcriptase (RT)-PCR and WB experiments
on mouse skeletal muscle tissues and primary myogenic cells extracted from the same animals. To investigate whether loss of Fam65b expression compromises the regeneration of skeletal muscles and muscle stem cell activity, the tibialis anterior (TA) muscles of both WT and KO mice were injured by injection of cardiotoxin (CTX). Muscles were harvested seven days after inducing the injury, sectioned and stained by Hematoxylin and Eosin to determine if muscle regeneration was occurring properly.
**METHODS**

*Genotyping*

Genomic DNA was extracted from tails of KO and WT mice as described by Zangala (2007). The isolated DNA was then amplified by PCR. Primer set 1 and 4 were used for amplifying WT DNA. The sequences were as follows: primer set 1, TTAGGCTTAGGAGCCTGTG (SUF, forward) and GGAAGGAGTCTGCAACTAACTGTG (TUR, reverse); primer set 4, AAGGGTTCAACCCAGTCTAAGG (TDF, forward) and GTCTCTGCTACCAAGTGACAAAGTGC (Fam1R, reverse) SUF, TUR and TDF primers were recommended by the KOMP repository that generated the ES KO cell line. Primer set 2 and 3 were utilized to amplify the DNA of KO mice. The sequences were as follows: primer set 2, TTAGGCTTAGGAGCCTGTG (SUF, forward) and GTCTGTCCTAGCTTCCTCACTG (LacZRev, reverse); primer set 3, TTCGGCTATGACTGGGCACAACAG (NeoF, forward) and GTCTCTGCTACCAAGTGACAAAGTGC (Fam1R, reverse).

Touchdown PCR was performed using equal amount of DNA (1 µg), 0.5 mM each of forward and reverse primer. Reactions run with primers 1, 4, and 3 were amplified in 2 phases. In phase I, the polymerase was activated for 3 min at 95°C, followed by denaturation at 95°C for 10s, successively lower annealing temperatures ($T_m$) between 65°C and 55°C (i.e., decreasing the $T_m$ of the reaction 1°C/cycle) for 30s, followed by extension at 72°C for 20s over the course of 10 cycles. In phase II,
denaturation was at 95°C for 10s, annealing at 55°C for 30s, followed by extension at 72°C for 20s for 30 cycles. A final elongation step was performed at 72°C for 5 min. The reaction with primer set 2 was run with no touchdown methodology and the following conditions were followed: denaturation at 95°C for 10s, annealing at 51.5°C for 30s, and extension at 72°C for 20s for 40 cycles. The end product of the PCR reactions were electrophoresed on 1% agarose gel and DNA was visualized by ethidium bromide staining.

**RNA Extraction and RT-PCR**

Total RNA was isolated from mouse gastrocnemius muscle tissue. In brief, individual muscle samples were immersed into 1 mL of trizol and homogenized. Samples were transferred into separate eppendorf tubes and 200 μL of chloroform was added to each tube to induce a phase separation. After centrifugation (12,000g for 15 min at 4°C), the aqueous layer was extracted and combined with 500 μL of isopropanol to precipitate the RNA; the samples were then frozen at -20°C for 1 hour. After centrifugation (12,000g for 10 min at 4°C), the supernatant was decanted, and 1 mL of 75% ethanol was added to each pellet. After a final spin (7,500g for 5 min at 4°C), the supernatant was decanted; the pellet of each sample was air-dried, resuspended in RNase-free water; RNA concentration and purity were analyzed via Nano drop.

For reverse transcription, the SuperScript III First Strand was used according to the manufacturer’s instructions to reverse transcribe RNA into cDNA (Invitrogen, Carlsbad, CA). RT-PCR was performed with 1μg of cDNA, and 0.5 μM of each forward
and reverse primer. Four primer sets were employed: primer sets 4-10 and 6-10 were used to amplify cDNA segments between exon 4 to 10, and exon 6 to 10 of the Fam65B gene, respectively; primer set 20-23 amplifies a cDNA fragment between exon 20 and 23, while the dystrophin primer set was utilized as positive control to amplify a segment of cDNA between exon 8 and 10 of the Dystrophin gene. The primer sequences were as follows: AGAAACTCTCGCCTGGGTGT (Fam65b exon 4, forward) and CACCTCTGCCGACCATACTT (Fam65b exon 10A, reverse); GACGCCTGGAGTTTCACATT (Fam65b exon 6, forward) and CAGGAAGACGTTTCTGCTC (Fam65b exon 10B, reverse); TGACAATGAGGTCAGCGAAG (Fam65b exon 20, forward) and AACTTCGGTTCCATGACGAC (Fam65b exon 23, reverse); ATGTTGCCAGGACATCTTCC (Dystrophin exon 8, forward) and CTCCTTGCTCGCAATGTA (Dystrophin exon 10, reverse).

The specific conditions for the PCR reactions where primer sets Fam65b 4-10, Fam65b6-10 and dystrophin were as follows: 95°C for 3 min, followed by 40 cycles of amplification (denaturation at 94°C for 45s, annealing at 57°C for 45s and extension at 72°C for 45s), and a final elongation step at 72°C for 5 min. For primer set 20-23, due to the lower T_m of both forward and reverse primers, the only parameter to be modified relative to the conditions outlined above was the annealing temperature, which was set at 54 °C. The end product of the PCR reactions were electrophoresed on 1% agarose gel and DNA was visualized by ethidium bromide staining.
Primary human and murine muscle cell isolation and FACS analysis of MCAM+ cells.

Mononuclear cells were isolated from either deidentified, discarded human fetal muscle samples or murine skeletal muscles. Collection was approved by the Committee of Clinical Investigation at Boston Children’s Hospital and Brigham and Women’s Hospital (Boston, MA). Skeletal muscle was minced and enzymatically dissociated with 0.5 mg/ml collagenase D and 0.6 U/mL dispase II (Roche Applied Science, Indianapolis, IN) for 45–75 min at 37°C as previously described (Lapan & Gussoni, 2012). Cells were filtered through cell strainers before being stored at -150°C for later use. Human and murine fetal muscle cells were cultured on sterile tissue culture-treated plates (Corning, Corning, NY) 24 hr prior to FACS analysis. Cells were washed once with 1X Hank’s Balanced Salt Solution (HBSS) and lifted using cell dissociation buffer (Invitrogen Gibco, Grand Island, NY). Samples were filtered and resuspended in warm 0.5% BSA/HBSS followed by incubation with anti-MCAM antibody conjugated with Alexa Fluor488 (Millipore, Billerica, MA) at a dilution of 1:100. Samples were washed, resuspended in 1g/mL propidium iodide/0.5% BSA/HBSS, and filtered through a 40 µm filter prior to FACS analysis and sorting.

