Ca\textsubscript{\(\alpha\beta_{1a}\)} in the Nucleus of Muscle Progenitor Cells:
Mechanisms, Functions, and Implications

BY

JACKSON R. TAYLOR

A Dissertation Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Neuroscience

December 2012

Winston-Salem, North Carolina

Approved By:

Osvaldo Delbono, M.D., Ph.D., Advisor

Ashok Hegde, Ph.D., Chair

Ronald Oppenheim, Ph.D.

David Riddle, Ph.D.

Cristina Furdui, Ph.D.
Dedication and Acknowledgements:

I dedicate this dissertation to my parents, Patrica Lord and Richard Taylor. They have both been immensely supportive to me throughout this whole process, and nurtured my scientific interests from an early age.

I would also like to acknowledge:

My advisor, Dr. Osvaldo Delbono, who put a tremendous amount of time and effort into my scientific and professional development.

All of the “Delbono Lab”: Laura Delbono, Tan Zhang, Zhong-Min Wang, and Alexander Birbrair. I truly could not have asked for more warm and caring group of people to spend the last six years with.

My thesis committee: Dr. Ron Oppenheim, Dr. David Riddle, Dr. Ashok Hegde, Dr. Cristina Furdui, and Dr. Christopher Turner. All of my committee members were very supportive and helpful in the preparation of this work. As Neuroscience Program director, Dr. Oppenheim was exemplary in his personal dedication to all Neuroscience students, myself included. Dr. Turner was instrumental in the development of my NRSA, which was funded largely in part due to his diligent efforts in the revision process.

Former Neuroscience Program secretary, Jody Dedo. Jody was not only immensely organized and effective at her job, she was one of the most comforting, understanding people I have ever met. She was truly a rare combination and her impact on the program was unforgettable.

Tamara Spence. Tamara has been there for me throughout my entire graduate school career. Her dedication to science is what motivated me to begin truly applying myself early on, and she has continued to serve as an ideal example of scientific integrity and enthusiasm.

Grad School Coordinator Susan Pierce, who put up with my redundant questions and last minute requests for all these years, always with a friendly smile.

And last but not least: my beloved dog Jabba Bear, who over the last few years has endured surgeries and chemotherapy with a brave face and a goofy smile, and to this day continues to beat the odds.
TABLE OF CONTENTS

List of Figures ........................................................................................................ v
List of Tables ........................................................................................................ vii
List of Abbreviations ............................................................................................. viii
Abstract: ................................................................................................................... xi

Chapter 1
(Introduction)

Part I: History of Caβ as a Caγ subunit ................................................................. 1
Part II: Novel function for Caβ in modern times ............................................... 10
References: .............................................................................................................. 19

Chapter 2
The Cavβ1a Subunit Regulates Transcription and Suppression of Myogenin in Muscle Progenitor Cells

Abstract: ................................................................................................................... 29
Introduction ............................................................................................................... 30
Results: ...................................................................................................................... 33
Discussion ................................................................................................................ 44
Materials and Methods: ......................................................................................... 50
Acknowledgements: ................................................................................................. 58
Supplemental Information Summary ................................................................. 58
References: .............................................................................................................. 60
Figure List: ............................................................................................................... 71
Supplemental Figures ............................................................................................ 80
List of Supplemental Tables .................................................................................. 88
Supplementary Tables for Chapter 2 ................................................................. 190
CURRICULUM VITAE .......................................................................................... 225

List of Figures

CHAPTER 1

Figure 1. Timeline of important Ca,β subunit discoveries ........................................... 15
Figure 2. Schematic of generalized and specialized Ca,β subunit functions ................. 16

CHAPTER 2

Figure 1. Cavβ1a expression in MPCs ........................................................................ 71
Figure 2. Cavβ1a-YFP and endogenous Cavβ1a translocate to the nucleus of myoblasts ................................................................................................................................. 72
Figure 3. Mapping of the CaVβ1a nuclear localization domain .................................... 73
Figure 4. Regulation of MPC proliferation by Cavβ1a in vitro and in vivo ..................... 74
Figure 5. ChIP-on-chip analysis of Cavβ1a ................................................................. 76
Figure 6. Microarray analysis of Cacnb1 wild type (+/+), heterozygous (+/-) and knockout (-/-) MPCs .................................................................................................................. 78
Figure 7. Cacnb1 modulates Myog expression in muscle progenitors ......................... 79
Figure S1. Representative images of Cavβ1a –YFP mutants with and without LMB treatment…………………………………………………………………………………81

Figure S2. shRNA-mediated knockdown of Cavβ1a impairs myoblast proliferation..82

Figure S3. FACS of muscle precursor cells from embryo……………………………………83

Figure S4. Analysis of cell death in Cavβ1a knockdown and Cacnb1 -/- cells. ........84

Figure S5. Extracted patterns of gene expression from Cacnb1 +/-, +/-, and -/- MPCs……………………………………………………………………………………85

Figure S6. Screening for CaVβ1a binding partners at the Myog promoter………………….86

Figure S7. Mechanism of CaVβ1a regulation of myogenesis………………………….87

CHAPTER 3

Figure 1. Ca,β1a-YFP does not co-IP with either Rem or HP1 in C2C12 myoblasts………………………………………………………………………………………102

Figure 2. Ca,β1a-YFP nuclear purification for Mass Spectrometry ……………………103

Figure 3. Categorization and validation of Ca,β1a-YFP nuclear binding partners identified by M.S………………………………………………………………………………104

Figure 4. Endogenous Lef1 does not co-IP with Ca,β1a-YFP…………………………105
Figure S1. Empirical determination of optimal YFP antibody : bead ratio and amount for Ca,β1a-YFP pull down………………………………………………………………………………106

Figure S2. Mitochondrial contamination in nuclear fractions………………………………………………………………………………107

Figure S3. Lef1 protein is dynamically expressed during myogenesis in C2C12 myoblasts………………………………………………………………………………………………108

Figure S4 Protein A/G and B contamination from commercially available agarose and sepharose beads used for IP……………………………………………………………………109

Figure S5 Crosslinking A/G beads to antibody avoids IgG and protein A/G contamination while preserving Ca,β1a-YFP pull down…………………………………………………………110

List of Tables

CHAPTER 1

Table I. Non-Ca, binding partners of Ca,β subunits………………………………………………17

CHAPTER 2.

Table SI. Inventory of primers used throughout the paper for various functions …….190

Table SII. Inventory of antibodies used throughout the paper for various experiments ………………………………………………………………………………………………192

Table SIII. List of buffers used in ChIP……………………………………………………………..193
Table SIV. Complete list of genes with promoter regions found to be enriched for Ca$_v$$\beta_{1a}$ binding. ..........................................................195

Table S5. List of genes with promoter regions found to be ChIP enriched for Ca$_v$$\beta_{1a}$ binding and also showing differential regulation in expression by microarray analysis.................................................................221

CHAPTER 3

Table I. Nuclear Cav$\beta_{1a}$-YFP binding partners identified by mass spectrometry......120
List of Abbreviations

AA...................................................................................................... amino acid
AID.............................................................................................. alpha interaction domain
BME.............................................................................................. β mercaptoethanol
Ca_v............................................................................................ voltage gated calcium channel
ChIP.............................................................................................. Chromatin Immunoprecipitation
DHPR.............................................................................................. dihydropyridine receptor
DM.............................................................................................. differentiation medium
DNA.............................................................................................. deoxyribonucleic acid
E_._.............................................................................................. embryonic day (post coitus)
E-C.............................................................................................. excitation-contraction
EMSA.............................................................................................. electrophoretic mobility shift assay
ER.............................................................................................. endoplasmic reticulum
FACS.............................................................................................. fluorescence assisted cell sorting
GDP.............................................................................................. guanine di-phosphate
GFP.............................................................................................. green fluorescent protein
GK.............................................................................................. guanylate kinase
GM.............................................................................................. growth medium
GTP.............................................................................................. guanine tri-phosphate
IgG.............................................................................................. immunoglobulin
kDa.............................................................................................. kilodalton
KO……………………………………………………………………………knockout
LMB……………………………………………………………………Leptomycin B
MS ………………………………………………………………………mass spectrometry
MAGUK…………………………………………………………membrane associated guanylate kinase
MPC……………………………………………………………………muscle progenitor cell
mRNA……………………………………………………………………messenger RNA
MW……………………………………………………………………molecular weight
NLS……………………………………………………………………nuclear localization sequence
PCR……………………………………………………………………polymerase chain reaction
qPCR……………………………………………………………………quantitative polymerase chain reaction
RGK……………………………………………………………………Ras Rad Gem/Kir
RNA……………………………………………………………………ribonucleic acid
RT………………………………………………………………………reverse transcriptase
RyR……………………………………………………………………ryanodine receptor
SDS-PAGE……………sodium dodecylsulfate polyacrylamide gel electrophoresis
SH3……………………………………………………………………sarcromere homology 3
shRNA…………………………………………………………short hairpin RNA
SR……………………………………………………………………sarcolemma
t-tubule…………………………………………………………transverse tubule
WB…………………………………………………………………..western blot
YFP……………………………………………………………………yellow fluorescent protein

x
Abstract:

Cavβ subunits are traditionally considered constituents of Cav complexes (Cav1 or 2, Cavβ, and Cav α2/δ), where they localize at the plasma membrane and serve to regulate channel expression and gating properties. Several recent publications also show Cavβ subunit localization in the nucleus. This phenomenon has been observed under a variety of conditions (different cell types, β subunit isoforms, co-expressed proteins, etc). However, the exact mechanisms responsible for Cavβ subunit nuclear shuttling, as well as a physiological role for this nuclear localization, remain major questions. Here we find that Cavβ1α enters the nucleus of myoblasts, and knockdown of Cavβ1α impairs myoblast proliferation in vitro and in vivo. To test if Cavβ1α may regulate gene expression, we conducted microarray experiments on RNA extracted from wild type, heterozygous, and Cavβ1–null mouse primary myoblasts. A large number of gene transcripts were found to be differentially regulated based on the relative amount of Cavβ1α expression. To identify specific Cavβ1α target genes, we performed ChIP–on-a-chip experiments to locate which promoter regions Cavβ1α bound to across the entire mouse genome. Nuclear binding partners of Cavβ1 were screened using affinity purification of Cavβ1α-YFP from myoblast nuclear fractions coupled with mass spectrometry. Our results support the idea of Cavβ subunits acting as transcription factors and regulating gene expression independently from Cav's, and suggest these functions may be particularly important to progenitor cell growth.
Chapter 1

General Introduction

Part 1: History of Ca,β as a Ca, subunit.

The β subunit of the voltage gated calcium channel (Ca,) made its first appearance in 1984, in a short article from the laboratory of William Catterall at the University of Washington (Curtis and Catterall, 1984). Here, the β subunit appeared in an acrylamide gel as a distinct silver-stained band of intermediate molecular weight (≈ 50 kDa), which had migrated in between the co-purified α (≈ 160 kDa) and γ (≈ 33 kDa) subunits of the voltage-sensitive calcium channel complex (at the time known as the dihydropyridine receptors due to their affinity for an antagonism by 1,4 dihydropyridines (Triggle and Janis, 1984)) in skeletal muscle (Figure 1). These three subunits, along with the soon-thereafter discovered α2 and δ subunits (Burgess and Norman, 1988; Leung et al., 1987) (transcribed and disulfide-bonded together, and essentially exist as a singular “α2/δ” subunit (De Jongh et al., 1990; Jay et al., 1991)), form the core components of the Ca, complex (Figure 2A). Each protein subunit was eventually found to embody a series of isoforms (coded by different genes) and splice variants (coded by the same gene but transcribed alternatively). All of the isoforms and splice variants of a given subunit family contain conserved structural and functional features of the subunit, while each also exhibits unique qualities, often tailored to the specific tissue in which they are expressed. Variations of this Ca, complex are ubiquitously expressed in excitable cell types throughout the body, where they generally serve to transduce an electrical signal (hence
“voltage gated”) into calcium influx (hence “calcium channel”). Calcium influx functions in a number of important cellular processes which include, but are not limited to; neurotransmitter release, muscle contraction, and intracellular signaling cascades. Thus these Ca\textsubscript{v}-complexes — in all their varieties and locations — serve in wide array of important physiological functions.

As the “pore forming” subunit responsible for all of the fundamental properties of the voltage-gated calcium channel (permeability, gating, pharmacological sensitivity, and voltage dependence) the $\alpha_1$ family of subunits (now formally known as Ca\textsubscript{v}1, Ca\textsubscript{v}2, or Ca\textsubscript{v}3 depending on their voltage sensitivity, and collectively Ca\textsubscript{v}s (Catterall et al., 2005)) have drawn the most research focus of any of the five subunits. However to be adequately functional, both Ca\textsubscript{v}1 and Ca\textsubscript{v}2 generally require at least the addition of a $\beta$ subunit (now formally known as Ca\textsubscript{v}$\beta_{1,2,3}$ or $\beta_4$ depending on the isoform, or Ca\textsubscript{v}$\beta$ collectively) and are therefore rarely studied in isolation (the low-voltage activated Ca\textsubscript{v}3 channels are not thought to associate with Ca\textsubscript{v}$\beta$ subunits). The importance of Ca\textsubscript{v}$\beta$ subunits lies in the fact that, in addition to fine tuning the conductance and gating properties of Ca\textsubscript{v}1 and Ca\textsubscript{v}2, Ca\textsubscript{v}$\beta$ subunits are necessary for trafficking and stable expression of these channels into the cell’s plasma membrane – a process in which the other 3 subunits are dispensable. Thus Ca\textsubscript{v}$\beta$ subunits have enjoyed a modest amount of popularity over the years in a number of biochemical, genetic, and electrophysiological studies, either as the main focus or as an important detail in dealing with Ca\textsubscript{v}1 and/or Ca\textsubscript{v}2.

The first Ca,β subunit was purified from rabbit skeletal muscle (Curtis and Catterall, 1984), specifically from sub-structures within the muscle fibers called transverse-tubules (t-tubules). T-tubules are deep invaginations that run perpendicular to the main axis of the muscle fiber. This subcellular localization would later underscore the unique function of this muscle-specific variant of Ca,β subunit, later termed Ca,β1a. A subsequent report from the Catterall laboratory put the original Ca,β subunit (now called Ca,β1a – the skeletal muscle specific variant) further “in its place” by proposing a model of the entire Ca,β-complex, based on the individual subunits’ biochemical properties (Takahashi et al., 1987), with the β subunit tightly bound to the cytosolic side of the transmembrane α1 subunit (α1s - now called Ca,1.1, also the skeletal muscle specific variant) in a 1:1 ratio (Figure 1). This model would eventually turn out to be an amazingly accurate depiction of the Ca,β-complex, not just in skeletal muscle, but in all tissues where it is found.

Early characterization of Ca,β1a was furthered by the laboratory of Kevin Campbell at University of Iowa, who developed the first monoclonal antibody to the protein (Leung et al., 1987). This antibody was successfully used to purify not only the Ca,β1a subunit, but also co-immunoprecipitate the Ca,1.1 subunit, thus reinforcing the notion that these two proteins were tightly bound. Densitometric analysis of the purified Ca,1.1 channel also suggested a 1:1:1:1 ratio of all of the subunits bound to Ca,1.1 (Leung et al., 1987). The same antibody was also used to stain Ca,β1a in situ, which verified its localization to the t-tubules and co-localization with Ca,1.1 (Jorgensen et al., 1989) (Figure 1). Interestingly, immunocytological data from this paper also suggested a much high expression of Ca,β1a (and Ca,1.1) in type II (fast) fibers than in type I (slow) fibers.
The primary sequence of Ca,β1a was determined in 1989 by Franz Hoffman’s group, who sequenced Ca,β1a peptide fragments and designed ribonucleotide probes based on these sequences to clone Ca,β1a cDNA (Ruth et al., 1989). Ca,β1a cDNA probes validated the expression of Ca,β1a mRNA in skeletal muscle. The probes also labeled a distinct, larger mRNA in brain tissue, thus revealing the existence of the first non-skeletal muscle Ca,β subunit, later found to be coded by the same gene and named Ca,β1b (Powers et al., 1992; Pragnell et al., 1991). Impressively, while the authors could not detect any complementary mRNA in cardiac tissue, they correctly speculated that this was due to the putative cardiac Ca,β subunit (then unknown) being of a significantly different sequence, rather than their absence from the tissue altogether. This work not only accomplished the impressive feat of sequencing the first Ca,β subunit, but also set the stage for the discovery and characterization of its many family members.

1990s: cloning and expression of additional Ca,β subunits yields functional insight.

Clues to the specific function of Ca,β subunits emerged in the early 1990s through the use of heterologous expression systems, where the recently cloned cDNA’s of Ca,β1a and other Ca, subunits could be used to express the proteins in a controlled manner. Co-expression of Ca,β1a was found to induce a 10-fold increase in radiolabeled dihydropyridine (a Ca,1.1 ligand) binding, compared to expression of Ca,1.1 alone, suggesting Ca,β1a drastically enhanced Ca,1.1 surface expression (Varadi et al., 1991). Co-expression of the other Ca,-subunits did not have such an effect. Additionally, Ca,β1a expression was found to be necessary for normal skeletal muscle-like activation and inactivation kinetics of Ca,1.1 (Lacerda et al., 1991; Varadi et al., 1991), which were greatly slowed down in the absence of Ca,β1a. In the following years, three additional
Ca,β subunits: Ca,β2 (cardiac & brain) (Hullin et al., 1992; Perez-Reyes et al., 1992), Ca,β3 (brain) (Castellano et al., 1993b; Hullin et al., 1992; Witcher et al., 1993), and Ca,β4 (brain; primarily cerebellar)(Castellano et al., 1993a) were cloned. All Ca,β isoforms showed the ability to modify three main characteristics of co-expressed Ca,1 or Ca,2 channels: inward current density (indicative of increase in total channel number at the membrane), voltage dependence of channel activation/inactivation, and gating activation/ inactivation kinetics. These experiments also demonstrated the non-exclusivity of Ca, - Ca,β subunit interactions, as each newly cloned Ca,β isoform was able to modify the Ca2+ currents of several different Ca, isoforms. This suggested a highly conserved mechanism for Ca, - Ca,β interaction and functional regulation.