Cell culture of myogenic cells

MCAM+ myogenic cells were cultured in growth medium (DMEM-high glucose, 20% fetal bovine serum, 100 U/mL penicillin, and 100g/mL streptomycin). At 80% confluence, myogenic cells were induced to differentiate in DMEM-low glucose, 2% horse serum, 100 U/mL penicillin, and 100g/mL streptomycin. Differentiation medium
was changed every day, and cells were monitored for myotube formation. Proliferating cells in growth medium with high glucose and serum were harvested and name “undifferentiated” samples, whereas the differentiating cells were harvested post the addition of DMEM-low glucose medium and labeled as “24hr” and “48hr” samples.

Protein lysates preparation and Western blot analysis.

Cell lysates were prepared by adding mammalian protein extraction reagent (M-PER) (Thermo Scientific, Pittsburgh, PA) containing protease inhibitors (Roche Applied Sciences, Indianapolis, IN) to cells that had been harvested. Cells were then spun at 13,200 rpm for 20 min at 4°C, and the supernatant was collected and stored at -80°C for WB analysis. Lysates derived from murine whole muscles were prepared by initially grinding the tissue with sterile mortar and pestle. Tissues were then transferred into Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA), resuspended with 400 μL of M-PER/protease inhibitor cocktail, and homogenized 3 to 4 times via 30s cycles. The samples were finally centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was stored at -80°C until use. Protein lysates were quantified using the Pierce BCA Protein Assay (Thermo Scientific, Pittsburgh, PA, USA) to ensure equal amount of loading for each samples. For WB analyses, 15 μg of total protein lysates were diluted with 2X sample loading buffer, heated for 3 min at 80°C and spun at 13,200 rpm, at 4°C for 2 min. Proteins were then separated using Precast NuPage 4 –12% Bis/Tris acrylamide gel (Invitrogen, Carlsbad, CA) SDS-PAGE. The proteins were wet-
transferred by using 0.45 μm pore size PVDF membranes (Invitrogen, Carlsbad, CA). Blocking of non-specific binding was performed with either 3% BSA or 5% BSA and 1% nonfat dry milk in 1 X TBS Tween (0.1%) for 60 min. Membranes were incubated in primary antibody diluted at 1:1000 in blocking solution overnight at 4°C. Blots were washed with 1 X TBS Tween (0.1%) and incubated with HRP-conjugated secondary antibody diluted at 1:2000 in blocking solution; then were washed in 1X TBS Tween (0.1%) and developed using ECL solution (Perkin Elmer, Waltham, MA). The primary antibodies used were hC6ORF32-M01 (1:1000; Novus Biologicals, Littleton, CO) Fam65B custom made (1:1000) and GAPDH (1:5000; Santa Cruz Biotechnology, Dallas, Texas). Secondary antibodies used were donkey anti-rabbit IgG HRP (1:10000; Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-mouse IgG HRP (1:10000; Jackson ImmunoResearch Laboratories, West Grove, PA).

Animal Husbandry

WT C57Bl/6 mice and Fam65b-/-, which had been generated in the C57Bl6 background, were housed and handled in compliance with the guidelines of the Boston Children's Hospital committee for animal research. All experimental procedures were performed as described in the protocol approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. The weights of KO and WT mice were also recorded monthly for a period of 15 months. Mice were euthanized at the age of 15 months. Quadriceps, gastrocnemius, triceps were utilized for RNA and protein analysis, while tails were used for DNA extraction. Tibialis Anterior muscles of both KO and WT mice were subject to
injury by injection of CTX (20 μL of 0.5 μg/μL stock) from Naja mossambica (Sigma-Aldrich, Natick, MA). Muscles were harvested 7 days after CTX injection, and were frozen for histological examination exactly as described by Meng et al. (2014).
RESULTS

Fam65B location within the Mus musculus genome is chromosome 13: 24,674,058-24,825,685. It is reported that 6 alternatively spliced transcripts are known to be protein coding: Fam65b-002 (633 aa, 14 exons), Fam65b-003 (1078 aa, 23 exons), Fam65b-006 (1053 aa, 23 exons), Fam65b-007 (672 aa, 15 exons), Fam65b-201 (1034 aa, 22 exons), Fam65b-202 (583 aa, 14 exons) (“Ensembl genome browser 54: M.musculus - Gene summary - Gene: Fam65b (ENSMUSG00000036006),” n.d.) (Figure 4). WB analysis of lysates human fetal myoblasts revealed that two major isoforms were expressed during myogenic differentiation: one being 1018 aa (105 Kd) and the other one being 591 aa (72 Kd) (Yoon et al., 2007).

**Figure 4. Fam65B protein coding transcripts in Mus musculus.** Six alternatively spliced transcript for the Fam65B gene are reported to be coding for protein. Adapted from Archive Ensemble Release 37.
Description of Fam65B Deletion Cassette.

Fam65b -/- mice were generated from the knock-out mouse project (KOMP) by using the Embryonic Stem Cell (ECS) clone 15686A-B6, in which exons 2-14 have been deleted. Therefore, the genomic regions coding for exon 1 and exons 15-23 remain part of the mice DNA. The deleted genomic sequence is 34,926 bp long. The deleted region was substituted via homologous recombination with a bacterial artificial chromosome (BAC) vector which contains both a B-galactosidase and a neomycin coding sequences (Velocigene, KOMP, Davis, CA). Figure 5 shows a representation of the deletion map.

**Figure 5.** Deletion strategy for Fam65B -/- ECS clone 15686A-B6. A map of the deletion cassette employed to delete portion of the Fam65B gene, spanning a region from the beginning of exon 2 to the end of exon 14. The BAC vector replacing the deleted area contains both a reporter and a selection cassette. Adapted from Velocigene, KOMP.
Genomic analysis of Fam65b-/- mice.