**Structural features of Ca,β and basis of Ca,1-Ca,β interaction (1994-2004).**

The structural basis of Ca,1-Ca,β interaction was first elucidated in 1994 by a duo of papers from the Campbell laboratory. The Ca,β interaction site of Ca,1 and Ca,2 channels was mapped to a conserved QQQXEXXLQGYXXWIXXXXE amino acid sequence residing in an intracellular loop located between the first and second major transmembrane elements of the channel, called the I-II loop (Pragnell et al., 1994) (Figure 1). This sequence would eventually be termed the alpha interaction domain (AID). A complimentary paper released later that year used a similar approach to locate the conserved sequence in Ca,β subunits required for Ca, interaction (De Waard et al., 1994), though the story would eventually prove more complex after the crystal structure of the two proteins was solved. This paper was also the first to identify the two structural domains conserved in all Ca,β subunits, along with three more variable NH2-terminal, middle, and COOH-terminal regions. These two conserved domains share 65% and 78%
homology across all isoforms respectively (and would later be identified as homologous functional domains). A conserved sequence, found in a short segment of the second domain of Ca,β (later called guanylate kinase domain), was found to be essential for enhancing Ca\(^{2+}\) current density, and also for Ca,1-Ca,β interaction (De Waard et al., 1994). Thus it was concluded that the same region of Ca,β necessary for interacting with Ca,s was also necessary for modulating all of their main effects on the channels.

While individual Ca,β isoforms varied in their specific “fine tuning” of Ca, properties such as voltage dependence and activation/inactivation kinetics, the most uniform function of Ca,β subunits seemed to be increasing Ca\(^{2+}\) current density, a correlate of Ca, expression. The exact nature of this function was resolved in 2000, when Michel De Waard’s research group in Marseille, France found that Ca,β subunits bind to and mask an endoplasmic reticulum retention signal in the I-II loop of nascent Ca,s. This signal masking allows the channel complexes to leave the endoplasmic reticulum and travel to the plasma membrane (Bichet et al., 2000), earning Ca,β subunits the title of “chaperones.” A further mechanism by which Ca,β subunits increase Ca, surface expression may occur by masking of a PEST sequence (sequences rich in Proline, Glutamine, Serine, and Threonine and are a signal for rapid protein degradation) on the channel, thus protecting it from being targeting for degradation (Catalucci et al., 2009).

3-D Structure of Ca,β and Ca,β-Ca,1 (1999-2004).

As noted earlier (De Waard et al., 1994), Ca,β subunits contain two highly conserved domains. Through the use of computer modeling, these two domains were found to share homology with Src3 homology (SH3) and guanlyate kinase (GK) domains (Hanlon et al.,
1999), which are found in a number of other proteins, namely those that function as scaffolds. SH3 domains are mainly thought to function as protein interaction domains, and bind to a PxxP amino acid motif (Mayer, 2001). GK domains catalyze the transfer of a phosphate group from ATP to GMP to make GDP, although this function is lost in Ca,β subunits and similar proteins, and thus the GK domain is instead thought to serve as a general protein-interaction module (Buraei and Yang, 2010). The inclusion of these two domains place Ca,β subunits in the membrane associated guanylate kinase (MAGUK) family of proteins, which include numerous synaptic proteins involved in scaffolding and channel clustering (Oliva et al., 2012). The identification of these two modular domains would serve as the foundation for future studies on the precise structure-function relationship of Ca,β subunits. Especially popular are studies making use of “split domain” Ca,β constructs, where the SH3 and GK domains were expressed separately and in tandem, in order to understand their individual and synergistic contributions to Ca, function (Chen et al., 2009; Gonzalez-Gutierrez et al., 2007; Leyris et al., 2009; Takahashi et al., 2004; Takahashi et al., 2005). In 2004 three separate groups reported solving the X-ray crystrallographic structures of Ca,β2 (Opatowsky et al., 2004; Van Petegem et al., 2004) and Ca,β3 (Chen et al., 2004) in complex with the Ca,β-interacting domain of Ca,1 (part of the I-II loop described earlier), and of Ca,β4 alone (Chen et al., 2004) (Figure 1). Three major findings were concluded from these studies (Richards et al., 2004). First, that the SH3 and GK domains of Ca,β interact intramolecularly (a common feature in MAGUK proteins). Second, that the region of Ca,β which directly interacts with Ca,1 is a hydrophobic groove in the GK domain distinct from the sequence originally thought to dictate the interaction (De Waard et al., 1994), although this
sequence is important for the structural integrity of Caβ and bridging of SH3 and GK domains. And finally, that the GK domain is not catalytically active.

*The specific role of Caβ1a in skeletal muscle.*

All Caβ subunits share certain qualities which give them fundamental abilities in regards to Ca regulation. And the understanding of these “core Caβ qualities” was a tremendous joint achievement accomplished by multiple outstanding research groups over many years. However, the qualities and functions which distinguish Caβ variants from one another is an equally important and fascinating topic. Perhaps the most elegant example of specific Caβ variant function lies in skeletal muscle, where the Caβ1a subunit is uniquely capable of facilitating the essential physiological process known as excitation-contraction (E-C) coupling.

E-C coupling is the process by which electrical activity is “coupled” into contractile activity. In skeletal muscle myofibers, E-C coupling occurs following depolarization (usually in response to stimulation from a motoneuron) of individual muscle cells, called the myofibers. This depolarization travels in a wave along the outer membrane, called sarcolemma, and down invaginations called t-tubules. Inside t-tubules reside Ca1.1 channels, which are different than other CaVs in that their main function is not to conduct Ca2+, but rather to physically shift position in response to a change in membrane potential. Ca1.1 channels are arranged in groups of four, called tetrads (Block et al., 1988), and these tetrads interact with large, individual Ca2+ channels called ryanodine receptors (RyR). The inward motion of Ca1.1 in response to membrane depolarization is analogous to a lock and key mechanism, whereby the Ca1.1 tetrad physically interacts
with a RyR and opens it. RyRs are located on the surface of intracellular Ca\(^{2+}\) stores, called the sarcoplasmic reticulum (analogous to endoplasmic reticulum), and their activation triggers massive intracellular Ca\(^{2+}\) release, which in turn results in the disinhibition of actin-myosin cross bridges and muscle contraction (reviewed in Melzer et al., 1995 and see Figure 2B).

*Embryonic lethality of Ca\(_{\alpha}\beta_{1a}\) KO*

Due to their important relationship with Ca\(_{\alpha}1.1\), it seems obvious that Ca\(_{\alpha}\beta\) subunits would also be important in E-C coupling. Nonetheless, the exact nature as to how and why the Ca\(_{\alpha}\beta_{1a}\) subunit was specifically and absolutely essential for proper E-C coupling took many years to fully appreciate. It was known from early works on the expression of newly cloned Ca\(_{\alpha}\beta_{1a}\) cDNA that Ca\(_{\alpha}\beta_{1a}\) was necessary for normal Ca\(_{\alpha}1.1\) function in skeletal muscle (Lacerda et al., 1991; Varadi et al., 1991). Creation of the Ca\(_{\alpha}\beta_{1}\) knockout mouse (termed β\(_{\text{null}}\)) (Gregg et al., 1996), however, truly underscored the necessity of Ca\(_{\alpha}\beta_{1a}\) in E-C coupling. β\(_{\text{null}}\) mice were found to be embryonic lethal, meaning they were found stillborn at birth. This was caused by complete lack of E-C coupling; presumably a result of the absence of Ca\(_{\alpha}1.1\) expression at the sarcolemma, as determined by immunofluorescent and charge movement (a measure of collective Ca\(_{\alpha}1.1\) movement within the membrane in response to depolarization) experiments. Importantly, activation or RyRs, which are downstream of Ca\(_{\alpha}1.1\), still elicited a contractile response in β\(_{\text{null}}\) mice, indicating this was a specific defect in Ca\(_{\alpha}\) function. Another noteworthy observation in the β\(_{\text{null}}\) mice was their severely underdeveloped and disorganized musculature at birth (Figure 1), which was attributed to lack of E-C coupling. It was later reported that both Ca\(_{\alpha}\beta_{1a}\) and Ca\(_{\alpha}\beta_{2a}\) isoforms could restore Ca\(_{\alpha}1.1\) expression in β\(_{\text{null}}\)
myotubes, however only Ca\(_v\beta_{1a}\) could restore charge movement, suggesting this to be a specific function of Ca\(_v\beta_{1a}\) in skeletal muscle (Beurg et al., 1999). Clues to the mechanism behind this specific Ca\(_v\beta_{1a}\) function emerged from studies in the zebrafish Ca\(_v\beta_{1a}\) knockout model relaxed (Red\(_{25}\)) (Granato et al., 1996; Zhou et al., 2006). In the relaxed zebrafish larvae, Ca\(_v\)1.1 is still expressed, albeit at a reduced level, in the t-tubules. However, these animals exhibited complete paralysis due to absence of E-C coupling. Closer examination by electron microscopy revealed that while Ca\(_v\)1.1 channels are still expressed, they are not arranged properly into tetrads (groups of four which are organized to precisely interact with a single RyR) (Schredelseker et al., 2005) (Figure 1). A follow up study using the same model found that while exogenous expression of other Ca\(_v\)\beta isoforms could restore Ca\(_v\)1.1 expression and charge movement in relaxed larvae, only Ca\(_v\beta_{1a}\) could rescue E-C coupling. Thus, Ca\(_v\beta_{1a}\) appears to be uniquely capable of facilitating the specific arrangement of Ca\(_v\)1.1 into tetrads necessary for E-C coupling. This notion is further supported by work showing a direct interaction between Ca\(_v\beta_{1a}\) and RyRs (Cheng et al., 2005), suggesting a role for Ca\(_v\beta_{1a}\) in establishing the macromolecular structure of the Ca\(_v\)1.1 tetrad – RyR complex.

**Part II: Novel function for Ca\(_v\)\beta in modern times.**

The first part of this introduction highlighted the elegant experiments, brilliant revelations, and incredible amount of hard work by many research groups that ultimately characterized the Ca\(_v\)\beta subunit as a calcium channel support protein. For the purposes of this dissertation, however, the point was to also emphasize the well-defined role of Ca\(_v\)\beta as a Ca\(_v\) subunit – and nothing more. In light of the monumental work that went into
defining Ca,β subunits in this role, it was very surprising then when Ca,βs began to turn up in an unexpected location, beginning speculation of entirely new functions for these proteins.

The non-cav binding era, RGKs and beyond (2001-present).

In 2001, the Ca,β1-3 subunits were reported to interact with Gem, a Ras-related small G-protein (Beguin et al.), in a GTP dependent manner. In this paper, Gem was found to completely inhibit Ca,1 expression at the plasma membrane via direct interaction with Ca,β subunits. This was significant in that it was the first report of any Ca,β subunit interacting with a non-Ca, protein, and also opened the door to an entirely new mechanism of Ca, regulation by Ca,β subunits. Researchers soon realized that all members of the RGK (Rad, Rem, Gem/Kir) family of proteins acted in similar fashion to inhibit Ca, function via Ca,β subunit interaction, and that this function was conserved across most Ca,β and Ca,1/2 isoforms (importantly, Ca,3 channels, which do not interact with Ca,β subunits, are not affected by RGK proteins (Finlin et al., 2003)). Whether RGKs inhibited Ca,s by reducing their surface expression or by effectively de-activating them while still in place was a subject of contention for many years before it was agreed that RGKs indeed act through both mechanisms, and even in Ca,β-independent manners (Yang and Colecraft, 2012). Several papers made the interesting observation that RGK proteins also seemed to have the ability to re-localize Ca,β subunits into the nucleus of various cell types (Beguin et al., 2006; Beguin et al., 2007; Leyris et al., 2009; Mahalakshmi et al., 2007; Yang et al., 2010), although the potential function of Ca,β subunits in the nucleus was not explored in detail by these works.
While RGK-mediated Ca,β nuclear localization garnered more attention, the first documented account of Ca,β subunits in the nucleus came from Henry Colecraft, then at Johns Hopkins University (now at Columbia University and also noteworthy as a large contributor to the RGK story), who observed Ca,β_1, Ca,β_3, and an especially strong signal of Ca,β_4 in the nucleus of isolated cardiomyocytes (Colecraft et al., 2002) (Figure 1). This work focused on Ca, regulation by Ca,β subunits and while Colecraft speculated on a potential novel function of Ca,β in the nucleus, he did not pursue this topic further. A major breakthrough on the subject of Ca,β nuclear localization and function came the following year, when Hibino et al. discovered that a novel truncated splice variant of Ca,β_4 lacking it’s GK domain (called Ca,β_{4c}) interacted with the well known chromatin silencing protein HP1γ in the nuclei of cochlear neurons (Figure 1). Even more striking, the authors showed that Ca,β_{4c} could regulate gene transcription in *in vitro* assays by inhibiting HP1γ (in effect, *dis*-inhibiting HP1γ target genes). Supporting the idea that the Ca,β nuclear localization sequence may fall in the SH3 domain, a Ca,β “split domain” electrophysiological study by Colecraft’s laboratory (Takahashi et al., 2005) noted the appearance of Ca,β-SH3 (but not Ca,β-GK) in the nucleus of HEK 293 cells. Several years later, Deborah Garrity’s research group found that the knockdown of Ca,β_4 in zebrafish embryos was shown to impair development in a channel-*independent* fashion (Ebert et al., 2008). This was demonstrated by the rescue of developmental impairment by overexpressing a mutant Ca,β_4 subunit which was deficient in Ca, binding. While not directly linked to nuclear localization, this finding of an important channel-independent function for Ca,β_4 further strengthened the possibility of an alternate function for Ca,β subunits within the nucleus. The following year, Bernhard Flucher’s laboratory at the
University of Innsbruck reported extensive evidence of Ca,β subunit nuclear localization and trafficking (Subramanyam et al., 2009). In this work, several Ca,β isoforms were found to actively traffic to the nuclei of skeletal muscle myotubes and primary hippocampal neurons, with Ca,β4 again being especially prevalent in the nucleus. Although no binding partners were explored, the group identified a non-canonical nuclear localization sequence (NLS) in the N-terminus of Ca,β4b, which was found to have some control over the proteins’ nuclear localization. Additionally, electrical activity in myotubes and neurons was found to promote nuclear export of Ca,β4b, specifically.

Overall the results of this paper suggest that although all Ca,β isoforms seem to be capable of nuclear entry, Ca,β4b possesses more potent means of nuclear entry (perhaps due to an intrinsic NLS), and is subject to more complex regulation than the other Ca,β isoforms. Most recently, another transcription factor, Pax6s, was found to interact with Ca,β3 and re-localize it to the nucleus of HEK 293 cells and possibly of human embryonic neurons (Zhang et al., 2010). Pax6s did not interfere with Ca,β3 regulation of Ca,1, while Ca,β3 was able to inhibit Pax6s transcriptional activity in vitro, providing another example of a Ca,β subunit acting as a transcriptional regulator inside the nucleus.

Overall these studies provide ample evidence for a non-Ca, function for Ca,β subunits inside the nucleus, however many important questions remain about the biological significance and generality of this phenomenon.