To characterize the Fam65b-/- mice and verify the gene deletion, we performed PCR analysis of genomic DNA extracted from the tails of 12 Fam65-/- mice. It should be noted that reactions 1 and 4 only amplify WT genomic DNA, while reactions 2 and 3 only amplify genomic DNA within the BAC deletion vector. Figure 6 is a diagram showing the adopted genotyping strategy and Table 1 lists the primers utilized for each individual PCR reaction and the size of the expected amplicon. Finally, images of the gels run for each PCR reaction are shown in Figures 7-10.

Figure 6. Genotyping strategy for Fam65b-/- and WT mice. The diagram shows how primers have been designed to amplify genomic amplicons which are either present with the DNA of WT mice or part of the BAC vector inserted into Fam65b-/- mice. Adapted from Velocigene, KOMP.
<table>
<thead>
<tr>
<th>Reaction 1</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
<th>WT</th>
<th>HET</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction 2</td>
<td>SUF</td>
<td>TuR</td>
<td>1030 bp</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Reaction 3</td>
<td>SUF</td>
<td>LacZ R</td>
<td>320 bp</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reaction 4</td>
<td>NeoF</td>
<td>Fam1R</td>
<td>698 bp</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reaction 4</td>
<td>TDF</td>
<td>Fam1R</td>
<td>1545 bp</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1.** PCR conditions for genotyping of WT and Fam65b-/- mice. A list of the primers utilized for specific PCR reactions and the size of the expected amplicons is provided. The genetic background where we expect to observe specific PCR products is also reported.

**Figure 7.** PCR reaction 1. The expected amplicon product of this reaction is 1030 bp. This amplicon is only amplified from DNA of a WT mouse and the heterozygous AF9 cell line. This amplicon is not amplified from DNA of Fam65b-/- mice.
Figure 8. PCR Reaction 2. The expected amplicon product of this reaction is 320 bp. This amplicon is only amplified from DNA of Fam65b-/- mice, the Fam65b-/- AB6 cell line and the heterozygous AF9 cell line. This amplicon is not amplified from DNA of a WT mouse.

Figure 9. PCR Reaction 3 and 4. The expected amplicon product of reaction 3 is 698bp; this amplicon is only amplified from DNA of Fam65b-/- mice and the heterozygous AF9 cell line. The expected amplicon product of reaction 4 is 1545bp and should be amplified only from the DNA of the WT mouse and of the heterozygous AF9 cell line. As reactions 3 and 4 utilize fwd and rev primers with similar T_m, they were run simultaneously.
Figure 10. PCR Reaction 4. The expected amplicon product of reaction 4 is 1545bp and is only amplified only from the DNA of the WT mouse and of the heterozygous AF9 cell line.

The results of this genomic analysis show that Fam65b-/ mice lack the region of the gene that has been replaced with the BAC vector. Reaction 1 employs a forward (fwd) primer upstream of the deleted region and a reverse (rev) primer that is within the region deleted in Fam65B-/ mice. As predicted, this reaction amplifies the expected 1030bp amplicon only from WT DNA or the heterozygous AF9 cell line. Similarly, reaction 4, which utilizes a rev primer downstream of the deleted region and a fwd primer within the deleted region only amplifies the expected 1545bp amplicon from the DNA of WT animals and the heterozygous AF9 cell line. Conversely, reactions 2 and 3 use primers that can only anneal with DNA regions within the BAC deletion vector. As expected, these reactions respectively amplify a 320bp amplicon and a 698bp amplicon only from DNA of Fam65b-/ mice, the Fam65b-/- AB6 cell line and the heterozygous AF9 cell line.
RNA isolation and RT-PCR analysis.

To confirm that the Fam65-/- mouse model is also a KO at the transcriptional level, total RNA was isolated from the quadriceps of Fam65b-/- and WT mice and converted into cDNA. RT-PCR was performed to identify the Fam65B muscle transcripts in both KO and WT mice. Four sets of fwd and rev cDNA primers were generated. Primer sets 4-10 and 6-10 respectively amplify Fam65b cDNA regions that span exons 4 to 10 and exons 6 to 10; consequently, as our KO model deletes genomic DNA from exon 2 to exon 14, these reactions should only amplify cDNA extracted from muscle tissue of WT mice. Instead, primer set 20-23 amplifies a Fam65b cDNA region that spans exons 20 to 23. As this segment is outside of the deleted area, a RT-PCR product could also be potentially generated from the cDNA of KO mice; indeed, if a promoter region, such as a TATA box and/or GCp islands, were to exist upstream of exon 20, a PCR product may then be generated in both KO and WT mice. Finally, a positive control for both WT and KO cDNA amplified the cDNA for Dystrophin exons 8 to 10. Dystrophin is encoded by 79 exons and its loss of function is responsible for Duchenne Muscular Dystrophy (DMD) (Roberts et al., 1993). Figures 11 and 12 show the gel electrophoresis of the PCR amplifications from the quadriceps of KO and WT mice in the presence and absence of dimethyl sulfoxide (DMSO) respectively.
Table 2. RT-PCR reaction conditions for transcriptional analysis of WT and Fam65b−/− mice. A list of the primers utilized for each specific RT-PCR reaction and the size of the expected amplicons is provided. The genetic mouse background where we expect to observe specific PCR products is also reported.

<table>
<thead>
<tr>
<th>Reaction 4-10</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction 6-10</td>
<td>Fam65b-exon 4</td>
<td>Fam65b-Exon 10/1</td>
<td>380 bp</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Reaction 20-23</td>
<td>Fam65b- exon 20</td>
<td>Fam65b-exon 23</td>
<td>334 bp</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Reaction 8-10</td>
<td>Dystrophin- exon 8</td>
<td>Dystrophin-exon 10</td>
<td>349 bp</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 11. RT-PCR Reactions of WT and KO with DMSO. Reactions 4-10 and 6-10 which are summarized in Table 2, were run in the presence of 0.16% vol. of DMSO. The 100bp ladder shows markers from 1.5 Kb to 0.1 Kb.
Figure 12. RT-PCR Reactions of WT and KO without DMSO. Reactions 4-10 and 6-10, which are summarized in Table 2, were run in the absence of DMSO. The 100bp ladder shows markers from 1.5 Kb to 0.1 Kb.