In addition to its nuclear binding partners, over the last decade many other non-nuclear, non- Ca, proteins have also been found to interact with Ca,β subunits. These include synaptic proteins Rim1 (Kiyonaka et al., 2007), Ahnak (Hohaus et al., 2002), Dynamin (Gonzalez-Gutierrez et al., 2007), MAP1a (Vendel et al., 2006), and Synaptotagmin1;
(Vendel et al., 2006); the signaling proteins: CaMKII (Grueter et al., 2006) and Akt (Catalucci et al., 2009); and other, non-Ca\textsubscript{v} ion channels: Slo1 (Zou et al., 2008) and hBest1 (Yu et al., 2008). It has even recently been shown that Ca\textsubscript{v}\textsubscript{β} subunits can form homo- and heterodimers and trimers with themselves (Lao et al., 2010). The nature of these interactions is summarized in Table 1. Besides these individual findings, a recent large-scale proteomics screening suggested that over 200 additional proteins may interact, both directly and indirectly, with the Ca\textsubscript{v}\textsubscript{β}-Ca\textsubscript{v2}-complex as part of a massive scaffold and signaling network (Muller et al., 2010).
Figure 1. Timeline of important Ca,β subunit discoveries. 1984 – first appearance of Ca,β, co-purified with Ca,α and γ subunits from rabbit skeletal muscle (Curtis and Catterall, 1984). 1987 – Early but accurate model of Ca, complex with Ca,β bound to Ca,α intracellularly (Takahashi et al., 1987). 1989 – immunofluorescent staining of Ca,β1a in the A and I bands of skeletal muscle (Jorgensen et al., 1989). 1994 – identification of the precise amino acid sequence and structural element on Ca,1 to which Ca,β binds (Pragnell et al., 1994). 1996 – generation of first Ca,β knockout mouse (Ca,β1a), which lacked E-C coupling and exhibited underdeveloped skeletal muscle at birth (T and F: tibula and fibula) (Gregg et al., 1996). 2002/2003 – early appearance of Ca,β in the nuclei of striated muscle (cardiomyocytes) and neurons (Colecraft et al., 2002; Hibino et al., 2003a). 2004- crystal structure of Ca,β3 in complex with the interacting domain of Ca,1(Chen et al., 2004). 2005 – absences of correct tetrad formation in relaxed Ca,β1 mutant zebrafish (Schredelseker et al., 2005). All images are re-printed in accordance with the expressed permission guidelines of their respective journals.
Figure 2. Schematic of generalized and specialized Ca$_{\beta}$ subunit functions. A) General model for ubiquitous Ca$_{\beta}$ actions. Ca$_{\beta}$ subunits bind to and mask an ER retention signal of Ca$_{\alpha}$s, allowing them to traffic to the plasma membrane. Here Ca$_{\alpha}$s may associate with the other members of the calcium channel complex: α2/δ and γ. Ca$_{\beta}$ subunits also appear in the nucleus of many cell types and appear to regulate gene transcription, though the general mechanism and function of this remains unknown. B) Model of Ca$_{\alpha}$$\beta_{1a}$ function in skeletal muscle. Ca$_{\alpha}$$\beta_{1a}$ subunits are found in transverse tubules as part of tertrameres of Ca$_{\gamma}$ complexes. Ca$_{\alpha}$$\beta_{1a}$ necessary for tetrad formation and located adjacent to RyR, with which they interact.
<table>
<thead>
<tr>
<th>Cavβ binding partner</th>
<th>Function of Interaction</th>
<th>Cell line/Tissue</th>
<th>Partner sequence responsible for interaction</th>
<th>Cavβ isoforms</th>
<th>Cavβ Sequence Responsible</th>
<th>Technique</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Phosphorylation of beta - protects alpha from proteolysis</td>
<td>primary cardiomyocytes</td>
<td></td>
<td>2a</td>
<td>Co-IP</td>
<td></td>
<td>Catalucci 2009</td>
</tr>
<tr>
<td>Ahnak</td>
<td>scaffolding, links channel complex to actin</td>
<td>cell free</td>
<td></td>
<td>1a, 2</td>
<td>GST pull down assay</td>
<td></td>
<td>Hohaus 2002</td>
</tr>
<tr>
<td>Cav1/2 i-ii loop</td>
<td>membrane targeting, channel modulation</td>
<td>cell free</td>
<td>QQXEXXLYGYYXXWIXXXE</td>
<td>all</td>
<td>GK/ BID</td>
<td>Co-IP</td>
<td>Pragnell 1991</td>
</tr>
<tr>
<td>CaMKII</td>
<td>phosphorylation of alpha and beta modulates current</td>
<td>HEK293/Cell free</td>
<td></td>
<td>1b, 2a</td>
<td>GST pulldown assay, tagged Co-IP</td>
<td></td>
<td>Greuter 2008</td>
</tr>
<tr>
<td>Gem</td>
<td>CaV inhibition</td>
<td>HEK293/Cell free</td>
<td></td>
<td>1b, 2a, 3</td>
<td>Y2h, tagged co-IP</td>
<td></td>
<td>Beguin 2001</td>
</tr>
<tr>
<td>Dynamin</td>
<td>CaV endocytosis</td>
<td>Xenopus oocytes</td>
<td></td>
<td>2a</td>
<td>SH3</td>
<td>Co-IP</td>
<td>Gonzalez, Guiterrez et al., 2007; Miranda-Laferte et al., 2011</td>
</tr>
<tr>
<td>hBest1</td>
<td>CaV inhibition</td>
<td>HEK293</td>
<td>AA's 352-380</td>
<td>1b, 2a, 4</td>
<td>SH3 implicated by requirement for PxxxF sequence for functional inhibition</td>
<td>tagged co-IP</td>
<td>Yu K 2008</td>
</tr>
<tr>
<td>HP1γ</td>
<td>inhibition of HP1γ silencing / scaffolding of HP1γ to silencing complexes</td>
<td>tsa201, brain</td>
<td></td>
<td>4c</td>
<td>SH3 (beta4c has no GK domain)</td>
<td>Y2h, tagged co-IP</td>
<td>Hibino et al., 2003; Tadmouri et al., 2012</td>
</tr>
<tr>
<td>pax6s</td>
<td>pax6s inhibition</td>
<td>HEK293/Cell free</td>
<td>s-tail</td>
<td>3</td>
<td>Y2h, GST pull down assay</td>
<td></td>
<td>Zhang 2010</td>
</tr>
<tr>
<td>Rad</td>
<td>CaV inhibition</td>
<td>HEK293/Cell free</td>
<td></td>
<td>1b, 2a, 3, 4</td>
<td>GST pull down assay</td>
<td></td>
<td>Finlin 03, Beguin 06</td>
</tr>
<tr>
<td>Rem</td>
<td>CaV inhibition</td>
<td>HEK293/Cell free</td>
<td></td>
<td>1b, 2a, 3, 4</td>
<td>GST pull down assay</td>
<td></td>
<td>Finlin 03, Beguin 06</td>
</tr>
<tr>
<td>Protein</td>
<td>Function</td>
<td>Cell Type</td>
<td>NMDG-AC1</td>
<td>Notes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>-----------</td>
<td>----------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rim1</td>
<td>Scaffolding/vesicle release</td>
<td>Cell free/Brain</td>
<td>AA's 1079-1463</td>
<td>2a,4b, 392-410, 49-86, 1125, 1225, not aid (still binds BADN)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RyR</td>
<td>E-C coupling</td>
<td>Cell free</td>
<td>AA's 3200-3600</td>
<td>1a, 2, gst pull down assay, Cheng 2005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slo1</td>
<td>CaV gating inhibition</td>
<td>chick ciliary ganglion/Cell free</td>
<td>calcium bowl (Thr911-Gln933) and noncanonical SH3(E637-D677)</td>
<td>1b, Both SH3 and GK interact; GK alone sufficient for CaV inhibition, y2h, gst pull down assay, co-IP, zou et al., 2008</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptotagmin 1</td>
<td>CaV interaction</td>
<td>cell free</td>
<td>3, 4a</td>
<td>y2h, gst pull down assay, Vendel et al., 2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP1a</td>
<td>CaV interaction</td>
<td>cell free</td>
<td>4a</td>
<td>y2h, gst pull down assay, Vendel et al., 2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cavβ</td>
<td>CaV current amplification</td>
<td>Cos-7</td>
<td>GK</td>
<td>1,2,3</td>
<td>GK, FRET, co-IP, far western, Lao et al., 2010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B56d</td>
<td>Scaffolding to PP2A and phosphatase activity</td>
<td>HEK293/primary hippocampal neurons</td>
<td>3,4</td>
<td>All except N-terminus</td>
<td>y2h, gst pull down assay, co-IP, Tadmouri et al., 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRα</td>
<td>DNA binding</td>
<td>HEK293</td>
<td></td>
<td></td>
<td>y2h, co-IP, Tadmouri et al., 2012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References:


Block, B.A., T. Imagawa, K.P. Campbell, and C. Franzini-Armstrong. 1988. Structural evidence for direct interaction between the molecular components of the


Leung, A.T., T. Imagawa, and K.P. Campbell. 1987. Structural characterization of the 1,4-dihydropyridine receptor of the voltage-dependent Ca2+ channel from rabbit


nuclear targeting of the calcium channel beta4b subunit in nerve and muscle cells.

*Channels (Austin).* 3:343-55.


Chapter 2

The Ca,β1a Subunit Regulates Transcription and Suppression of Myogenin in Muscle Progenitor Cells

Jackson Taylor¹,², Tan Zhang¹, Maria Laura Messi¹, Zhong-Min Wang¹, Claudia Hereñú³, Pei-Fen Kuan⁴ and Osvaldo Delbono*,¹,²

Department of Internal Medicine-Gerontology¹, Neuroscience Program²

Wake Forest School of Medicine, Winston-Salem, NC, 27157, USA¹,²

Department of Histology, National University of La Plata, La Plata, Argentina³

Department of Biostatistics, The University of North Carolina, Chapel Hill, NC, 27514, USA⁴

This work is currently under revision for re-submission to the Journal of Cell Biology, and is formatted according to the style of that journal.
Abstract:

Voltage-gated calcium channel (Ca_v) β subunits have long been defined by their classical role as auxiliary subunits to high voltage-activated Ca_v’s (Ca_v1 & Ca_v2). More recent evidence has identified Ca_v β subunits in the nucleus and suggested a role in transcriptional regulation; however this link has not been well explored. Here we show that Ca_v β_1a regulates gene expression in muscle progenitor cells, independently of Ca_v expression, and plays an important role in MPC self renewal and muscle development. Ca_v β_1a is expressed in MPCs, before the expression of Ca_v1.1, where it shuttles to the nucleus. Loss of Ca_v β_1a expression in these cells leads to impaired proliferation in vitro and in vivo. Ca_v β_1a regulates a number of genes by direct promoter binding, including the key Muscle Regulatory Factor myogenin. Loss of Ca_v β_1a expression during development leads to precocious myogenin expression and MPC depletion in vivo, resulting in decreased skeletal muscle mass.
Introduction

Skeletal muscle progenitor cells (MPCs; also called myoblasts) are capable of rapid proliferation during development or following activation in response to muscle damage in adults, after which they can differentiate and fuse together into mature myofibers. Initially identified as “satellite” cells located adjacent to myofibers (Mauro, 1961), but beneath the basal lamina, these cells were later discovered to be the primary cell type responsible for muscle development and regeneration following injury (Charge and Rudnicki, 2004; Collins et al., 2005). Unlike mature myofibers, muscle progenitor cells are capable of DNA replication and cell division, but also maintain a commitment to the skeletal muscle fate (Collins et al., 2005; Moss and Leblond, 1971). Upon reaching a certain density or following serum withdrawal in vitro, MPCs exit the cell cycle and begin to express genes associated with mature skeletal muscle (e.g. troponin and myosin heavy chain), while fusing together into multinucleated myotubes and eventually functional myofibers. Several key transcription factors regulate and define MPCs during different periods of myogenesis. Pax7 is expressed in both quiescent satellite cells in adult muscle and actively dividing MPCs during muscle regeneration and development. At the onset of differentiation, Pax7 expression is down-regulated and replaced by myogenin, a key muscle transcription factor thought to turn on many of the genes associated with mature skeletal muscle (Charge and Rudnicki, 2004; Yablonka-Reuveni et al., 2008). Although the core set of transcription factors involved in MPC regulation are well defined, questions still remain about how these factors themselves are regulated. Specifically, recent studies have highlighted the importance of temporal control of myogenic transcription factors by other proteins during both embryonic myogenesis
(Schuster-Gossler et al., 2007; Van Ho et al., 2011; Vasyutina et al., 2007) and adult muscle regeneration (Brack et al., 2008).

The voltage-gated calcium channel (Ca,) β subunit (Ca,β) was initially purified from rabbit skeletal muscle as part of the dihydropyridine receptor (now termed Ca,1.1) complex (Curtis and Catterall, 1984). A total of four Ca,β genes have been cloned (Cacnb1-4), each with multiple splice variants (reviewed in Buraei and Yang, 2010). These cytoplasmic proteins bind to an intracellular loop on high voltage-activated Ca,s (Ca,1 & Ca,2) (Pragnell et al., 1994). Ca,s contain an endoplasmic reticulum retention signal which is masked by Ca,β binding, allowing both proteins to traffic to the plasma membrane (Bichet et al., 2000). Ca,β interaction with Ca,s also serves to modulate gating properties of the channel (Lacerda et al., 1991; Varadi et al., 1991). Ca,β subunits contain two protein-interaction domains: Src 3 homology (SH3) and guanylate kinase (GK), which act both together and individually to regulate channel function (Miranda-Laferte et al., 2011; Takahashi et al., 2004; Takahashi et al., 2005).

Ca,β1a is the dominant β subunit isoform/splice variant in skeletal muscle, where it associates with Ca,1.1 in transverse tubules. Ca,β1a plays a critical role in excitation-contraction (EC) coupling, the process of converting an electrical stimulus to mechanical response, through several mechanisms. First, it is necessary for proper calcium channel expression in the transverse tubules (Gregg et al., 1996), although calcium currents can be partially restored in Ca,β1a deficient myotubes by transfection of other Ca,β subunits (Schredelseker et al., 2005). Ca,β1a also interacts with ryanodine receptors (RyR) located in the adjacent sarcoplasmic reticulum (SR), and this interaction is thought to facilitate the mechanical interaction between DHPR and RyR in response to sarcolemmal
depolarization (Cheng et al., 2005; Rebbeck et al., 2011); which leads to RyR opening and calcium release from the SR. Finally, Ca_{v}\beta_{1a} specifically functions to organize Ca_{v}1.1 into defined groups of four (tetrads), which pair to a single RyR (Schredelseker et al., 2009; Schredelseker et al., 2005). This strict geometrical organization and 4:1 stoichiometry is apparently necessary for proper EC-coupling. The β1null mouse (Gregg et al., 1996) and the relaxed (redts25) zebrafish (Zhou et al., 2006) both show paralysis due to total lack of EC-coupling, underscoring the importance of Ca_{v}\beta_{1a} in this process. The β1null mouse (hereafter called Caenb1^{-/-}) also shows severely reduced skeletal muscle mass at birth, attributed to a lack of activity during development.

In the last decade, over a dozen additional, non-calcium channel binding partners have been described in the literature for multiple Ca_{v}\beta subunit isoforms (Beguin et al., 2001; Buraei and Yang, 2010; Catalucci et al., 2009; Gonzalez-Gutierrez et al., 2007; Grueter et al., 2006; Hibino et al., 2003a; Hidalgo and Neely, 2007; Hohaus et al., 2002; Kiyonaka et al., 2007; Yu et al., 2008; Zhang et al., 2010). Several additional reports show the nuclear localization of all four Ca_{v}\beta subunits, either alone (Colecraft et al., 2002; Subramanyam et al., 2009) or when co-transfected with members of the RGK family of proteins (Beguin et al., 2006; Leyris et al., 2009). Two studies have demonstrated transcriptional regulation by Ca_{v}\beta subunits in vitro; Ca_{v}\beta3 (Zhang et al.) and Ca_{v}\beta_{4c} (Hibino et al., 2003b), however little biological relevance was concluded. Despite numerous suggested alternate roles, no clear Ca_{v}-independent function for Ca_{v}\beta subunits has been defined.

We sought to define the physiological role for Ca_{v}\beta subunits in the nucleus, specifically in MPCs. Here we report that a Ca_{v}\beta subunit, Ca_{v}\beta_{1a}, regulates proliferation in MPCs via
Ca$_v$-independent mechanisms. Specifically, Ca$_v$$\beta_{1a}$ interacts with chromatin in the promoter regions of numerous genes, including the key regulator of myogenesis: myogenin.

Results:

**Ca$_v$$\beta$ expression in muscle progenitor cells**

While Ca$_v$$\beta$ subunits have been studied in myotubes, specifically the muscle-specific splice variant Ca$_v$$\beta_{1a}$ (coded by the Cacnb1 gene), little is known about their expression in MPCs (myoblasts). A microarray study suggested that while Cacnb1 mRNA expression increases during differentiation, it still expresses in significant quantities in sub-confluent, proliferating C2C12 myoblasts (Tomczak et al., 2004). Therefore we sought to characterize Cacnb1 expression at the mRNA and protein level in C2C12 and primary mouse myoblasts (see Figure S1). RT-PCR using primers to the muscle specific Cacnb1 splice variant A ($\beta_{1a}$), which codes for the Ca$_v$$\beta_{1a}$ protein, detected Ca$_v$$\beta_{1a}$ mRNA in sub-confluent myoblasts, which increased during myogenic differentiation (Figure 1A). We next characterized Ca$_v$$\beta_{1a}$ subunit protein expression in C2C12 and primary myoblasts, using two different Ca$_v$$\beta_{1a}$ antibodies. A protein band close to the expected molecular weight of Ca$_v$$\beta_{1a}$ (52 kDa), appeared specifically in both C2C12 and primary myoblasts, using both antibodies, compared to isotype IgG controls (Figure 1B, Ca$_v$$\beta_{1a}$ antibody clone H-50 was used for the remainder of experiments). Ca$_v$$\beta_{1a}$ specific shRNA-mediated knockdown of this protein band further confirmed its identity as Ca$_v$$\beta_{1a}$ (Figure 1C). Additionally, while the antibody we used did label brain and cardiac protein bands (presumably other Ca$_v$$\beta$ isoforms), these were different molecular weights (Figure
1C). To examine Cavβ1a expression and localization during myogenesis, we analyzed cytosolic and membrane lysates collected from proliferating (sub-confluent in growth medium: GM) and differentiating C2C12 cells (96 hrs in differentiation medium: DM) by western blot (Figure 1D). Cavβ1a appeared in the cytosolic, but not plasma membrane fractions, of proliferating myoblasts while the Cav1.1 protein was completely absent, as expected (Bidaud et al., 2006). The absence of Cavβ1a in the membrane fraction suggests it does not interact with any channels, Cav1.1 or otherwise, in myoblasts, indicating a non-channel function. Cytosolic Cavβ1a protein expression increased mildly during differentiation, but showed a large increase in the membrane fraction after 48 hrs in DM, concomitantly with the appearance of Cav1.1, re-affirming the classical role of Cavβ1a as a Cav1.1 binding partner. Immunostaining of Cavβ1a showed a very faint nuclear and perinuclear signal in proliferating and early-fusing myoblasts, however in fully differentiated myotubes a strong punctuate signal was observed, presumably corresponding to Cavβ1a associated with Cav1.1 at the plasma membrane (Figure 1E).

These results support the idea that myoblasts do not express Cavαs, yet still express Cavβ1a. Thus Cavβ1a exists in a spatially and temporally separate pool from its constituent Cavα, raising the likelihood of Cavα-independent functions in MPCs.