As predicted, reactions 4-10 and 6-10 are only observed in cDNA of WT mice, both in the presence and absence of DMSO. Nevertheless, it should be noted that a non-specific band of about 1.4 Kb is present in the WT lane of reactions 4-10 and 6-10 (Figures 11 and 12).

As aforementioned, reaction 20-23 amplifies a region from exon 20 to 23 of the Fam65b gene which is located downstream of the deleted region. If a single promoter region is expected to exist upstream of the transcription start site of the Fam65b gene, the product of this reaction would only be amplified from the cDNA of WT mice. Nevertheless, as the expected amplicon (334bp) for this reaction is observed both in the KO and WT lanes, the presence of a putative transcription start site downstream of the deleted region and upstream of exon 20 cannot be excluded. Consequently, an online promoter-scan program was used to identify potential transcriptional promoter regions.
downstream of the Fam65B deletion cassette ("Promoter 2.0 Prediction Server," n.d.); four entries were found, respectively within intron 15, 16, 17 and 19 of the Fam65b gene. If this explanation were to be true, we cannot exclude the possibility that shorter Fam65b isoforms may also be translated and could possibly be identified as protein-coding transcripts by WB analysis. Table 3 shows the location of these putative promoter sites and the sizes of the corresponding encoded proteins.

<table>
<thead>
<tr>
<th>Highly Likely Promoters Downstream of Deletion Cassette</th>
<th>Genomic Location</th>
<th>Putative Protein Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24,801,658 (intron 15)</td>
<td>340 AA (~37.4 Kd)</td>
</tr>
<tr>
<td>2</td>
<td>24,804,358 (intron 16)</td>
<td>284 AA (~31.2 Kd)</td>
</tr>
<tr>
<td>3</td>
<td>24,806,758 (intron 17)</td>
<td>236 AA (~25.9 Kd)</td>
</tr>
<tr>
<td>4</td>
<td>24,810,858 (intron 19)</td>
<td>131 AA (~14.4 Kd)</td>
</tr>
</tbody>
</table>

Table 3. Promoter locations downstream of Fam65B deletion cassette. The genomic location of putative promoter regions downstream of the Fam65B deletion cassette and the expected size for the putative proteins are listed.

Finally, as explained above, amplification of a region of the dystrophin cDNA, was conducted as positive control for both WT and KO mice. Indeed, both in the presence and absence of DMSO, this amplicon is transcribed both in KO and WT mouse quadriceps.

Analysis of Fam65B protein expression

WB studies were conducted on lysates prepared from both fully differentiated skeletal muscle tissues and cultured myoblasts undergoing differentiation. As previous
studies have shown that Fam65B expression in primary human fetal myoblasts reaches its maximum 24 to 48 hours after switching them into differentiation medium, cells were approximately harvested at these time points (Yoon et al., 2007; Balasubramanian et al., 2014). Protein lysates harvested from differentiating human myoblasts were used as a positive control. The lysate of human myoblasts showed two major bands (~70 Kd and ~110 Kd), while several bands were identified on the lysates isolated from Fam65B/- and WT mice. To identify the best conditions for the WB analysis of murine lysates, different combinations of primary antibody (Ab) and blocking solutions were utilized. Table 4 summarizes the different conditions tested.

<table>
<thead>
<tr>
<th>Blocking Solution</th>
<th>Primary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 13a</td>
<td>3% BSA</td>
</tr>
<tr>
<td>Figure 13b</td>
<td>3% BSA</td>
</tr>
<tr>
<td>Figures 14a &amp; 14b</td>
<td>5% BSA + 1% NFDM</td>
</tr>
<tr>
<td>Figure 15</td>
<td>5% BSA + 1% NFDM</td>
</tr>
</tbody>
</table>

**Table 4. Summary of western blots experimental condition.** A list of the primary antibodies and blocking solutions used for each WB is provided. Secondary antibodies were chosen against the species in which the primary antibodies were raised. NFDM= non fat dry milk; BSA= bovine serum albumin.
Figure 13. Fam65b expression analysis in murine skeletal muscle tissue lysates with MO1 Ab. Lanes labeled as WT and 2681 KO are lysates isolated from quadriceps of two mice. Lanes labeled as 12 Hr and 24 Hr correspond to lysates harvested from primary human fetal myoblasts. 

a) Mouse monoclonal antibody (MO1) raised against the full length of Fam65B was used as primary Ab (1:1000) in blocking solution. b) Rabbit monoclonal antibody raised against GAPDH was used as primary Ab (1:5000) in blocking solution.

It should be noted that though bands of ~200 Kd, ~ 100 Kd and ~ 25 Kd present in lane KO 2681 (Fam65b -/-) decreased in intensity relative to the WT lane, none of the band disappeared (see Figure 13a). Furthermore, while the ~100 Kd band may correspond to the Fam65 protein, it is more likely that the ~ 200 Kd band represents either a dimeric form of Fam65b or a cross-reacting protein. In order to compare Fam65b protein sequence to the ones of other proteins and see whether they share significant homology, an online database, called Basic Local Alignment Search Tool (BLAST) was utilized (“NCBI Blast: Protein Sequence (1078 letters),” n.d.). The search showed that dysferlin, a cell membrane protein whose function is critical for maintenance of plasmalemma integrity and for regeneration of skeletal muscle, shares 28% homology with Fam65b (Han et al., 2011). Furthermore, previous studies in our lab have shown that Fam65b co-immunoprecipitates with dysferlin (Balasubramanian et al., 2014). For these
reasons, the potentially cross-reacting band with a molecular weight of ~ 200 Kd may be dysferlin. Instead, the ~ 25 Kd band may represent a short isoform of Fam65b due to the presence of an alternative promoter downstream of the deleted area, located in intron 17 of the Fam65b gene (see Table 3). Furthermore, because the primary Ab (MO1) was raised in mouse, and the tissue lysates are also murine in origin, MO1 can cross-react with light (25 Kd) and heavy chains (50 Kd) of IgGs present in murine lysates. Therefore, for studying Fam65b protein expression in mouse tissue, a custom-made Ab raised in rabbit was also utilized.