**Nuclear Caαβ1α**

As other Cavβ subunits have been observed in the nucleus of various cell types, we hypothesized Cavβ1a may localize there in MPCs as well. Because our antibodies did not appear sensitive for immunofluorescent detection in C2C12 myoblasts, we constructed a
recombinant adenoviral vector (RAd) to overexpress a Ca₅β₁a-YFP plasmid (Leuranguer et al., 2006) in these cells. Ca₅β₁a-YFP shows a predominantly cytoplasmic localization (Figure 2A), although some cells also exhibited substantial fluorescence in the nucleus. However, after 3 hrs of treatments with the CRM1 nuclear export channel blocker Leptomycin-B (LMB), all cells exhibited Ca₅β₁a-YFP fluorescence predominantly in the nucleus (Figure 2B). Ca₅β₁a antibody staining of these treated cells overlapped with YFP fluorescence in the nucleus (data not shown). To further confirm the translocation of Ca₅β₁a-YFP into the nucleus of C2C12 myoblasts, we obtained pure cytosolic and nuclear fractions from Ca₅β₁a-YFP-expressing cells, and analyzed Ca₅β₁a-YFP protein expression by western blot (Figure 2C). Ca₅β₁a-YFP was clearly present in the nucleus, even without LMB treatment. In both untreated and LMB treated cells, Ca₅β₁a-YFP could be immunoprecipitated from nuclear fractions with an YFP antibody, further indicating the specific localization of Ca₅β₁a-YFP (Figure 2C). It is important to note that the expected and observed size of Ca₅β₁a-YFP is nearly 80 kDa (Figure 2C), far above the size for passive diffusion into the nucleus (<50k Da), suggesting that Ca₅β₁a-YFP is rapidly and actively transported into the nucleus of myoblasts. Furthermore, when examined carefully, the Ca₅β₁a-YFP band in nuclear fractions appears to be of a slightly higher molecular weight than cytosolic Ca₅β₁a-YFP, suggesting a possible post-translational modification is required or induced by nuclear translocation.

We next wanted to determine if the endogenous Ca₅β₁a protein entered the nucleus of myoblasts. Immunostaining for endogenous Ca₅β₁a following LMB treatment in non-transfected cells did not detect any clear nuclear enrichment (data not shown), likely due to a lack of sensitivity or epitope masking. To circumvent these issues, we performed a
nuclear fractionation protocol and western blot analysis which is more sensitive and avoids epitope masking (Figure 2D). Compared to untreated cells, Ca,β1a is enriched in the nuclear fraction of 6 and 12 hr LMB treated MPCs. As Ca,β1a expression levels change in cytoplasmic and membrane fractions with myogenic differentiation, we also examined if Ca,β1a nuclear expression changes during this process (Figure 2E).

Interestingly, while cytoplasmic Ca,β1a increases modestly with differentiation, nuclear Ca,β1a appears to decline, further underscoring a possible MPC-specific role for Ca,β1a in the nucleus. Close examination reveals that, like Ca,β1a-YFP, endogenous Ca,β1a also seems to exist as a slightly higher molecular weight species (Figures 2D and 2E) within the nucleus, supporting the notion of a post translational modification. In sum these data strongly support the notion that Ca,β1a shuttles to the nucleus of myoblasts.

Ca,β1a possesses variable N and C-terminal domains, as well as conserved SH3, HOOK, and GK domains. We wanted to determine which part of Ca,β1a endows its ability to enter the nucleus, and whether this is a specific ability of Ca,β1a, or a mechanism conserved in all Ca,β subunits. Other Ca,β subunits have been reported to enter the nucleus (Buraei and Yang, 2010), and data suggests that this ability may lie in the SH3 domain. The truncated Ca,β4c protein which does not possess its GK domain was shown to enter the nucleus (Hibino et al., 2003a). Also, when split domain Ca,β2 constructs were transfected into HEK cells, the SH3 fragment showed partial nuclear localization while the GK fragment did not (Colecraft et al., 2002). However, Ca,β1a also possesses a yeast nuclear localization sequence (NLS) KRKGRFKR (Hicks and Raikhel, 1995) in its N-terminal domain which is not highly conserved in other Ca,β subunits, and thus Ca,β1a may have a specific ability to shuttle to the nucleus. We created various truncation
mutants of the Ca$_v$β$_{1a}$-YFP protein and tested their individual ability to enter the nucleus in the presence and absence of LMB (Figure 3A and S1). All of the constructs except for Cavβ$_{1a}$161-524 (lacking N-terminus and SH3 domains) (Figure 3B) showed enriched nuclear localization after LMB treatment, including the mutant lacking the putative yeast NLS. Several constructs lacking the GK domain showed strong nuclear localization without LMB; however this could be attributed to low molecular weight. One possible exception is the Ca$_v$β$_{1a}$-101-274 construct (Figure 3C), which contains SH3 and middle regions, and has a predicted molecular weight (47 kDa when fused to YFP) close to the 50 kDa barrier. Thus it appears the nuclear localization domain of Ca$_v$β$_{1a}$ lies somewhere in the SH3 or middle region, and is likely conserved in other Ca$_v$β subunits. This region could harbor a non-canonical NLS or may facilitate Ca$_v$β subunits interaction with another protein that does possess an NLS.

**Effects of altering Ca$_v$β$_{1a}$ expression on MPCs**

Loss of the Cacnb1 gene in mice leads to a noticeable deficit in muscle mass in prenatal stages (Gregg et al., 1996). Though this was attributed to lack of EC-coupling in myofibers, we hypothesized it may be due in part to the loss of Ca$_v$β$_{1a}$ in MPCs as well. Therefore, we knocked down Ca$_v$β$_{1a}$ to examine its effects on C2C12 myoblast proliferation *in vitro*. Because C2C12 myoblasts are not excitable and do not express Ca$_v$1.1 (Figure 1D) (Bidaud et al., 2006), any effect from reduction of Ca$_v$β$_{1a}$ likely reflects a Ca$_v$-independent function of Ca$_v$β$_{1a}$. Stable transfection of cells with Ca$_v$β$_{1a}$ shRNA achieved substantial reduction of Ca$_v$β$_{1a}$ protein (Figure 1C). When observed in
culture, Ca$_v$β$_{1a}$ shRNA-transfected cells grew more slowly than controls. To verify this observation, clonal cultures of scrambled-control and Ca$_v$β$_{1a}$-shRNA transfected C2C12 myoblasts were plated at equal densities then counted 24 and 48 hrs later. Two out of three Ca$_v$β$_{1a}$-shRNA transfected cultures showed significantly fewer cells at 24 and 48 hrs compared to scrambled controls (Figure S2). Similarly, knockdown of Ca$_v$β$_{1a}$ in primary MPCs resulted in significantly impaired growth over 7 days in culture compared to controls, especially at later time points (Figure 4A). Ca$_v$β$_{1a}$ knockdown cells showed more frequent AnnexinV-FITC staining than control cells in vitro, although the difference was not significant (p=0.07, Figure S4A). To further evaluate the role of Ca$_v$β$_{1a}$ in MPC proliferation, we cultured MPCs from Cacnb1$^{-/-}$ embryos using Fluorescence Activated Cell Sorting (FACS)(Figure S3). MPCs derived from Cacnb1$^{-/-}$ embryos also showed significantly less cell proliferation after 4 days in culture (Figure 4B) compared to heterozygous controls. Together these data suggest that loss of Ca$_v$β$_{1a}$ expression severely affects MPC self-renewal.

MPCs are prone to spontaneous differentiation even under proliferative conditions, thus we hypothesized that overexpression of Ca$_v$β$_{1a}$ may enhance proliferation in wild type MPCs by protecting against differentiation. To test this idea, we transfected primary MPCs with an EGFP control plasmid (Figure 4C) or Ca$_v$β$_{1a}$-YFP (Figure 4D), and then stained for the well established marker of proliferation, Ki67(Gerdes et al., 1984). After 24 hrs in culture, a significantly higher percentage of Ca$_v$β$_{1a}$-YFP transfected cells were also Ki67 positive, compared to EGFP/Ki67 positive cells (Figure 4E). Thus increasing Ca$_v$β$_{1a}$ expression level appears to enhance proliferation of MPCs, possibly by protecting
against differentiation, further supporting the concept that Ca\textsubscript{v1\alpha} plays a critical role in MPC self renewal and expansion.

Due to the effects of loss of Ca\textsubscript{v1\alpha} expression on MPC proliferation \textit{in vitro}, we hypothesized that some of the deficits in muscle mass seen in \textit{Cacnb1}\textsuperscript{-/-} mice are due to impaired MPC proliferation, beyond the already known loss of Ca\textsubscript{1.1} function and EC-coupling. As \textit{Cacnb1}\textsuperscript{+/+} mice do not survive past birth, they must be studied at the embryonic stages. We chose to examine E13.5 embryos, as this time corresponds to the early stages of limb muscle development in mice, before complete innervation and large scale muscle formation (Ontell et al., 1995; Platzer, 1978). Thus effects from loss of EC-coupling during development could be minimized, as most of the muscle cells at this time are newly formed myotubes or still in the progenitor phase. Hematoxylin and Eosin (H&E) staining of hind limbs from E13.5 embryos showed that the relative area of nascent muscle bundles was markedly smaller in \textit{Cacnb1}\textsuperscript{-/-} embryos compared to wild type, while the overall number of the muscle bundles was similar (Figure 4F-H). Thus the deficit in muscle mass previously observed in \textit{Cacnb1}\textsuperscript{+/+} mice (Gregg et al., 1996) occurs very early during muscle development. To test whether impaired MPC growth contributed to the lower muscle mass seen in \textit{Cacnb1}\textsuperscript{+/+} embryos, we stained cross sections of \textit{Cacnb1}\textsuperscript{+/+} (Figure 4I) and \textit{Cacnb1}\textsuperscript{-/-} (Figure 4J) hind limbs for the MPC marker Pax7. Compared to \textit{Cacnb1}\textsuperscript{+/+}, \textit{Cacnb1}\textsuperscript{-/-} mice had a significantly lower number of Pax7\textsuperscript{+} cells per \(\mu m^2\) at the same time during development (Figure 4K). To see if decreased Pax7\textsuperscript{+} cell number was caused by increased cell death, we measured AnnexinV-FITC/7AAD staining in E12.5 embryos by flow cytometry (Figure S4B).

Similar to our in vitro results with Ca\textsubscript{v1\alpha}-shRNA in primary MPCs, \textit{Cacnb1}\textsuperscript{-/-} cells did
not show an increase in any markers of apoptosis or necrosis. Overall, these results suggest that lack of Ca₃β₁a expression impairs MPC proliferation during development in vivo, and that the Cacnb1 deletion causes loss of muscle mass at least in part due to impaired MPC proliferation, rather than increased cell death.

**Chromatin binding of Ca₃β₁a**

Our results demonstrate that Ca₃β₁a enters the nucleus of myoblasts and loss of Ca₃β₁a expression impairs proliferation. Therefore, we hypothesized that Ca₃β₁a may act as a transcription factor. To test this question, we performed chromatin immunoprecipitation (ChIP) on-a-chip assays (Figure 5). A Ca₃β₁a antibody was used to immunoprecipitate chromatin from C2C12 myoblasts, and enriched regions compared to IgG control were determined using CMARRT (Kuan et al., 2008). Enriched Ca₃β₁a binding was detected in the promoter regions of 952 genes (Table S3). Binding peaks were enriched closest to the transcription start site (TSS) of most genes (Figure 5A), with the vast majority falling inside or upstream of the genes coding region (Figure 5B). Motif analysis provided a consensus motif of CCTTTAATCCAG (e value 9.5e-102) (Figure 5C). Comparison to known motifs showed significant overlap with Tcf1 (TAATC, p = 0.0036) and Lef1 (TTTGAT, p = 0.0075) binding sites, suggesting a possible involvement of Ca₃β₁a with the canonical Wnt signaling pathway. Functional annotation of enriched peaks revealed Ca₃β₁a binds to the promoter regions of a broad set of genes, including many involved in signal transduction and stress response (Figure 5D). Normalized log2 (Ca₃β₁a/IgG) Ca₃β₁a binding peaks at the promoter regions of genes of interest, namely transcription
factors with known involvement in development, were visualized in the UCSC browser (Figure 5E). Secondary validation of these genes was tested by ChIP-PCR with a GFP/YFP antibody in untransfected and Ca,β1a-YFP transfected myoblasts. Nearly all of the promoter regions tested showed >2 fold enrichment, across multiple primer pairs, in the Ca,β1a-YFP transfected cells, with the exception of negative controls (Figure 5F and 5G). Thus Ca,β1a is shown to bind directly to the promoter region of numerous genes in separate experimental designs.

**Global gene regulation by cancb1**

To further complement our ChIP-on-chip data, we used microarray analysis to examine changes in global gene expression in the presence or absence of Ca,β1a, but utilizing primary MPCs harboring the Cacnb1 deletion. Pure MPC cultures were isolated from Cacnb1+/+, Cacnb1+/−, and Cacnb1−/− mice (Figure S3), and total RNA was extracted and used from microarray analysis (Figure 6 and Table S4). We identified genes of interest as those that showed significant fold change in a Cacnb1 dose-dependent manner(Figure S5). Specifically, we identified 1104 genes that increased with decreasing Cacnb1 expression (negatively regulated by Cacnb1) (Figure 6A) and 1888 genes that decreased with decreasing Cacnb1 expression (positively regulated by Cacnb1) (Figure 6B). A gene negatively regulated by Cacnb1 (increased in Cacnb1−/−) of particular interest was the transcription factor myogenin (Myog), which is known to play a critical role in skeletal muscle development. Further functional analysis highlighted many genes involved in cell cycle regulation and muscle development (Figure 6A and 6B). Genes with promoters
enriched for Ca$_{\text{v}1\alpha}$ binding, which also showed positive or negative regulation by Ca$_{\text{v}1\alpha}$, are listed in Table S5.

**Cacnb1 regulates Myog**

Myogenin acts as a switch for MPCs to transition from proliferation to differentiation, and previous studies have shown that precocious expression of myogenin can lead to MPC pool depletion (Schuster-Gossler et al., 2007; Van Ho et al., 2011). We therefore hypothesized that if Myog is inhibited by Ca$_{\text{v}1\alpha}$, then this may explain the impaired MPC growth we observed when Ca$_{\text{v}1\alpha}$ was knocked down/out due to aberrant myogenin expression in these cells. To confirm our microarray results, we generated myogenic explant cultures from E18 embryos and quantified myogenin expression using immunocytochemistry (Figure 7A-C) and real time RT-PCR (Figure 7D). After 3 days, cultures from Cacnb1$^{-/}$ embryos had significantly more myogenin positive cells than Cacnb1$^{+/+}$ (Figure 7C). Myogenin mRNA was also approximate 7-fold higher overall in Cacnb1$^{-/}$ cultures compared to Cacnb1$^{+/+}$ and Cacnb1$^{+/}$ (Figure 7D). Similarly, primary MPC cultures transfected with Ca$_{\text{v}1\alpha}$-shRNA showed a significantly higher percentage of myogenin positive cells compared to controls (Figure 7E). To test whether loss of Cacnb1 increases myogenin in MPCs *in vivo*, we measured myogenin mRNA in the hind limb buds of E11.5 Cacnb1$^{+/+}$, Cacnb1$^{+/}$ and Cacnb1$^{-/}$ embryos (Figure 7F), at a time which pre-dates differentiated muscle formation in the developing limb (Taher et al., 2011). Myogenin mRNA was approximately 10-fold and 25-fold higher in the limb buds of Cacnb1$^{+/}$ and Cacnb1$^{-/}$ embryos, respectively, compared to wild type controls.
Together these results demonstrate that loss of Ca$_{\alpha}\beta_{1a}$ results in increased myogenin mRNA and protein, thus validating our microarray data, and also suggesting that Ca$_{\alpha}\beta_{1a}$ acts to inhibit myogenin expression. To test whether this inhibition was dependent on Ca$_{\alpha}\beta_{1a}$ nuclear entry, we made use of Ca$_{\alpha}\beta_{1a}$-YFP mutants which showed the strongest and weakest nuclear localization (Figure 3). Compared to full length Ca$_{\alpha}\beta_{1a}$-YFP, Ca$_{\alpha}\beta_{1a}$-101-274-YFP (lacking GK domain and constitutively nuclear) showed an enhanced ability to suppress myogenin expression in differentiating C2C12 myoblasts, while Ca$_{\alpha}\beta_{1a}$-161-524-YFP (lacking SH3 domain and constitutively cytoplasmic) was much less effective at inhibiting myogenin expression (Figure 7G).

We next sought to determine how Ca$_{\alpha}\beta_{1a}$ regulates myogenin expression, hypothesizing that Ca$_{\alpha}\beta_{1a}$ may act via actions on the Myog promoter. To initially test this idea, we made use of a luciferase reporter gene linked to a core region of the myogenin promoter (-184+44; Myog-luc) (Berkes et al., 2004). Myoblasts co-transfected with Myog-luc and Ca$_{\alpha}\beta_{1a}$–shRNA showed significantly higher luciferase activation than controls (Figure 7H). Thus, loss of Ca$_{\alpha}\beta_{1a}$ protein enhances activation of the Myog promoter. Conversely, when Myog-luc was co-transfected with Ca$_{\alpha}\beta_{1a}$-YFP, we saw decreased reporter gene activation compared to EGFP transfected controls (Figure 7I), suggesting Ca$_{\alpha}\beta_{1a}$ represses myogenin via direct or indirect actions on the Myog promoter region. After two days in differentiation medium, Ca$_{\alpha}\beta_{1a}$-YFP no longer supressed Myog-luc activity, indicating that the Myog promoter escapes Ca$_{\alpha}\beta_{1a}$ regulation after terminal differentiation (Figure 7I). Although our ChIP-on-chip data suggested a possible binding of Ca$_{\alpha}\beta_{1a}$ to Lef1 consensus binding regions, and the -184+44 Myog promoter contains a similar motif (TTGATGTGCAG), western blotting showed that Lef1 protein is not expressed in
myoblasts (Figure S6A), thus making it unlikely that Ca\textsubscript{\textbeta}1\textalpha acts on the *Myog* promoter via interactions with Lef1. We also tested whether several other proteins known to bind to the *Myog* promoter region might interact with Ca\textsubscript{\textbeta}1\textalpha. MyoD (Cheng et al., 1992), Mef2 (Edmondson et al., 1992), and Pbx1 (Berkes et al., 2004) all failed to co-precipitate with Ca\textsubscript{\textbeta}1\textalpha-YFP (Figure S6B), and expression of Ca\textsubscript{\textbeta}1\textalpha-YFP did not alter the sub-nuclear localization of MyoD or Mef2 (Figure S6C), indicating that Ca\textsubscript{\textbeta}1\textalpha does not act on the *Myog* promoter in concert with any of these proteins. Interestingly, mutation of both E-boxes (CANNTG) (Berkes et al., 2004) within the -184+44 *Myog*-luc construct did significantly impair Ca\textsubscript{\textbeta}1\textalpha-YFP’s ability to inhibit reporter gene activation, suggesting Ca\textsubscript{\textbeta}1\textalpha may interact with some other E-box binding proteins, other than MyoD. ChIP-qPCR (Figure 7J) of Ca\textsubscript{\textbeta}1\textalpha-YFP showed specific enrichment of the proximal *Myog* promoter compared to 5’ and 3’ regions of the gene. Electrophoretic mobility shift assays (EMSA) (Figure 7K) also indicated binding of Ca\textsubscript{\textbeta}1\textalpha-YFP to both the *Myog* promoter and the ChIP-on-chip consensus DNA motif, with Ca\textsubscript{\textbeta}1\textalpha-YFP causing a specific shift of these probes, which was further super-shifted by an anti-YFP antibody. Together these results provide strong evidence that Ca\textsubscript{\textbeta}1\textalpha acts locally at the *Myog* promoter in vivo, like through involvement of other, as of yet unidentified, proteins.

Discussion

Ca\textsubscript{\textbeta}1\textalpha has long been known solely for its essential role in skeletal muscle EC Coupling. Though a few reports have hinted at novel roles for the protein, a well defined biological function for any Ca\textsubscript{\textbeta} subunit outside of Ca\textsubscript{\textalpha}-regulation has yet to be determined. Here we
have described such a role for Ca\textsubscript{v}β\textsubscript{1a} in skeletal MPCs: as a transcriptional regulator of myogenesis via localized repression of the *Myog* promoter.

Here we report for the first time that the Ca\textsubscript{v}β\textsubscript{1a} subunit enters the nucleus of MPCs. An important precursor to this finding was the observation that Ca\textsubscript{v}β\textsubscript{1a} is expressed in C2C12 and primary myoblasts before the expression of Ca\textsubscript{v}1.1 and only localizes to the plasma membrane after the appearance of Ca\textsubscript{v}1.1. This finding suggests that Ca\textsubscript{v}β\textsubscript{1a} plays a Ca\textsubscript{v}-independent role in MPCs. The idea of Ca\textsubscript{v}-independent roles for Ca\textsubscript{v}β subunits have been suggested previously, an idea mainly tied to their nuclear translocation. Ca\textsubscript{v}β\textsubscript{4c} and Ca\textsubscript{v}β\textsubscript{3} subunits have been shown to regulate transcription *in vitro* by direct suppression of Hp1 (Hibino et al., 2003a) and Pax6(s) (Zhang et al., 2010), respectively, in a Ca\textsubscript{v}-independent fashion. While not linked to nuclear translocation, a few hints at a biological role for Ca\textsubscript{v}-independent functions of Ca\textsubscript{v}β subunits have been reported. (Berggren et al., 2004) found that knockout of the *Cacnb3* gene caused higher glucose-stimulated insulin release from pancreatic β cells, attributed to a possible interaction of the Ca\textsubscript{v}β\textsubscript{3} subunit with intracellular IP3 receptors. Garrity and colleagues have also shown effects of morpholino-mediated Ca\textsubscript{v}β knockdown in zebrafish models: knockdown of Ca\textsubscript{v}β\textsubscript{4} prevented normal epiboly in early zebrafish embryos, which could be rescued by a Ca\textsubscript{v}-binding deficient mutant Ca\textsubscript{v}β\textsubscript{4} (Ebert et al., 2008). Interestingly, this group also found impaired cardiac progenitor cell proliferation in 24-30 and 30-36 hr post-fertilization zebrafish embryos following Ca\textsubscript{v}β\textsubscript{2} knockdown (Chernyavskaya et al., 2012). The latter finding is especially compelling in the context of our findings that Ca\textsubscript{v}β\textsubscript{1a} knockdown/gene knockout in muscle progenitor cells impairs their proliferation *in vitro* and *in vivo*. 
An important question that remains is how does Ca\textsubscript{v}β\textsubscript{1a}, and Ca\textsubscript{v}β subunits in general, travel to the nucleus? And once there, how does Ca\textsubscript{v}β\textsubscript{1a}, locally act on the Myog promoter? One likelihood is that Ca\textsubscript{v}β subunits bind to other proteins, either in a conserved or isoform specific fashion. Earlier works with other Ca\textsubscript{v}β subunits offered several different protein binding partners that may be responsible for their nuclear translocation, including the aforementioned Hp1 and Pax6(s) proteins, as well as the RGK (Rad,Rem,Gem/Kir) family of proteins (reviewed in Buraei and Yang, 2010). In our own assays, neither Hp1 nor the RGK protein Rem co-precipitated with Ca\textsubscript{v}β\textsubscript{1a}-YFP (data not shown). We did not see evidence of Ca\textsubscript{v}β\textsubscript{1a} interaction with Lef1 or several other proteins known to also regulate the Myog promoter (MyoD, Mef2, Pbx1), leaving this an open question. The apparent importance of E-box motifs for Ca\textsubscript{v}β\textsubscript{1a} regulation of Myog may offer insight for future screening studies.

Another other possibility is that Ca\textsubscript{v}β enters the nucleus based on an NLS specific to one or more isoforms of the protein. We believe this is less likely as Ca\textsubscript{v}β subunits do not possess classical a NLS, and although they do possess a lysine/arginine rich sequence similar to a known yeast NLS (Hicks and Raikhel, 1995), truncation of this sequence does not affect nuclear localization of our Ca\textsubscript{v}β\textsubscript{1a}-YFP constructs. Our truncation mutants offer some insight by highlighting the importance of the SH3 domain, along with the dispensability of the GK domain, for Ca\textsubscript{v}β\textsubscript{1a}-YFP nuclear translocation, support this notion. Previous studies which have shown that fluorescently tagged Ca\textsubscript{v}β2-SH3, but not Ca\textsubscript{v}β2-GK, localizes to the nucleus (Takahashi et al., 2005), and also that the truncated splice variant Ca\textsubscript{v}β\textsubscript{4c} (containing SH3 but not GK domains) localizes to the nucleus (Hibino et al., 2003a). The apparent importance of the SH3 domain in nuclear
localization across Ca,β isoforms suggests that this phenomenon is conserved. However the apparent individualized preference of Ca,β isoforms for nuclear binding partners argues in favor of isoform specific mechanisms of nuclear translocation. The SH3 domain contains a PxxP binding motif (Buraei and Yang, 2010), and may therefore bind multiple, NLS-containing proteins in a tissue-specific fashion, offering a possible way to reconcile these findings. Still, further experiments are necessary to uncover the precise nature of Ca,β-protein interactions and nuclear translocation. Findings from these studies will yield vital information on the role of Ca,β1a and other Ca,β subunits as transcription factors.

Reduced skeletal muscle mass has been associated with loss of the Cacnb1 gene since the original creation of the knockout mouse (Gregg et al., 1996). This phenotype was viewed as similar to that seen in the dysgenic (Ca,1.1 mutant) (Chaudhari, 1992; Knudson et al., 1989; Pai, 1965), and dyspedic (RyR mutant) (Takeshima et al., 1994), and therefore attributed to lack of EC-coupling during development. While the initial report looked at mice in the late prenatal stage (∼E18), we observed visible deficits in Cacnb1 /− muscle mass as early as E13.5, and clear differences in gene expression at E11.5. While EC-coupling may occur at E13.5, the differences were already quite large at this stage, suggesting a sustained impairment in muscle development, likely at the MPC level. In support of this idea, we also observed fewer Pax7+ MPCs in Cacnb1 /− hindlimbs; a deficit which cannot be explained by lack of EC-coupling. We also detected a higher level of myogenin mRNA in the limb buds of E11.5 mutant mice, at a time that pre-dates myotube formation (Taher et al., 2011) and thus EC-coupling. It is plausible that Ca,ss may play some other role in MPC function, thus explaining the impaired proliferation in
the absence of \textit{Cacnb1} or following shRNA-mediated knockdown of Ca\textsubscript{\textit{v}}\textbeta\textsubscript{1a}. However this appears unlikely as our earlier data shows the complete absence of Ca\textsubscript{\textit{v}}1.1 expression and the absence of Ca\textsubscript{\textit{v}}\textbeta\textsubscript{1a} membrane localization in C2C12 myoblasts. Additionally, a previous report found no differences in the growth rate of MPCs isolated from wild type and dysgenic mice (Pincon-Raymond et al., 1991). Thus any alterations seen in Ca\textsubscript{\textit{v}}\textbeta\textsubscript{1a} knockdown/Cacnb1\textsuperscript{\textminus} MPCs is likely due to Ca\textsubscript{\textit{v}}-independent functions.

A key concept in the field of skeletal muscle development and regeneration is the need for precise balance between progenitor cell self-renewal and differentiation. Because terminal differentiation (e.g., myogenin expression) of MPCs is tightly associated with their exit from the cell cycle, anti-differentiation signals need to stay in effect while MPC populations multiply enough to provide sufficient substrate for the formation of an adequate number of myofibers. Notch signaling is thought to be a key regulator of MPC self-renewal. Impaired Notch signaling during development leads to early depletion of MPC pools and subsequent hypomorphic skeletal muscle development (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). In adult mice, inhibition of Notch following muscle injury caused impaired regeneration due to depleted MPC pools (Brack et al., 2008). A similar phenomenon was also recently observed in \textit{Nrg1} knockout mice (Van Ho et al., 2011). A hallmark feature in several of these examples (Schuster-Gossler et al., 2007; Van Ho et al., 2011) is a decreased number of Pax3/Pax7\textsuperscript{+} MPCs, accompanied by an increase in myogenin\textsuperscript{+}, and presumably terminally differentiated cells. This is consistent with our findings that Cacnb1\textsuperscript{\textminus} mice show fewer Pax7\textsuperscript{+} MPCs in hind limbs at E13.5, which is preceded by significantly higher myogenin mRNA in hind limb buds at E11.5.
Based on our data, we propose a mechanism for this pathway by which \( \text{Ca}_v \beta_{1a} \) acts at the \( \text{Myog} \) promoter region to suppress its transcription (Figure S7).

It is important to note that while \( \text{Ca}_v \beta_{1a} \) inhibits myogenin expression in MPCs, \( \text{Ca}_v \beta_{1a} \) expression actually increases during myogenic differentiation \textit{in vitro}, a period which is well known to be marked by a transient increase in myogenin expression. Thus under normal circumstances it seems that the \( \text{Myog} \) promoter finds a way to escape \( \text{Ca}_v \beta_{1a} \)-mediated inhibition during differentiation. The apparent decline in \( \text{Ca}_v \beta_{1a} \) nuclear localization with myogenic differentiation offers a partial mechanism for this escape, although some visible \( \text{Ca}_v \beta_{1a} \) apparently remains in the nucleus of differentiated myotubes, suggesting additional regulatory mechanisms. Thus the identification of co-factors involved in \( \text{Ca}_v \beta_{1a} \) regulation of the \( \text{Myog} \) promoter will be important in addressing this question as well.
Materials and Methods:

**RT-PCR and real time PCR:** Total RNA was isolated from cells and tissue using TRIZOL Reagent. Primer sequences used for RT-PCR and ChIP-PCR, as well as product numbers for real time Taqman primers (Applied Biosystems) are listed in Table S1. For real time PCR, gene expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). mRNA was primed with random hexamers and reverse transcribed with Reverse Transcriptase III (Invitrogen). RT-PCR was performed on a GeneAmp PCR System 3700 (Applied Biosystems) under the default parameters for 35 cycles. For real time PCR, samples were prepared using Taqman Gene Expression Master Mix (Applied Biosystems) and Taqman probes (Supplemental table 1) on a Stratagene MX3000P, using the default parameters for 35 cycles.

**Protein isolation and western blot:**

Cytosolic, nuclear, and membrane fractionation and SDS-PAGE were performed as described (Leung et al., 1987; Taylor et al., 2009; Washabaugh et al., 2007). For cytosolic and membrane fractions, C2C12 cells were collected with a rubber scraper, rinsed in ice-cold PBS, and lysed in ice-cold Buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monobasic, 1 mM MgCl$_2$, 0.5 mM EDTA, 303 mM sucrose with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) using a handheld glass homogenizer. Homogenate was centrifuged at 100,000 x g for 90 min at 4°C in a Beckman Type Ti.70i rotor. The supernatant was saved as the cytosolic fraction.
The pellet was rinsed with ice cold PBS and resuspended with a glass homogenizer in fresh Digitonin buffer (1% digitonin (w/v), 185 mM KCl, 1.5 mM CaCl₂, 10 mM HEPES pH 7.4 with complete protease inhibitor cocktail) as the membrane fraction.

For cytosolic and nuclear fractions, C2C12 myoblasts were lysed in Ontell buffer (Washabaugh et al., 2007) (19mM NaCl, 1.5mM MgCl₂, 20mM HEPES pH 7.4, 1mM DTT, 20% glycerol, 0.1% Triton X-100) (300ul buffer per 100mg tissue) using 50-100 strokes glass homogenizer. Lysate was centrifuged at 1000 x g at 4°C for 10m and supernatant was taken as cytosolic fraction. Pellet was rinsed twice in Ontell buffer then resuspended in Buffer 2 (0.5M sucrose 5mM MgCl₂, 10mM Tris, 1mM DTT, 0.6M KCl) as the nuclear fraction.

Protein concentration was measured using Bradford or bicinchoninic protein assays with bovine serum albumin standards.

For western blotting, proteins samples were mixed with 2x Laemmli Buffer (2% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris, and 5% β-mercaptoethanol) (Laemmli, 1970) (samples to be probed for Ca,1.1 were loaded in 8M Urea buffer – 8M Urea, 20% SDS, 50 mM Tris, 0.004% BPB, 2M Thiourea, 1mM DTT), boiled at 95°C for 5m and separated by SDS-PAGE using 10% polyacrylamide gels with 4.5% stacking gels. Proteins were transferred to PVDF membranes and blotted using 5% non-fat dry milk in TBS + 0.02% Tween-20 for all antibody incubation steps. Proteins were visualized with ECL Plus (GE).

Antibody clone, dilution, and product numbers are listed in Table S2.
Construction of recombinant adenoviral vector RAd-Caβ1a-YFP: cDNA for Caβ1a-YFP (Genbank accession number: M25514.1, generously donated by Dr. K. Beam, Colorado State University (Leuranguer et al., 2006)) was inserted into a RAd vector by a variant of the two plasmid method (Hitt et al., 1998) using the AdMax® plasmid kit (Microbix). cDNA coding for Caβ1a-YFP was excised from plasmid Caβ1a-YFP (with EcoRI and HpaI at the 5´and 3´end, respectively) and inserted in the multiple cloning site (MCS) of shuttle pDC516 (one of the shuttle plasmids in the kit) which contains an expression cassette consisting of the mouse cytomegalovirus promoter (mCMV) and the simian virus 40 (SV40) polyadenylation signal, immediately upstream and downstream of the MCS, respectively. Downstream this cassette, pDC516 also contains an frt recognition site for the yeast FLP recombinase. The second plasmid of the kit, the genomic plasmid pBHGfrt(del)E1,3 FLP, consists of the entire genome of adenovirus 5 (Ad5), with deletions in the E1 and E3 regions. Upstream of the E1 deletion, pBHGfrt(del)E1,3FLP contains an expression cassette for the gene for yeast FLP recombinase and immediately downstream the E1 deletion, there is a frt recognition site. Both plasmids were cotransfected in HEK293 cells, a line stably transfected with a portion of the Ad5 E1 genomic region. In cotransfected HEK293 cells, FLP recombinase is readily expressed and efficiently catalyzes the site-directed recombination of the expression cassettes of pDC516 into the left end of pBHGfrt(del)E1,3 FLP, thus generating the genome of the desired recombinant adenoviral vector, RAd-Caβ1a-YFP (Figure). The newly generated RAd was rescued from HEK293 cell lysates, plaque
purified, and then purified by ultracentrifugation in CsCl gradient and dialyzed. Final virus stock was titrated by a serial dilution plaque assay. RAd-GFP control was purchased from the UNC Vector Core Facility (University of North Carolina at Chapel Hill).