Figure 14. Fam65b expression analysis in murine skeletal muscle tissue lysates with custom made Ab. Lanes labeled as WT and KO are lysates isolated from murine quadriceps. Lanes labeled as 12 Hr and 24 Hr correspond to lysates harvested from differentiating primary human fetal myoblasts. a and b) Rabbit Polyclonal Custom made antibody raised against a peptide of Fam65B was used as primary Ab (1:1000) in blocking solution. Both experiments were performed by following the same protocol, yet lysates extracted from additional mice were analyzed in b.

As in previous WBs potentially cross-reacting bands were observed, in the WBs illustrated in figure 14a and 14b, the concentration of BSA was increased to 5% and 1% of non-fat dry milk (NFDM) was also added. Figure 14a shows that the bands of 200 Kd,
100 Kd and 25 Kd detected in the WT lysate are of greater intensity relative to those detected in the KO lysate. Nevertheless, this result could not be reproduced in Figure 14b. Indeed, only the expression of the band at 25 Kd is reduced in tissue lysates of KO mice relative to those of WT mice. Instead, none of the other bands disappeared in the lanes of KO tissue lysates. For this reason, as it is known that lysates of myogenic cells cultured in vitro are more enriched for Fam65b than lysates that have been directly prepared from differentiated skeletal muscles, myogenic cells were isolated from both KO and WT mice via FACS as described in the methods section. Cells were cultured and induced to differentiate before being harvested either 36 or 48 hours after switching them into differentiation medium.

**Figure 15. Fam65b expression analysis in lysates of murine myoblast with MO1 Ab.** Lanes labeled as WT4, WT5, KO4 and KO5 are lysates isolated from murine myoblast, 48 hours after differentiation had been induced. Lanes labeled as undifferentiated and 24 Hr humans correspond to lysates harvested from primary human fetal myoblasts before and after differentiation. a) Mouse monoclonal antibody (MO1) raised against the full length of Fam65B was used as primary Ab (1:1000).
Figure 15 shows that bands of ~ 200 Kd and ~ 55 Kd are equally expressed in lysates harvested from both WT and KO differentiating murine myoblasts. Yet, the band of ~ 120 Kd, which may correspond to the longest Fam65b isoform, is only present in WT 5 (murine myoblasts 48 hours post differentiation) and human fetal myoblasts lanes; this ~ 120 Kd is absent in WT 4, KO4 and KO5 (murine myoblasts 48 hours post differentiation respectively isolated from WT and Fam65b-/- mice). This result may be encouraging, but it remains important to answer why the WT4 lane lacks this 120Kd band. A worthwhile experiment would be to re-genotype and conduct RT-PCR analysis for the WT4 clone to fully ensure that it was not misgenotyped at the beginning of this study.

Fam65B and its potential role in adiposity

To completely characterize Fam65b-/- mice and observe any relevant phenotypic changes, the weight of both KO and WT animals were recorded monthly over the course of 15 months. It should be noted that the weights of Fam65b-/- male and female mice are significantly greater than those of their WT counterparts (Figure 16). The difference in weight between WT and KO mice remains significant for a longer period of time in the male gender, where the KO background is significantly heavier up to 12 months of age (p value < 0.05). Instead, in females, though Fam65b-/- animals are remarkably heavier up to 7 months of age (p value 0.01), statistical significance is lost after this time point.
Figure 16. Fam65/- and WT mice weight chart. The weight of Fam65 -/- KO and WT were recorded over a period of 15 months.

As this significant difference in weight between WT and KO backgrounds has been recorded, it is plausible to think that the loss of Fam65b expression may be responsible for the observed phenotype. For this reason, it would be interesting to utilize new cohorts of Fam65b/- and WT mice in weight studies.
DISCUSSION

The present work investigates the functionality of the Fam65b KO mouse model at the gene, transcript and protein levels, with the aim to better comprehend the role of this protein during the differentiation of myogenic cells. Because it is thought that Fam65b plays an important role in facilitating myoblast fusion and myotubes formation, which are critical steps of myogenic differentiation, the availability of a Fam65b KO model would allow us to show whether the lack of expression of this protein compromises proper skeletal muscle development and regeneration. Furthermore, the generation of a functional Fam65b KO mouse model would enable our lab to temporally define the expression of Fam65b relative to other MRFs, such as MRF4 and myocyte enhancer factor 2C (MEF2C), and to understand whether the ablation of Fam65b also affects the ability to express these other myogenic proteins.

In this work, PCR analysis of 12 Fam65b KO mice has showed that partial deletion of the Fam65B gene was performed successfully by inserting a deletion cassette via homologous recombination between exon 2 and exon 14 (see Figures 7-10). Furthermore, RT-PCR analysis showed that the transcript corresponding to the deleted region of the Fam65b gene is not transcribed in KO mice (see Figures 11-12). Since these results show that Fam65b KO mice lack the genomic region between exon 2 and exon 14 of Fam65b, and that they do not transcribe the mRNA corresponding to this specific genomic region, it was initially thought that a true KO model had been generated.
Nevertheless, Figures 11 and 12 also show that Fam65b-/ mice transcribe a mRNA region (exon 20 to exon 23) which is downstream of the deletion cassette. This finding could be explained by the presence of a putative transcription start site downstream of the deleted region and upstream of exon 20. Indeed, analysis via a promoter-scan program revealed the presence of four putative transcription start sites downstream of the deletion cassette (see Table 3). The presence of a promoter within intron 17 would potentially result in the expression of a Fam65B truncated isoform of 25.9 Kd. Because a band of approximately this molecular weight is also detected via WB analysis (see Figures 13a, 14a and 14b), this finding further suggests that the presence of additional promoter regions downstream of the deletion cassette may indeed exist.

Although initial genomic and transcriptional analysis of this KO model revealed promising results, experiments studying Fam65b expression in KO mice have been more problematic. Because previous WB analysis of human fetal myoblast performed by Yoon et al. (2007) showed that the two major isoforms of Fam65B in muscle tissue were 105Kd and 70Kd, molecular bands of approximately these sizes were expected to be present in muscle lysates of WT mice, and to be absent in Fam65b -/- mice. Nevertheless, bands of 200Kd, 120 Kd, 55 Kd and 25 Kd were identified in protein lysates of skeletal muscle tissues of both WT and KO mice (see Figures 13a, 14a and 14b). Although the expression of these isoforms was decreased in KO mice compared to WT ones (see Figures 13a and 14a), none of the bands were absent in KO samples when protein extraction was directly performed in skeletal muscle tissue. Because Fam65b shares 28% homology with dysferlin (which has a molecular weight of ~ 200 Kd), and given the fact
that these two proteins also co-immunoprecipitate, the 200 Kd band may correspond to dysferlin (Balasubramanian et al., 2014; “NCBI Blast: Protein Sequence (1078 letters),” n.d.). This hypothesis implies that dysferlin is cross-reacting with the Fam65b antibody, and that the immunogenic site is being shared by the two proteins. Furthermore, since both the MO1 Ab and the custom-made Ab were previously generated to recognize the human Fam65b protein, it is possible that some of the bands identified in murine skeletal muscle lysates are cross-reacting bands.