**Histology and immunofluorescent staining:** Animal housing and procedures were approved by the Animal Care and Use Committee of Wake Forest University Health Sciences. For timed embryo studies, breeding pairs were placed together overnight and separated the following morning. Pregnancy was confirmed by sustained weight gain over the next 10 days. Genotypes were confirmed by PCR. E13.5 embryo hindlimb sections were processed as previously described (Le Grand et al., 2004) for cryosectioning. For immunofluorescent staining, cells and 10μM transverse cryosections were fixed, permeabilized with 0.3% Triton X100, blocked in 5% goat serum and incubated overnight with primary antibodies at 4°C (see Table S2). Slides were mounted with Dako Fluorescent Mounting Medium (Dako). Hematoxylin Gill No. 2 and Eosin Y (Sigma) solutions were used for H&E staining, and slides were mounted with Cytoseal (Thermo Scientific). LMB (LC Laboratories) was added to culture medium at 20 nM for 3 hrs before fixation or lysate collection. Images were captured on an Olympus IX81 microscope with a Hamamatsu Orca TC² camera at room temperature and analyzed using Metamorph Basic software. All counting experiments were performed with the experimenter blind to experimental conditions

**Cell Death Analysis and Flow Cyometry:** Cells were isolated from hindlimbs of E12.5 embryos by enzymatic digestion, pre-plated for 1 hour on plastic, and stained for
AnnexinV-FITC and 7AAD for 15 minutes at room temperature. Flow cytometry was performed on an Accuri B6 Flow Cytometer (BD Biosystems).

**Molecular cloning:** Ca₄β₁a-YFP truncation mutants were cloned by PCR using primer pairs containing EcoR1 and SalI restriction enzyme digest sequences (listed in Table S1) and inserted into the pEYFP_n1 vector (Clontech). Sequencing confirmed all constructs (DNA sequencing laboratory, WFUHS).

**FACS, primary cell culture and transfection:** C2C12 (ATCC) myoblasts were maintained in Dulbecco’s Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS). FACS was performed as described (Griffin et al., 2010) in a FACSAria (BD Biosciences). Embryos were harvested at E18.5 under sterile conditions. Skeletal muscle was dissected from the limbs, minced, washed in ice-cold PBS, and incubated in a Collagenase type II/Dispase I solution for 1 hr at 37°C. Cells were then tritutrated, passed through a 40 μm nylon-mesh filter, washed, and labeled with α7-integrin APC and CD31/CD45-FITC-conjugated antibodies in PBS with 1% FBS for 30m at 4°C and washed twice before FACS. MPCs were cultured on laminin (Invitrogen) coated dishes in Ham’s F-10 medium with 20% FBS and basic Fibroblast Growth Factor (5 ng/ml, Promega) (Rando and Blau, 1994). Differentiation was induced upon C2C12 myoblasts or MPCs reaching 90% confluence by placing cells in DMEM with 2% horse serum. Lipofectamine 2000 (Invitrogen) was used for transfection. 5 Ca₄β₁a specific shRNA sequences from the RNAi Consortium (Open Biosystems) were individually tested in C2C12 myoblasts selected with 3ng/ul puromycin (Sigma). TRC 69052 showed the highest efficacy of Ca₄β₁a protein knockdown and was used for further experiments.
MISSION SHC002 non-targeting shRNA (Sigma) was used as a control. Explant cultures were prepared from E18.5 mice as described (Smith and Merrick, 2010).

**Microarray:** Total RNA was prepared from FACS sorted primary Cacnb1 +/-, +/-, and -/- MPCs and hybridized to GeneChip Mouse Genome 430A 2.0 Arrays (Affymetrix) according to manufacturer’s instructions at the WFUHS Microarray Core Laboratory. CEL files were analyzed using Partek Genomics Suite (Partek) and grouped into 9 categories of expression based on fold change between the three experimental groups. Functional annotation was performed using DAVID v6.7 software using GOTERM_BP_2 (Huang et al., 2009).

**Luciferase assay:** C2C12 myoblasts were infected with RAd Caβ1a-YFP or RAd-GFP and 24 hrs later transfected with pMyog-firefly luciferase, (Berkes et al., 2004) (a kind gift from Dr. S. Tapscott) and β-actin-renilla luciferase. Cells were checked for equal density and viral transfection and harvested 12 hrs later at confluence in GM, or after 48 hrs in DM and normalized firefly to renilla luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega).

**ChIP on chip and ChIP-PCR/ qPCR:** Chromatin was immunoprecipitated from sub-confluent C2C12 myoblasts using a Caβ1a antibody (H-50, Santa Cruz) or control rabbit IgG (Santa Cruz), hybridized to GeneChip Mouse Promoter 1.0R Arrays (Affymetrix) according to the manufacturer’s protocol. For ChIP buffer recipes, see Supplementary Table 3. Briefly: 5x10^7 C2C12 myoblasts were fixed in formaldehyde at a final concentration of 1% for 10m, quenched with 2.5 M glycine, washed with PBS, and collected with a rubber cell scraper. The pellet was washed 3 time in Lysis Buffer, re-
suspended in Pre-IP dilution buffer, and sonicated for 10 x 60s on ice. Average chromatin fragment size was confirmed on an agarose gel to be 200-1000 bp before proceeding. Chromatin was pre-cleared with protein A Sepharose beads then incubated overnight with antibodies at 4°C. The following day chromatin was incubated with protein A Sepharose beads for 4 hrs at room temperature. Beads were pelleted and washed with ChIP washes 1-2 (twice each for 5m), ChIP wash 3, TE, and finally chromatin was eluted twice with Elution Buffer at 65°C. Crosslinking was reversed by incubation with Proteinase K overnight at 65°C, and DNA purified using cDNA Cleanup Columns (Affymetrix). DNA was randomly primed using Sequenase and Primer A

\[ GTTTCCCAGTCACGGTC(N)\text{HPLC purified} \] and amplified using Primer B

\[ GTTTCCCAGTCACGGTC \] and Taq Polymerase. Amplification of fragments sized 200bp-2000bp was confirmed on an agarose gel. Samples were purified on a GeneChip Sample® Cleanup Module (GE). For ChIP-on-chip, DNA was fragmented, labeled, and hybridized to Mouse Promoter 1.0R Arrays (Affymetrix) according to the manufacturer’s protocol.

ChIP-PCR/qPCR was performed using an anti-GFP antibody (see Table S2) for immunoprecipitation, following the same protocol used for ChIP on chip. Primers were designed using NCBI Primer-BLAST. ChIP-PCR was performed on a GeneAmp PCR System 3700 (Applied Biosystems) under the default parameters for 35 cycles. ChIP-qPCR was performed on a Stratagene MX3000P, using the default parameters for 40 cycles.
**EMSA:** Nuclear protein extracts were prepared from Cos7 cells (Holden and Tacon, 2011), expressing either GFP or Ca\(_{\text{v}}\)\(\beta_{1a}\)–YFP, and incubated with infrared dye-labeled oligos (see Table S1) for 30 minutes at room temperature, and an additional 30 minutes with antisera or an equivalent volume of PBS. Samples were run on 4-12% non-denaturing TBE gels.

**ChIP-on-chip analysis:** The average log2 (Ca\(_{\text{v}}\)\(\beta_{1a}\)/IgG) intensity value of the 2 biological replicates was computed for each probe position. Regions enriched for Ca\(_{\text{v}}\)\(\beta_{1a}\) relative to IgG were determined using CMARRT (Kuan et al., 2008) on the average log2 (Ca\(_{\text{v}}\)\(\beta_{1a}\)/IgG) at the FDR level of 0.05. Peak annotation, distance to TSS and position to nearest gene were performed using ChIPpeakAnno (Zhu et al., 2010) and the Galaxy web platform (Blankenberg et al., 2010; Goecks et al., 2010). Refseq ID conversion to gene names and functional annotation were performed using DAVID v6.7 using GOTERM_BP_ALL (Huang et al., 2009). For distance to TSS and peak position relative to nearest gene, peak coordinates were lifted over from mm8 to mm9 and then mapped to either the nearest or overlapping genes using the prebuilt transcription start sites annotation library for mouse genome TSS.mouse.NCBIM37. Peaks (log2 Ca\(_{\text{v}}\)\(\beta_{1a}\)/IgG intensity) were visualized in BedGraph format using the UCSC Genome Browser (Kent et al., 2002). Motif Analysis was performed with MEME software (Bailey et al., 2009) and TOMTOM (Gupta et al., 2007).

For peak position, peaks not overlapping with a gene were tied to the nearest feature when generating the pie chart. “Overlap Start” and “Overlap End” correspond to peaks
which overlap with gene transcription start and end site, respectively. “Upstream” and “Downstream” correspond to peaks which are upstream of gene transcription start site and downstream of gene transcription end site, respectively. “Inside” corresponds to peaks which are within gene region. “Inside” corresponds to peaks which covers the entire gene (especially for very short genes).

**Data Analysis:** Data are presented as means ± s.e.m. Data were analyzed using Sigma Plot v11.0 with Student t-test or one-way ANOVA repeated measures, with Holm-Sidak test applied *post hoc* when appropriate. An alpha value of *P* < 0.05 was considered significant.

Acknowledgements:

We would like to thank Dr. Ronald Gregg and Dr. Weichun Lin for developing and providing the *Cacnb1*−/− mice, Dr. Jeff Chou for microarray data analysis, and Lou Craddock, James Wood, and Beth Hollbrook for technical assistance. This work was supported by the NIH/NIA.

**Supplemental Information Summary**

Table S1. Inventory of primers used throughout the paper for various functions (RT-PCR, cloning, shRNA, etc).
Table S2. Inventory of antibodies used throughout the paper for various experiments (western blot, immunocytochemistry, immunohistochemistry, ChIP). Dilutions are included.

Table S3. List of buffers used in ChIP.

Table S4. Complete list of genes with promoter regions found to be enriched for Ca,β1a binding. Related to Figure 5 of the main text.

Table S5. List of genes with promoter regions found to be ChIP enriched for Ca,β1a binding and also showing differential regulation in expression by microarray analysis. Subdivided into ChIP enriched genes that are upregulated in cacnb1 -/- (left hand column) and ChIP enriched genes that are downregulated in cacnb1 -/- (right hand column)
References:


Bichet, D., V. Cornet, S. Geib, E. Carlier, S. Volsen, T. Hoshi, Y. Mori, and M. De Waard. 2000. The I-II loop of the Ca2+ channel alpha1 subunit contains an
endoplasmic reticulum retention signal antagonized by the beta subunit. Neuron. 25:177-90.


Cheng, W., X. Altafaj, M. Ronjat, and R. Coronado. 2005. Interaction between the dihydropyridine receptor Ca2+ channel beta-subunit and ryanodine receptor type
1 strengthens excitation-contraction coupling. *Proc Natl Acad Sci U S A.*
102:19225-30.


neurotransmitter vesicle anchoring to presynaptic Ca2+ channels. Nat Neurosci. 10:691.


Schuster-Gossler, K., R. Cordes, and A. Gossler. 2007. Premature myogenic
differentiation and depletion of progenitor cells cause severe muscle hypotrophy
in Delta1 mutants. Proc Natl Acad Sci U S A. 104:537-42.

Smith, J., and D. Merrick. 2010. Embryonic skeletal muscle microexplant culture and

Subramanyam, P., G.J. Obermair, S. Baumgartner, M. Gebhart, J. Striessnig, W.A.
nuclear targeting of the calcium channel beta4b subunit in nerve and muscle cells.

6:e28358.

kinase-like properties of beta-subunits required for modulation of voltage-

SH3/guanylate kinase domain interaction regulates multiple properties of voltage-
gated Ca2+ channels. J Gen Physiol. 126:365-77.

Noda. 1994. Excitation-contraction uncoupling and muscular degeneration in


Figure List:

Figure 1. Ca₄β₁₄ expression in MPCs. (A) mRNA expression of Ca₄β₁₄ in primary MPCs cultured in GM (myoblasts) and after 24, 48, or 96 hrs in DM (-RT: non-reverse transcribed control). (B) Expression of Ca₄β₁₄ protein in C2C12 myoblasts and primary MPCs detected by western blot using antibody clones H-50 and C1C3. A distinct band was detected (arrowheads) with a molecular weight of approximately 55 kDa. (C) Western blot for Ca₄β₁₄ expression in C2C12 stably transfected with scrambled control or Ca₄β₁₄ specific shRNA. Brain and heart protein lysates were also run as negative controls. (D-E) C2C12 myoblasts were grown to confluence in GM, then switched to DM for analysis at 24 hr intervals. (D) Western blot for Ca₄β₁₄ and Ca₄.1.1 in cytosolic and membrane fractions. Troponin T is a marker of myogenic differentiation and Ponceau S stain shows equal loading. (E) Immunofluorescent staining for endogenous Ca₄β₁₄ (green) and DNA (Hoechst stain, blue). Images taken at 100x objective, scale bar = 100μm.
Figure 2. Ca\(_{\beta 1a}\)-YFP and endogenous Ca\(_{\beta 1a}\) translocate to the nucleus of myoblasts. (A) C2C12 myoblasts transfected with Ca\(_{\beta 1a}\)-YFP, and (B) following treatment with LMB. (C) Detection and immunoprecipitation of Ca\(_{\beta 1a}\)-YFP in the nuclear fraction of untreated and LMB treated C2C12 myoblasts by western blot. (D) Western blot for endogenous Ca\(_{\beta 1a}\) in C2C12 myoblasts. Cytosolic and nuclear fractions of C2C12 myoblasts treated with LMB for 0, 6, and 12 hrs. (E) Comparison of Ca\(_{\beta 1a}\) protein levels in cytoplasmic and nuclear fractions in myoblasts vs. myotubes. Tubulin and GAPDH are cytosolic markers, and HP1 and H3 are nuclear proteins. Figures are representative of at least 2 independent experiments. Images taken at 20x objective, scale bar = 100 µm.
Figure 3. Mapping of the \(\text{Ca}_\text{V}\beta_1\alpha\) nuclear localization domain. A) Diagram of constructed \(\text{Ca}_\text{V}\beta_1\alpha\)-YFP truncation mutants and respective cytoplasmic and nuclear intensity in untreated and LMB treated cells. Conserved SH3 and GK domains are noted in dark blue, putative NLS highlighted in purple, and YFP sequence in green. Construct names indicate amino acid positions of truncation, with \(\text{Ca}_\text{V}\beta_1\alpha\)1-524 as full length \(\text{Ca}_\text{V}\beta_1\alpha\). Table reflects relative intensity of cytoplasmic (Cyto) and nuclear \(\text{Ca}_\text{V}\beta_1\alpha\) B) Enlarged image of \(\text{Ca}_\text{V}\beta_1\alpha\)161-524, which is absent from the nucleus after LMB treatment. Enlarged image of \(\text{Ca}_\text{V}\beta_1\alpha\)101-274, which is present in the nucleus without LMB treatment. Images taken at 20x objective.

* Predicted molecular weight < limit for passive nuclear entry
Figure 4. Regulation of myoblast proliferation by Ca,β1a in vitro and in vivo. (A) Quantification of myoblast growth for 7 days after transfection with either scrambled control shRNA or Ca,β1a-targeted shRNA (western blot of Ca,β1a knockdown is inset). (B) Quantification of MPCs cultured from Cacnb1+/− and Cacnb1−/− embryos for 4 days. (C–E) Primary mouse myoblasts transfected with EGFP (C) or Ca,β1a-YFP (D) and stained 24 hrs later for Ki67 (red) (E) Quantification of Ki67+/EGFP and Ki67+/Ca,β1a-YFP cells expressed as a percentage of total EGFP or Ca,β1a-YFP + cells. (F–H) H&E staining of early muscle bundles in E13.5 Cacnb1+/+ (F) and Cacnb1−/− (G) embryos.
(N=3). Eosin positive bundles were traced, averaged and normalized to overall cross section size (H). (I-J) Staining for the myoblast marker Pax7 (green) in 10μm transverse sections of hindlimbs from E13.5 Cacnb1+/+ (I) and Cacnb1−/− (J) embryos (N=3). (K) Quantification of (I) & (J). Data are ± SEM, *p < 0.05. Images taken at 20x objective, scale bar = 100 μm.
Figure 5. ChIP on chip analysis of Ca\textsubscript{\(\beta\)}\textsubscript{1a}. (A) Histogram of Ca\textsubscript{\(\beta\)}\textsubscript{1a} binding distance from transcription start site. (B) Distribution of features of each Ca\textsubscript{\(\beta\)}\textsubscript{1a} peak relative to overlapping or nearest genes. (C) Consensus Ca\textsubscript{\(\beta\)}\textsubscript{1a} DNA binding motif. (D) Functional
annotation of genes bound by \( \text{Ca}_\gamma\beta_{1\alpha} \). Top 20 categories are shown. (E) Representative log2 (\( \text{Ca}_\gamma\beta_{1\alpha}/\text{IgG} \)) binding peaks on genes of interest in UCSC genome browser. Orange peaks indicate positive log2 \( \text{Ca}_\gamma\beta_{1\alpha}/\text{IgG} \) values and presumed sites of \( \text{Ca}_\gamma\beta_{1\alpha} \) chromatin binding, while blue indicates negative enrichment. (F-G) Validation of ChIP-chip identified target genes by chromatin immunoprecipitation using a GFP antibody in control and \( \text{Ca}_\gamma\beta_{1\alpha}-\text{YFP} \) transfected C2C12 myoblasts. # indicates separate primer pairs used to test multiple sites on each promoter region. *indicates negative controls. Immunoprecipitated DNA intensity was normalized to input for control and \( \text{Ca}_\gamma\beta_{1\alpha}-\text{YFP} \) (G).
Figure 6. Microarray analysis of *Cacnb1* wild type (+/+), heterozygous (+/-) and knockout (-/-) MPCs. Genes were selected based on dose-dependent correlation with *Cacnb1* expression. Genes said to be up-regulated by *Cacnb1* are lowest in -/- cells and functionally annotated in (A), while genes said to be down-regulated by *Cacnb1* are highest in -/- cells and functionally annotated in (B). GOTERM “other” (1640 for A and 234 for B) was omitted from charts in order to improve visibility of other categories. Genes of interest involved in cell cycle and muscle development are listed below pie charts. See also Figure S3 and Table S4.
**Figure 7.** *Cacnb1* modulates *Myog* expression in muscle progenitors. Representative images from *Cacnb1*<sup>+/+</sup> (A) and *Cacnb1*<sup>−/−</sup> (B) E18.5 hindlimb explants cultures after 3
days in vitro. Cultures were then fixed and stained for myogenin (C) (n=5 and 3) or analyzed by quantitative PCR (n=3 each, note that Cacnb1+/+ and Cacnb1+/− are pooled) (D). (E) Quantification of myogenin positive cells in control and Ca,β1a-shRNA treated primary MPC cultures (n=3 each). (F) qPCR for Myog mRNA from hind limb buds dissected from Cacnb1+/+ and Cacnb1+/− E11.5 embryos (n=4 each). (G) Quantification of myogenin expression in differentiating (1d DM) C2C12 myoblasts, transfected with full length, nuclear (101-274), or cytoplasmic (161-524) Ca,β1a-YFP constructs (n=6 each). (H) Myog-luc expression in control and Ca,β1a-shRNA transfected C2C12 myoblasts. (I) Myog-luc expression in GFP or Ca,β1a-YFP expressing myoblasts (n=5) and myotubes (n=6). (J) ChiP-qPCR showing relative fold enrichment of Ca,β1a-YFP pulldown of different regions of the Myog promoter. d5′ = distal 5’ prime region; Pro = promoter; CR = mid sequence coding region, d3′ = distal 3’ region. (K) Gel shift assay using GFP protein (control) or Ca,β1a-YFP protein from Cos7 nuclear extracts. 200x comp is cold competitor, Mouse IgG is non-specific antibody. A specific shift can be seen in lanes 2 and 8, and supershift induced by YFP antibody seen in lanes 5 and 11. Images taken at 20x objective, scale bar = 100 μm. Data are ± SEM, *p < 0.05.
Supplemental Figures

Supplemental Figure 1

Figure S1. Representative images of Ca\(_{\beta 1a}\) –YFP mutants with and without LMB treatment. Images taken at 20x objective.
Figure S2. shRNA-mediated knockdown of Caβ1a impairs myoblast proliferation. C2C12 myoblasts were transfected with pLKO.1 (vector) or Caβ1a sequence-specific shRNA, and selected with puromycin. Individual clones were isolated and expanded (c1-c3), and compared to a vector-transfected pool of cells to control for freezing and extended passaging. Caβ1a knockdown was evaluated by western blot and the actin-normalized Caβ1a intensity is presented in A.U. (average of 2 experiments). The proliferation rate of each group was evaluated by plating cells at equal densities and quantifying the number of cells per mm² using Metamorph counting software (the average of 10 fields per dish, n=3 per group). Data represent mean ± SE (*p≤0.05 compared to vector pool). Related to Figure 4 of the main text.
Figure S3. FACS of muscle precursor cells from embryos. (A) Gating strategy for α7 integrin-APC, CD45-FITC, CD31-FITC labeled mononucleated cells isolated from skeletal muscle. Gate was set to isolate the α7 integrin-APC+, CD45-FITC−, CD31-FITC− population. (B) Pax7 staining (red) of sorted cells immediately after FACS. Approximately 50% were Pax7+. (C) and (D) after several weeks in culture, >95% of cells were myoD (C) or Pax7 (D) positive. Related to Figures 1, 4, & 6 of the main text.
Supplementary Figure 4

Figure S4. Analysis of cell death in Ca,β1a knockdown and Cacnb1 +/- cells. (A) Control and Ca,β1a shRNA treated primary myoblasts were stained with AnnexinV-FITC and quantified by fluorescent microscopy (n=3 each). (B) Cells were isolated from hindlimbs of E12.5 Cacnb1 +/- (n=4) and Cacnb1 -/- (n=3) mice by enzymatic digestion, pre-plated for 1 hour on plastic, stained for AnnexinV-FITC and 7AAD, and analyzed by flow cyometry. AnnexinV is a marker of apoptosis, while 7AAD is a marker of general cell death.
Figure S5. Extracted patterns of gene expression from *Cacnb1* +/-, +/-, and +/- MPCs. (A) Patterns of differentially regulated genes in *Cacnb1* +/-, and -/- MPCs, compared to *Cacnb1* +/+ (Red). (B) Heat map of all 9 patterns, green color indicates down regulation compared to control (*Cacnb1* +/+ ) while red indicates up-regulation. Horizontal lines are representative of individual genes. Related to Figure 6 of the main text and Table S4 of the supplementary.
**Figure S6.** Screening for Cavβ1α binding partners at the Myog promoter. (A) Western blot analysis of Lef1 protein in proliferating myoblasts (MB) and 1, 2, and 4 day differentiated myotubes. (B) Pbx, MyoD, or Mef2 are all expressed in proliferating myoblasts, but do not co-immunoprecipitate with Cavβ1α-YFP. All blots are from the same membrane. Cavβ1α-YFP is specifically precipitated by YFP antibody, but not non-specific control IgG (top row). (C) Immunfluorescent images of MyoD (top) and Mef2 (bottom) localization (red) in Cavβ1α-YFP cells. Arrow points to Cavβ1α-YFP expressing cell, arrow head points to un-transfected cell. (D) Relative inhibition by Cavβ1α-YFP, compared to GFP transected control cells, of wild type and E-box mutant (Δ) Myog–luc reporter gene activity. N=4. Data represent mean ± SE (*p≤0.01).
Figure S7. Mechanism of CaVβ1a regulation of myogenesis. In Cacnb1+/+ MPCs, CaVβ1a enters the nucleus to suppress the Myog promoter, allowing Pax7+ MPCs to proliferate in sufficient quantity before entering terminal differentiation and fusing into myotubes. In Cacnb1+/− MPCs, Myog is not suppressed, causing precocious differentiation and underdeveloped skeletal muscle at later time points.
List of Supplemental Tables

Table S1. Inventory of primers used throughout the paper for various functions (RT-PCR, cloning, shRNA, etc).

Table S2. Inventory of antibodies used throughout the paper for various experiments (western blot, immunocytochemistry, immunohistochemistry, ChIP). Dilutions are included.

Table S3. List of buffers used in ChIP.

Table S4. Complete list of genes with promoter regions found to be enriched for Ca,β1a binding. Related to Figure 5 of the main text.

Table S5. Complete list of genes found to be differentially regulated by microarray analysis, between our three experimental groups. Genes are subdivided into the 9 different patterns shown in Figure S3. Related to Figure 6 of main text.

Table S6. List of genes genes with promoter regions found to be ChIP enriched for Ca,β1a binding and also showing differential regulation in expression by microarray analysis. Subdivided into ChIP enriched genes that are upregulated in cacnb1 +/- (left hand column) and ChIP enriched genes that are downregulated in cacnb1 +/- (right hand column).
Chapter 3

A proteomics based screening for nuclear binding partners of Ca\(_v\beta_1\alpha\) in muscle progenitor cells

Jackson Taylor\(^{1,2}\), Tan Zhang\(^1\), Jiang Qian\(^3\), Cristin Furdui\(^3\), Claudia Hereñú\(^4\), and Osvaldo Delbono\(^*\),\(^{1,2}\)

Department of Internal Medicine-Gerontology\(^1\), Neuroscience Program\(^2\), Molecular Medicine Program\(^3\)

Wake Forest School of Medicine, Winston-Salem, NC, 27157, USA\(^{1,2}\)

Department of Histology, National University of La Plata, La Plata, Argentina\(^4\)

This work is currently in preparation for submission (journal yet to be determined).
Introduction

Voltage gated calcium channel (Caᵥ) β subunits are part of the membrane-associated guanylate kinase (MAGUK) family of proteins (Hanlon et al., 1999) and are well known for their essential role in Caᵥ regulation (Buraei and Yang, 2010). Caᵥβ subunits enhance Caᵥ surface expression, and modulate their voltage-dependence and activation and inactivation kinetics (reviewed in Birnbaumer et al., 1998; Buraei and Yang, 2010). Like all MAGUK proteins, Caᵥβ subunits contain Src3 homology (SH3) and guanylate kinase (GK) domains, which endow them with many of their functional properties. Both domains can act as protein interaction modules, so it is not surprising then that Caᵥβ subunits have recently been found to interact with over a dozen additional proteins besides Caᵥ1 and Caᵥ2 (Beguin et al., 2001; Catalucci et al., 2009; Finlin et al., 2003; Gonzalez-Gutierrez et al., 2007; Grueter et al., 2006; Hibino et al., 2003a; Hohaus et al., 2002; Kiyonaka et al., 2007; Vendel et al., 2006; Yu et al., 2008; Zhang et al., 2010; Zou et al., 2008).

The appearance of Caᵥβ subunits in the nucleus (Beguin et al., 2006; Colecraft et al., 2002; Hibino et al., 2003a; Zhang et al., 2010) is especially curious, where their function is largely unknown. Caᵥβ subunits do not possess a classical nuclear localization sequence (Subramanyam et al., 2009), so it stands to reason that they enter the nucleus via interaction with one or more additional proteins which do possess such a sequence. This idea is supported by the fact that several proteins have been found to interact with Caᵥβ subunits and localize them to the nucleus (Beguin et al., 2006; Hibino et al., 2003a;
Zhang et al., 2010). However, there is very little consistency amongst individual Ca,β isoforms, their binding partners, and their behavior inside the nucleus of various cell types. Yet all Ca,β isoforms seem to be capable of entering the nucleus, pointing to some type of highly conserved mechanism. The fact that Ca,β nuclear localization occurs in many different cell types suggests an intrinsic mechanism that does not rely on binding to specific transcription factors, which are often expressed only in certain cell types. Regardless of whether or not they are transported by other nuclear proteins, identifying additional proteins which interact with Ca,β inside the nucleus will yield important insight on Ca,β nuclear function.

We sought to better understand Ca,β nuclear localization by using immunoprecipitation (IP) and mass spectrometry (MS) to identify Ca,β1a nuclear binding partners. Although the yeast two-hybrid method has been used successfully by other groups to identify novel binding partner of Ca,β subunits (Hibino et al., 2003a; Tadmouri et al., 2012; Zhang et al., 2010), this method does not discriminate between different subcellular compartments. Because Ca,β subunits interact with many cytoplasmic proteins, it is important to avoid these proteins during the screening. We recently found that Ca,β1a is expressed in muscle progenitor cells (MPCs), where it enters the nucleus and regulates gene expression (Taylor et al., 2012). Additionally, because MPCs do not express Ca,αs, we decided they would be a particularly good model to screen for Ca,β nuclear interactions, as there would be less non-nuclear “bait” for Ca,β subunits within the cell.
Results:
All of our interaction assays followed the same basic model: an adenovirus was used to
overexpress a yellow fluorescent protein (YFP) – tagged Ca_\(\text{v}_1\)\(\beta1\)a proteins (Ca_\(\text{v}_1\)\(\beta1\)-YFP) in
C2C12 myoblasts (an MPC cell line), which was then IP’d using a highly specific
GFP/YFP antibody (or a non-immunized immunoglobulin (IgG) as a control). This
method produced a highly enriched and specific pull-down of Ca_\(\text{v}_1\)\(\beta1\)-YFP, both from
whole cell lysates and purified nuclear extracts (see materials and methods and Figure
2a).

In order to identify a mechanism for Ca_\(\text{v}_1\)\(\beta1\)a nuclear localization in MPCs, we first tested
whether Ca_\(\text{v}_1\)\(\beta1\)a interacted with two proteins, Rem and HP1\(\gamma\), previously reported to
interact with other Ca_\(\text{v}_1\)\(\beta\) subunits in the nucleus of other cell types. Rem is an RGK
protein (\(\text{Ras, Rad, Gem, Kir}\)) reportedly expressed in C2C12 myoblasts (Finlin et al.,
2003), and found to re-localize Ca_\(\text{v}_1\)\(\beta3\) to the nucleus of HEK 293 cells (Beguin et al.,
2006). All RGK proteins interact with Ca_\(\text{v}_1\)\(\beta1\)-3 subunits in a GTP-dependent manner
(Beguin et al., 2001), and so this was considered when testing the interaction by the
addition of GDP\(\beta\)S (negative control), or GTP\(\gamma\)S (stable analog of GTP). We were able to
confirm Rem’s expression in C2C12 myoblasts, however no Rem protein was co-
precipitated with Ca_\(\text{v}_1\)\(\beta1\)-YFP, even in the presence of GTP\(\gamma\)S (Figure 1a). Although we
could not distinguish between HP1 \(\alpha/\beta/\gamma\) isoforms, at least one isoform of HP1 protein of
approximately 20-25 kDa was abundant in nuclear fraction of myoblasts as measured by
a pan-HP1 antibody. However none of this protein was co-precipitated with Ca_\(\text{v}_1\)\(\beta1\)-YFP
from the nuclear fraction (Figure 1b).
Because we could not detect an interaction with either of the previously reported Ca,β subunit - nuclear interacting partners, we decided to pursue a broad, proteomics-based screening approach (Figure 2a), based on a modified paradigm described above. Ca,β1α-YFP was first enriched in the nucleus by the CRM1 nuclear export channel-blocking drug Leptomycin B (LMB). Cells where then fractionated into cytosolic and nuclear fractions, and the purity of nuclear fractions was validated by the absence of tubulin and GAPDH and the abundance of HP1 (in this case used as an established nucleus specific marker). The nuclei were then lysed and IP’d as described above, only that the unbound fractions were swapped between GFP/YFP antibody conjugated beads and IgG conjugated beads (Figure 2a) in order to maximize the amount of Ca,β1α-YFP IP’d. This technique enabled us to gather the extra Ca,β1α-YFP leftover from the control IgG beads, thus providing a bigger pool of the protein from which to locate novel binding partners. The beads were then washed extensively and bound proteins were eluted and separated by SDS-PAGE. Coomassie blue staining revealed similar overall protein profiles between IgG and GFP/YFP IP groups, although a distinct band of approximately 80 kDa corresponding to Ca,β1α-YFP was visible only in the GFP/YFP lane (Figure 2b). The bands from the GFP/YFP lane were excised and analyzed by M.S. (with the exception of the large 50 kDa band corresponding to IgG heavy chain left over from the antibodies). A total of 83 unique proteins were identified from the gel pieces, which are grouped by functional annotation in Figure 3a (entire list available in Supplementary Table 1). Importantly, a number of peptide fragments corresponding to Ca,β subunits were identified in the mid-range molecular weight pieces (60-100 kDa), verifying the strong enrichment of Ca,β1α-
YFP. A number of mitochondrial proteins were also found, suggesting mitochondrial contamination of the nuclear fractions (confirmed in Figure S2).

Many of the proteins co-precipitated with Ca$_v$β$_{1a}$-YFP were structural, such as nuclear envelope and cytoskeletal proteins. This is likely due to a high abundance of these proteins in the nucleus leading to their non-specific co-purification with Ca$_v$β$_{1a}$-YFP. For secondary validation, we focused on proteins known to be localized in the nucleus and involved in transcription in some way. Histone H1, actin, and Lamin B were all tested, but could not be validated by standard co-immunoprecipitation (Figure 3b). A particularly interesting candidate was Lef1, which is a transcription factor involved in the Wnt signaling pathway. Not only was Lef1 the only transcription factor found to co-purify with Ca$_v$β$_{1a}$-YFP, but a recent study from our lab found that Ca$_v$β$_{1a}$ binds to a consensus Lef1 binding motif (TTTGAT) (Taylor et al., 2012). Initial attempts to verify the Ca$_v$β$_{1a}$-YFP – Lef1 interaction in C2C12 myoblasts failed to detect any Lef1 in the I.P.’d fraction with Ca$_v$β$_{1a}$-YFP; however Lef1 also could not be detected in the input (Figure 3b). Further exploration revealed that Lef1 protein is not expressed at detectable levels in myoblasts, however its expression immediately increases after 24 hours in differentiation medium, in both the nucleus and cytoplasm, with the expression level dropping back down as differentiation continued (Figure S3). In lysates from myoblasts differentiated for 24 hours, Ca$_v$β$_{1a}$-YFP still did not co-precipitate Lef1 (Figure 4a).

Based on its dynamic expression during myogenic differentiation, we hypothesized that Lef1 may be regulated by proteosomal degradation in Ca$_v$β$_{1a}$ dependent manner. To test this hypothesis, we treated proliferating myoblasts and 24 hour-differentiated myoblasts with the proteosome inhibitor MG132 (Rock et al., 1994), to see if this enhanced Lef1
expression and facilitated co-precipitation of Lef1 with Ca$_v$β$_{1a}$-YFP. IP with a YFP antibody successfully pulled down Ca$_v$β$_{1a}$-YFP protein as expected. Interestingly MG132 seemed to enhance Ca$_v$β$_{1a}$-YFP protein levels; however no Lef1 protein could be detected (Figure 4b). Conversely, IP with a Lef1 antibody did not pull down of Ca$_v$β$_{1a}$-YFP (Figure 4b). In this particular assay, Lef1 was also undetectable in the input fractions suggesting some other technical problem, likely with our Lef1 antibody (Figure 4b). Although not entirely conclusive, overall the evidence suggests that Ca$_v$β$_{1a}$ and Lef1 do no interact, or interact in a very unstable and transient manner.