Consequently, with the objective of minimizing cross-reacting bands, two strategies were undertaken: the concentration of blocking solution was increased to 5% BSA and 1% NFDM, and myogenic cells - which are more enriched for the Fam65b protein - were isolated from both KO and WT mice via FACS. Under these conditions, although the 55 Kd and 200 Kd bands were present in both KO and WT samples, the 120 Kd band, which may correspond to the long isoform of Fam65b, was only present in WT 5 clone muscle cells and absent in the KOs (see Figure 15). Nevertheless, as Figure 15 also shows that the WT 4 clone lacks the 120 Kd band, it would be important to conduct once more PCR and RT-PCR analysis to ensure that this clone was not initially misgenotyped.

Moreover, to further improve the accuracy of our experiments, additional strategies could be employed. The use of protein G magnetic beads to pre-clear IgG light and heavy chains would enable us to still utilize the mouse-raised MO1 Ab in whole murine skeletal muscle lysates and reduce the presence of cross-reacting bands. This step could also be performed for myogenic cells isolated by FACS; yet it is well known that
the amount of IgGs is significantly lower in purified cell lines than in whole skeletal muscle tissue. Furthermore, since the commercially available Abs and our custom-made Ab have all been designed to detect the human Fam65b protein, it would be worthwhile to design a new Ab that specifically recognizes an epitope of the murine Fam65b protein.

Finally, if further WBs of protein lysates of either differentiated skeletal muscles or myogenic cells from Fam65b−/− were insufficient to fully demonstrate the validity of this knock-out strategy, another technique should be employed to generate a functional KO model. A valid alternative may be to use the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas9 technology. The enzyme Cas9 is a bacterial nuclease directed by a single guide RNA (sgRNA) sequence that specifically base-pairs with a 20-bp DNA target; an additional requirement for the selection of Cas9 target loci is the presence of a protospacer adjacent motif (PAM) sequence which is located 3’ of the 20-bp target sequence (F. A. Ran et al., 2013; Hsu, Lander, & Zhang, 2014). Upon complementary binding of the guide RNA to the target DNA, the Cas9 induces genome editing by introducing double-stranded breaks (DSB) in the target region. The DNA cleavage promotes one of two possible damage repair mechanisms: the error prone non homologous end joining (NHEJ), which can be utilized to mediate gene knockouts, or the high fidelity homologous directed repair (HDR), which introduces precise modifications when an exogenous repair template is also being supplied. NHEJ, by generating DSB within a coding exon, can induce frameshift mutations and premature stop codons (Cong et al., 2013). Furthermore, to increase the specificity of the NHEJ pathway and minimize off-target activity, a double nicking strategy can be used to introduce DSBs; indeed,
while the WT Cas9 nuclease is guided by an sgRNA, the D10A mutant Cas9 nickase (Cas9n) is directed by two appropriately oriented sgRNAs to promote the formation of single-stranded nicks on both target DNA strands (F. A. Ran et al., 2013; H. Yang, Wang, & Jaenisch, 2014). As single-stranded nicks are repaired without indel formation, DSBs can only occur if both sgRNAs are able to locate target sequences within a defined space. Thus, this strategy, by doubling the number of bases that need to be recognized at the target site, significantly increases the specificity of genome editing. As our aim is to ablate the expression of Fam65b, the induction of NHEJ would allow us to generate a functional KO and better assess how this protein contributes to the differentiation of myogenic cells. A possible experimental strategy would be to transfect C2C12 cells with an expression plasmid bearing the oligo pairs encoding for both a Fam65b-specific sgRNA scaffold backbone and Cas9. Finally, after testing the efficacy of the targeted cleavage via the SURVEYOR nuclease assay or sequencing analysis, transfected cells can be clonally expanded to create isogenic cell lines with the specific mutation. A scheme of this editing strategy is shown in Figure 17.
Figure 17. Model of CRISPR Cas9 editing strategy. sgRNA and Cas9 sequences are first cloned into an expression vector. The pSpCas9 plasmid is then transfected into cells, and the targeted DNA modifications are detected via sequencing. Cell clones bearing the desired mutation are isolated via FACS, and finally expanded to generate isogenic cell lines. Adapted from Ran et al. (2013).

Alternatively, the CRISPR/Cas9 system could be employed to generate a Fam65b KO mouse model. This technology has been employed to introduce loxP sites into specific exon regions, so that CRE recombinase-mediated excision can then be performed to delete the genomic area of interest (H. Yang et al., 2013, 2014). This strategy requires creation of a vector donor which bears oligo nucleotide sequences corresponding to the
loxP sites, in addition to those encoding for both a Fam65b-specific sgRNA scaffold backbone and Cas9. The plasmid construct would be introduced into the cytoplasm of a mouse zygote, which is then transferred into the oviduct of a pseudopregnant mouse. This method has been successfully employed to generate mutant mice and rats models for Duchenne Muscular Dystrophy, and we think it may help to understand how the lack of function in Fam65b affects the differentiation of myogenic cells in vitro and in vivo (Nakamura et al., 2014).

As previously explained, studies have shown that the downregulation of Fam65b via RNAi does not affect MyoD expression levels, while resulting in a significant reduction of myogenin expression (Yoon et al., 2007). These results suggest that Fam65b acts downstream of MyoD, yet upstream of myogenin in the differentiation of mononuclear myogenic cells. The availability of a functional Fam65b KO murine cell line or mouse model would allow us to define the temporal expression of this protein relative to other myogenic transcriptional regulators like MRF4 and MEF2C. Previous studies have reported that MRF4 KO mice, while possessing a wide range of phenotypic variance in muscle development, show augmented myogenin expression; this experimental outcome has led to the belief that MRF4 may act in parallel to myogenin, and has a role in terminal differentiation similar to myogenin (Patapoutian et al., 1995; Francetic & Li, 2011). We hypothesize that ablation of Fam65b expression may result, as it occurs with myogenin, in the reduction of MRF4 expression levels. Instead, MEF2C has a more longitudinal expression span during myogenic differentiation (Panda et al., 2014). Indeed, chromatin immuno-precipitation (ChIP) experiments have revealed that
MyoD upregulates the transcription of MEF2C by binding to an A/T rich enhancer regions during early skeletal muscle differentiation (Dodou, Xu, & Black, 2003; Potthoff et al., 2007); at the same time, MEF2C, by binding to promoter regions of MRF4 and myogenin, amplifies and maintains their expression, thus contributing to the later stages of myotube development (Buchberger, Ragge, & Arnold, 1994; Naidu, Ludolph, To, Hinterberger, & Konieczny, 1995). Consequently, in order to understand at which stage of skeletal myogenesis we can place Fam65B expression relative to other myogenic regulatory factors like MyoD, MRF4, myogenin and MEF2C, differentiating myoblasts from both KO and WT Fam65b mice should be harvested at different time points, and WB analysis of Fam65B and these other MRFs should be performed. Furthermore, to establish whether the transcriptional activity of Fam65b is controlled by key myogenic transcription factors like MyoD or Myf5, which are responsible for the initiation of myogenic differentiation, ChIP analysis may also be carried out.

Previous studies from our lab have also shown that Fam65b, by directly interacting with HDAC6, may play a role in regulating microtubule dynamics (Balasubramanian et al., 2014); it now remains to be elucidated whether this function may contribute to the migration of individual myoblasts toward one another, thus facilitating myotube formation and the progress of myogenic differentiation. It is known that HDAC6, by modulating the levels of acetylation of α-tubulin and cortactin, is important for the regulation of cell motility (J. Ran et al., 2015).

In Hela cells, cortactin, a protein which interacts with f-actin at the leading edge of migrating cells to form lamellipodia and membrane ruffles, is a target of HDAC6.
Utilization of HDAC inhibitors or HDAC6 siRNA resulted in cortactin hyperacetylation and inhibition of its translocation to the cell periphery; this prevented cortactin from associating with F-actin, and thus impaired cell motility (X. Zhang et al., 2007). Thus, it would be interesting to study, by either co-immunoprecipitation or the proximity ligation assay (PLA), whether Fam65B interacts with cortactin and F-actin, and whether Fam65b protein contributes to regulate cortactin acetylation levels in differentiating myoblasts.

Moreover, it should be pointed out that cortactin translocation to the cell periphery is mediated by the small GTPase Rac1; at this location, cortactin has been shown to promote the activation of the actin-nucleating complex Arp2/3 and facilitate the formation of lamellipodia networks (Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001; Head et al., 2003). It is also known that the Rho family of guanine nucleotide exchange factors (GEFs) is involved in cytoskeletal rearrangement, formation of focal adhesion, and regulation of cell polarity. As Fam65B shares 30% homology with the GTPase Rac1 (NCBI, Aceview), it may, in conjunction with Rac1, regulate the activity of cortactin within the cell. These experiments would allow us to understand more precisely the role of Fam65B in the migration of myoblasts, and whether this function contributes to differentiation.

Finally, as our data indicate that Fam65KO mice are significantly heavier than WT mice, and that this phenotype is consistently observed across both genders during the first seven months of age, it would be interesting to investigate whether Fam65b plays a regulatory role in fat metabolism. For example, since the important role of the mammalian target of rapamycin (mTOR) signaling pathway as a regulator of adipose
tissue mass is well established, the expression levels of mTOR and its downstream effector AKT, also known as protein kinase B (PKB), may be analyzed in both WT and KO mice (Laplante & Sabatini, 2009; Blanchard et al., 2012).

Furthermore, it is noteworthy that cytoskeletal modifications and microtubule rearrangements are important for the differentiation of pre-adipocyte into mature adipocytes (Kanzaki & Pessi, 2002; Takenouchi et al., 2004). Importantly, the levels of acetylated α-tubulin are significantly increased during cellular adipogenesis, and the acetylation state of this protein is regulated by acetyltransferase MEC-17 and HDAC6 (W. Yang et al., 2013). Furthermore, C57BL/6 mice fed on a high fat diet revealed elevated levels of acetylated α-tubulin and MEC-17; additionally, knock-down of HDAC6 in fibroblasts (3T3-L1 cells) leads to both elevated acetylated α-tubulin and increased content of lipid droplets (W. Yang et al., 2013). Furthermore, it has been reported (unpublished data) that HDAC6 KO mice gain significantly more weight than WT mice, and that cells lacking HDAC6 increase glucose uptake while downregulating fatty acid oxidation (Kapur, 2015). As Balasubramanian et al. (2014) showed that downregulation of Fam65b induced a reduction in the translated levels of HDAC6, this negative-feedback response may result in increased levels of acetylated α-tubulin and differentiated adipocytes, thus potentially explaining the phenotype that we observed in Fam65KO mice.

Other studies have also shown that down-regulation of HDAC6 results in the hyperacetylation and destabilization of the chaperone heat shock protein 90 (Hsp90) (Kovacs et al., 2005). Because Hsp90 needs to be in its deacetylated state to bind and
generate a stable complex with the glucocorticoid receptor (GR), downregulation of HDAC6 indirectly compromises the stability of GR, which becomes defective in ligand binding and transcriptional activation (Kovacs et al., 2005). It is well known that proper function of the GR is critical for maintaining metabolic homeostasis (Rose, Vegiopoulos, & Herzig, 2010); therefore, it would be interesting to investigate whether Fam65b, by regulating the levels of HDAC6, can regulate the acetylation levels of tubulin and Hsp90, and thus play a critical role in the body energy balance.

This series of experimental questions will allow us to better define the role of Fam65b in both myogenic differentiation and cellular metabolism; for this reason, we believe that the characterization of the KO mouse model described in this thesis will enable us to design additional experiments. The present studies represent an important step towards future accomplishments in this line of research. Furthermore, it is important to point out that no previous studies have so far characterized a Fam65b KO mouse model in the context of skeletal muscle differentiation. Only Gao et al. (2015), who analyzed the function of Fam65b in the polarization of neutrophils, reported to have investigated the motility of neutrophils isolated from Fam65b KO mice, and that those immune cells exhibited defective directionality in their chemotaxis patterns. Nevertheless, the characterization of their Fam65b KO mouse model at the gene, transcript and protein levels was not reported. Consequently, as the complete characterization of a Fam65b KO model has yet to be described in the literature, the importance of the work undertaken in this thesis is significant. We here report that a Fam65b KO mouse model was generated by replacing the genomic region of Fam65b
spanning exon 2 to exon 14 with a deletion cassette. Although genomic and transcriptional studies have shown that a KO mouse model may have been generated, WB analysis of Fam65b expression remains inconclusive. Indeed, while lysates of murine myoblast isolated from KO clones lack the 120 Kd isoform of Fam65b, WBs of murine skeletal muscle tissue lysates can still detect the presence of the main Fam65b isoforms. Furthermore, the present study, by reporting that Fam65b KO mice are significantly heavier than WT mice and that this phenotype is observed in both genders during the first seven months of age, highlights a potential link between the loss of Fam65b expression and weight gain. Finally, as a definitive conclusion about the functionality of the generated KO mouse model cannot still be made, further work is required to answer these questions and additional steps will be taken to generate a new KO model via the CRISPR-Cas9 technology.
REFERENCES


Potthoff, M. J., Arnold, M. A., McAnally, J., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. (2007). Regulation of Skeletal Muscle Sarcomere Integrity and


http://doi.org/10.1002/(SICI)1097-4652(199912)181:3<499::AID-JCP14>3.0.CO;2-1


http://doi.org/10.1038/270725a0


CURRICULUM VITAE

ALESSANDRO COLLETTA

Year of Birth: 1989
Current Address: 14 Buswell Street (Unit 312), Boston, MA 02215.
Phone: (734) 355-1135 Email: alecolle@bu.edu

EDUCATION

University of Michigan, Ann Arbor, MI
Bachelor of Science, Microbiology, April 2012. GPA: 3.82
Boston University School of Medicine – Graduate Medical Sciences Division, Boston, MA
M.S. in Medical Sciences (MAMS), August 2016. GPA: 4.00

RESEARCH EXPERIENCE

Research Thesis for Master in Medical Sciences • Boston Children’s Hospital, Skeletal Muscle Stem Cells Lab, Boston, MA, September 2015 – May 2016

• Investigating the role of the cytoplasmic protein Fam65b in the formation of multinucleated myotubes via cell fusion, and the potential implications of this process in human muscle diseases.

Optional Practical Training • University of Michigan, Biomedical Analytical Chemistry Lab, Ann Arbor, MI, June 2012 - August 2014

• Collaborated with a biomedical firm, Biocreed, to study the antimicrobial/antibiofilm properties of Foley catheters impregnated with S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide (NO) donor.

• Utilized silicone rubber catheters doped with SNAP and CarboSil, a thermoplastic urethane copolymer, to study the prevention of biofilms by bacterial strains causing urinary infections.

• Studied the electrochemical modulated production of NO through polymeric materials from a stable nitrite electrolyte reservoir, with application in controlling biofilm formation and preventing thrombosis on intravascular catheters.

Research Assistant • University of Michigan, Biomedical Analytical Chemistry Lab, Ann Arbor, MI, January 2012 - June 2012
• Investigated the production of NO in the nasal breath as non-invasive mode to detect airway disease. The quantification of exhaled NO was performed by utilizing the reaction of NO with oxyhemoglobin to form nitrate. Ion-selective electrodes were employed to measure nitrate levels.

Research Assistant • University of Michigan, Rheumathoid Arthritis Lab, Ann Arbor, MI, September 2009 - October 2011

• Investigated how the shared epitope (SE), a RA genetic risk factor, dysregulates the immune system by suppressing Treg cells and up-regulating T helper 17 cells. These aims were accomplished by means of a collagen-induced arthritis mice model, cell culture, flow cytometry and ELISA techniques.

• Employed fluorescence based essays to study how the SE, by upregulating NO and reactive oxygen species (ROS) levels, augments RA severity by inducing osteoclastogenesis.

• Screened cyclic SE-peptidomimetic compounds that inhibit signaling between the SE and its cell-surface receptor calreticulin.

Research Assistant • Internship at University of Piemonte Orientale "Amedeo Avogadro", Novara, IT. June - August 2009

• Investigated whether carriage of the 7-repeat polymorphism of the dopamine receptor D4 (DRD4) gene may influence the histologic outcome in a cohort of untreated Hepatitis C virus (HCV) patients with persistently normal transaminases (PNALT).

• Learned to clone the HCV Core Protein and express it transiently in mammalian cells.

VOLUNTEER EXPERIENCE

Volunteer • Outreach Van Project, Boston University Medical Center, Boston, MA, June 2015 - May 2016.

• To outreach to the medically underserved populations in the Greater Boston area by providing food, clothing items and heath care services.

Volunteer • Emergency Room, University of Michigan Hospital, Ann Arbor, MI, January 2010 - January 2012
• Assisted Emergency Department Staff in the provision of support services for recovered patients and their families.

Volunteer • Internal Medicine Department, “Madonna Del Popolo” Hospital, Omegna, Italy. June 2010 - August 2010

• Gave moral and physical support to elderly patients.

PUBLICATIONS


CONFERENCES AND PRESENTATIONS


• Denise de Almeida, Ying Liu, Alessandro Colletta, Song Ling, Joseph Holoshitz. Immune Regulation by the Rheumatoid Arthritis Shared Epitope. Oral Presentation at the Autumn Immunology Conference 2010.

PROGRAMS AND ORGANIZATIONS

Peer Mentor ● University of Michigan Health Science Scholars Program, Ann Arbor, MI September 2008 - May 2010

• Assisted first-year Health Science Scholars Program students in their transition to college.
• Shadowed health care professionals at the UM Cardiovascular Center and Emergency Room.

AWARDS

The National Society of Collegiate Scholars ● University of Michigan, Ann Arbor, MI
• Awarded to the Top 10% of their class.

Graduation Honors ● University of Michigan, Ann Arbor, MI
• Graduated with Distinction honors.