Discussion:
We sought to identify nuclear binding partners of Ca$_v$β$_{1a}$ in myoblasts, both to understand this proteins tissue specific function in the nucleus (Taylor et al., 2012), and also to gain insight into nuclear localization mechanisms and functions shared by all Ca$_v$β isoforms. Using a combination of fluorescent labeling, adenoviral-mediated overexpression, pharmacological nuclear enrichment, sub-cellular fractionation, and immunoprecipitation, we were able to purify large quantities of Ca$_v$β$_{1a}$ from the nuclei of myoblasts and identify co-precipitated proteins by mass spectrometry. Overall the technique was successful in principle, and provided several intriguing and novel candidates as Ca$_v$β$_{1a}$ nuclear binding partners. However, technical issues also limited the effectiveness of this method, which was underscored by the failure to validate any of the candidate proteins identified. Still, it seems possible to circumvent these issues, and thus the overall approach may still be useful for future attempts at identifying Ca$_v$β$_{1a}$ (or other Ca$_v$β subunit) nuclear binding partners.

**Technical problems and workarounds**
When examined by immunolabeling (western blot for Ca,β1a-YFP), the YFP antibody seemed to be highly specific compared to non-immunized IgG control (Figure 1, Figure 2a). However, coomassie blue staining revealed similar overall protein bands from both IgG and YFP antibodies (Figure 2b). Ideally, the IgG antibody should not bind to any protein, and thus it appears both antibodies have a high capacity for non-specific binding.

The lysates were pre-cleared with protein A/G beads before immunoprecipitation, suggesting this is not an effect of the beads but rather the antibodies. A possible solution may be pre-clearing the lysate with several non-immunized IgG proteins conjugated to beads, before proceeding with the actual I.P. Another obvious source of contamination in both groups was the presence of IgG heavy (50 kDa) and light (25 kDa) chains – remnants of the IgG and YFP antibodies after the sample was denatured. In addition, the A/G protein from the beads also appears to contaminate the sample after denaturing (Figure S4). Cross-linking the antibody to the beads presents a solution to both problems, whereby the protein (in this case Ca,β1a-YFP) can be eluted without disturbing the IgG or protein A/G complex, and then separated. Preliminary data suggests the method will drastically reduce IgG heavy/light chain and protein A/G contamination (Figure S5), while still allowing ample IP and elution of Ca,β1a-YFP. Alternatively, magnetic beads already crosslinked to GFP antibodies are commercially available and present another effective way to purify Ca,β1a-YFP, while avoiding antibody and bead contamination.

The abundance of mitochondrial proteins following MS analysis of our supposed nuclear fractions was striking. This suggests a rather large contamination of mitochondria into our nuclear fractions, which we confirmed by western blotting for the mitochondrial specific protein VDAC1/porin (Figure S2). It appears that more traditional methods of
nuclear isolation based on ultracentrifugation in a high density sucrose gradient may reduce or avoid this contamination. Our initial attempts using this method did indeed substantially reduce mitochondrial contamination. However, Ca₅β₁a-YFP was also almost non-existent from nuclei isolated this way (data not shown), suggesting that either the ultracentrifugation process, or the longer time needed for this method in some way expels the protein from the nucleus. Specially formulated buffers, designed to lyse mitochondria while keeping nuclei intact, may present another option to remove mitochondrial contamination while maintaining our original nuclear isolation protocol.

**Future directions**

The lack of evidence for a conserved nuclear chaperone could mean that Ca₅β subunits possess a noncanonical NLS, which has yet to be identified. Still, due to their SH3 and GK interaction modules and abundance of known interacting partners outside the nucleus, it is highly unlikely that Ca₅β subunits act in isolation inside the nucleus.

Ca₅β₁a was recently found by our laboratory to bind to several DNA motifs (Taylor et al., 2012), as assessed by DNA microarrays. These oligonucleotides present an alternative method to purify Ca₅β₁a from the nucleus, specifically in the context of chromatin binding. A technique demonstrated by the Mann laboratory uses streptavidin conjugated beads to pull down biotinylated oligonucleotide sequences and their associated proteins, which can then be analyzed by MS (Mittler et al., 2009). Using this method, Ca₅β₁a DNA binding motifs could be biotinylated and highly purified with their associated protein complexes. This method would serve as an excellent secondary validation that the Ca₅β₁a
protein indeed binds to these sequences, and also offers a way to screen specifically for other proteins found in complex with Ca,β1a while bound to DNA.

Although we could not verify its interaction with Ca,β1a, Lef1 remains a potential binding partner for Ca,β1a. Besides detection in the MS assay, our recent publication shows that Ca,β1a binds to DNA sequences which match the Lef1 consensus binding motif (Taylor et al., 2012), suggesting Ca,β1a may interact with Lef1 at this sequence in vivo. In addition, our novel finding that Lef1 is dynamically regulated during myogenesis suggests its importance in this process, as our recent research also suggests for Ca,β1a (Taylor et al., 2012). Our attempts to verify Ca,β1a – Lef1 interaction were thorough, but not exhaustive. Many protein-protein interactions are only verified by overexpressing tagged versions of both proteins in cell lines permissive to high protein expression. Thus experiments to see if overexpressed and tagged Lef1 co-precipitate with Ca,β1a-YFP should also be performed. Since Lef1 could not be detected in myoblasts by western blot, the question remains whether Lef1 is completely silent in these undifferentiated cells, or is expressed but rapidly degraded, and if Ca,β1a interaction plays any role in this. We initially detected Lef1 in a screen using nuclear lysates from undifferentiated myoblasts; however it is possible that what was detected came from a small subset of precociously differentiating cells, a fairly common occurrence in culture. Our attempt to see if MG132 treatment enhanced Lef1 expression in myoblasts suffered from technical issues and needs to be repeated. PCR assays could also be performed to see if Lef1 mRNA is expressed in myoblasts. If Lef1 is completely silent in proliferating myoblasts, then we can effectively dismiss its role in Ca,β1a nuclear localization and function in these cells, though it may still play some important function after the onset of differentiation.
Materials and Methods:

**Cell culture:** C2C12 myoblasts (ATCC) were cultured in Dulbecco’s Modified Essential Medium (DMEM) with 10% fetal bovine serum. For differentiation procedure, myoblasts were allowed to reach 90% confluence and then switched to DMEM with 2% horse serum. LMB (LC Labs) was diluted in ethanol and added directly to culture medium at a final concentration of 20 nM for 3 hours. Adenovirus was added directly to culture medium at a final concentration of $2 \times 10^{13}$ plaque forming units for 24 hours. MG132 (Sigma) was added directly to culture medium at a final concentration of 10 μM for 12 hours.

**Construction of recombinant adenoviral vector RAd-Ca$_v$β$_{1a}$-YFP:** cDNA for Ca$_v$β$_{1a}$-EYFP (Genbank accession number: M25514.1, generously donated by Dr. K. Beam, Colorado State University (Leuranguer et al., 2006)) was inserted into a RAd vector by a variant of the two plasmid method (Hitt et al., 1998) using the AdMax® plasmid kit (Microbix, Ontario, Canada). RAd-GFP control was purchased from the UNC Vector Core Facility (University of North Carolina at Chapel Hill).

**Cell Fractionation:** Nuclear fractions were isolated as previously described (Washabaugh et al., 2007). Briefly, cells were harvested and resuspended in Ontell buffer (Washabaugh et al., 2007), homogenized with a handheld glass homogenizer, and centrifuged at 2000 x g for 10 minutes. Supernatant was saved as cytosolic fraction. The pellet was washed twice in Ontell buffer, then resuspended in 0.6 M KCl buffer (Horovitz-Fried et al., 2008). Debris was pelleted and supernatant was taken as nuclear fraction. Chromatin was sheered using a handheld glass homogenizer. For mass
spectrometry experiments, 10 x 150mm dishes of 90% confluent myoblasts were used for each group, yielding approximately 500mg of cells for starting material. Whole cell lysates were extracted in I.P. lysis buffer (see below). Isolation of skeletal muscle and myoblast nuclei by sucrose gradient centrifugation was performed as previous described (Edelman et al., 1965).

**Immunoprecipitation:** Protein was immunoprecipitated according to (Beguin et al., 2001; Zhang et al., 2009). Protein A/G+ beads (Santa Cruz) were conjugated to mouse anti-GFP/YFP (Clone 3E6, Invitrogen) or mouse IgG overnight before incubation with cell lysates. Cell lysates were pre-cleared with A/G+ beads before incubation with antibody conjugated beads overnight in I.P. lysis buffer (50 mM Tris-HCl (pH 7.5),100 mM NaCl,10 mM MgCl2, 1mM DTT, 0.15% Triton X100). The optimal ratio of beads to YFP antibody was determined to be 40ul:20ul (2ug) / 100ug of protein (Figure S1). Beads were washed extensively in lysis buffer containing 0/015% TritonX100, before protein was eluted in Laemmli buffer (Laemmli, 1970) at 95oC. For Rem IP experiments, 10 μM GDPβS (negative control) or GTPγS, were added immediately following cell lysis.

**SDS-PAGE:** Samples were separated by SDS-PAGE in 10% polyacrylamide gels as described (Taylor et al., 2009) with all samples in Laemmli buffer (Laemmli, 1970). For mass spectrometry experiments, special care was taken to avoid keratin contamination. Gels were fixed in 45% methanol/ 10% glacial acetic acid solution immediately following SDS-PAGE.
Western blotting and antibodies: Western blotting was performed according to standard techniques. Briefly, proteins were transferred to PVDF membranes, blocked in 5% non-fat dry milk in TBS + 0.2% Tween20 (TBST), and incubated in primary antibodies overnight at 4°C in blocking solution. Non-immunized rabbit and mouse IgGs and Ca,β1a (clone H50) antibodies were purchased from Sigma. Mouse anti-GFP/YFP antibody (clone 3E6) was purchased from Invitrogen. Rabbit anti-Lef1 was purchased from Cell Signaling.

Mass Spectrometry: To visualize protein bands, gels were stained in 3% Coomassie Brilliant Blue R250 solution (Sigma). Gel pieces of 2-3mm in height were excised with a sterile razor blade on a light box, collected in individual eppendorf tubes, and digested in trypsin (100 ng/ul) using standard protocols (Aitken and Learmonth, 2002). Tryptic peptides were separated and analyzed by nano-LC (Dionex Ultimate 3000 System) coupled to a Thermo ESI LTQ mass spectrometer in data dependent mode. Peptides were identified with Bioworks 3.3 software. Functional annotation was performed by DAVID v 6.7 software (Huang et al., 2009).
Figure 1. Ca\textsubscript{v,1a}-YFP does not co-IP with either Rem or HP1 in C2C12 myoblasts.
(A) Ca\textsubscript{v,1a}-YFP was IP’d from myoblasts left either untreated, or treated with GDP\textsubscript{β}S (negative control), or GTP\textsubscript{γ}S (stable analog of GTP shown to be necessary for Ca\textsubscript{v,β}-RGK interactions). Rem was easily detected in input fractions (right), but was absent from IP fractions (left). (B) Similarly, HP1 protein was abundant in myoblast nuclear fractions (left), but did not co-purify with Ca\textsubscript{v,1a}-YFP (right) and did not decline in the YFP unbound fraction (middle).
Figure 2.  

**Figure 2. Ca,β1a-YFP nuclear purification for MS** (A) Experimental design and validation for Ca,β1a-YFP nuclear enrichment, purification, and immunoprecipitation. LMB was used to concentrate Ca,β1a-YFP in the nucleus of C2C12 myoblasts, which were fractionated into cytosolic and nuclear fractions. Nuclear fraction purity was confirmed by absence of tubulin and presence of HP1. Pre-clearing with protein A/G beads did not remove Ca,β1a-YFP from the nuclear fraction, while YFP antibody specifically pulled down all Ca,β1a-YFP. When loaded directly, the bead-antibody complexes retained some contamination (HP1 and Ca,β1a-YFP bound to IgG), but after washing the final YFP antibody eluted fraction was specifically enriched for Ca,β1a-YFP. (B) Coomassie blue staining of IgG and YFP eluted proteins (far right group in A) from myoblast nuclear fractions. Arrow points to ≈80 kDa band presumably corresponding to Ca,β1a-YFP. BSA (67 kDa) shows the sensitivity of protein band detection to be ≈100 ng. IgG heavy and light chain contamination from both antibodies is visible at ≈50 kDa and ≈25 kDa, respectively. YFP lane was used for MS analysis.
Figure 3

Figure 3. Categorization and validation of Ca,β1a-YFP nuclear binding partners identified by MS (A) Functional annotation of all proteins identified by MS “Cell death” category mostly corresponds to mitochondrial proteins. (B) Standard co-IP of Lef1, Actin, Histone H1, and Lamin B, which were identified by MS analysis, but failed to co-IP with Ca,β1a-YFP. Lef1 also did not appear in the input of myoblasts.
Figure 4. Endogenous Lef1 does not co-IP with Ca,β1a-YFP. (A) C2C12 cells overexpressing Ca,β1a-YFP were differentiated for 24 hours before fractionation into cytosolic and nuclear fractions and standard co-IP analysis. Lef1 could be detected in the cytosolic input but not nuclear, but did not appear to co-IP with Ca,β1a-YFP. (B) IP with a Lef1 antibody was performed on cell extracts from four groups indicated (top). Western blotting was unable to detect Lef1 protein in any case. The IP was performed using YFP antibody in the same groups (bottom). Ca,β1a-YFP protein was easily detected and IP’d, while Lef1 was not.
Supplementary Figure 1

Supplementary Figure 1. Empirical determination of optimal YFP antibody : bead ratio and amount for Ca,β1a-YFP pull down.
Supplementary Figure 2. Mitochondrial contamination in nuclear fractions. KCl nuclear extraction used for MS screening shows a high level of the mitochondrial protein VDAC/porin (far right) compared to other, sucrose gradient ultra-centrifugation based methods (lanes 2,3,4). Nuclear fraction in lane 2 has substantially more protein loaded than the cytosolic fraction in lane 1 (data not shown), suggesting a very low relative level of mitochondrial contamination.
Supplementary Figure 3. Lef1 protein is dynamically expressed during myogenesis in C2C12 myoblasts. C2C12 cells were collected as either sub-confluent, proliferating myoblasts (MB), or after 1, 2, or 4 days in differentiation medium (DM), and fractionated into cytosolic and nuclear fractions. Lef1 protein was absent in myoblasts, but showed strong expression after 1 day in DM, especially in the cytosol.
Supplementary Figure 4

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/G beads loaded directly with or without any denaturing buffer or heat</td>
</tr>
<tr>
<td>2</td>
<td>A/G beads loaded with loading buffer and heat</td>
</tr>
<tr>
<td>3</td>
<td>Sepharose beads loaded with loading buffer and heat</td>
</tr>
<tr>
<td>4</td>
<td>Sepharose beads filtered flow through</td>
</tr>
<tr>
<td>5</td>
<td>Sepharose beads loaded buffer + 35°C</td>
</tr>
<tr>
<td>6</td>
<td>Sepharose beads loaded buffer + 95°C</td>
</tr>
<tr>
<td>7</td>
<td>Mouse IgG (50 and 25 kDa, respectively)</td>
</tr>
<tr>
<td>8</td>
<td>Loading buffer</td>
</tr>
</tbody>
</table>

Supplementary Figure 4. Protein A/G and B contamination from commercially available agarose and sepharose beads used for IP. Lanes 1 & 6: A/G beads loaded directly with or without any denaturing buffer or heat produced a substantially sized protein band corresponding to the expected molecular weight of protein A/G. Lane 2: elution with loading buffer and heat also resulted in protein A/G extraction, even after separation from the beads, while the beads still retained some of their dissociable protein as well (Lane 3). A similar but not as pronounced effect was seen with Sepharose beads containing protein A (Lanes 4&5). Mouse IgG produced expected heavy and light chain fragments (50 and 25 kDa, respectively, lane 7). Loading buffer alone did not house any contaminating proteins (Lane 8). Protein was visualized with Coomassie Brilliant Blue staining. BME = β-mercaptoethanol.
Supplementary Figure 5. Crosslinking A/G beads to antibody avoids IgG and protein A/G contamination while preserving Caβ1a-YFP pull down. (A) Western blot of IP fractions from C2C12 myoblasts expressing Caβ1a-YFP. IgG and YFP antibodies were crosslinked to A/G beads, used to IP Caβ1a-YFP, and eluted sequentially using 0.1M glycine, 1M glycine, and standard elution (Laemmli) buffer. The majority of Caβ1a-YFP protein (80 kDa) was eluted by 0.1M glycine. Staining with anti-mouse secondary antibody only detected mouse antibody fragments in the Laemmli buffer eluted fractions, suggesting glycine elution does not disrupt the IgG-bead crosslinking. (B) Silver staining of duplicate gel used for (A). Very little protein contamination, especially of protein A/G (lane 7) or IgG (lane 8) can be seen in the glycine elution groups, while a substantial amount is present in the Laemmli buffer (LB) lanes.
References:


Table List:

Table I. List of binding proteins identified by MS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Location</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLEC*</td>
<td>plectin</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>NES*</td>
<td>nestin</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>PLEC*</td>
<td>plectin</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>KRT8</td>
<td>keratin 8</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>NEFM</td>
<td>neurofilament, medium polypeptide</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>VIM*</td>
<td>vimentin</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>DES*</td>
<td>desmin</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>IMMT</td>
<td>inner membrane protein, mitochondrial</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>NDUFS1</td>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Location</td>
<td>Function</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>HSPA1L*</td>
<td>heat shock 70kDa protein 1-like</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>HSPA5</td>
<td>heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>HSPA8*</td>
<td>heat shock 70kDa protein 8</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>POR</td>
<td>P450 (cytochrome) oxidoreductase</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>HSPA9</td>
<td>heat shock 70kDa protein 9 (mortalin)</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>KRT1*</td>
<td>keratin 1</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>TOMM70A</td>
<td>translocase of outer mitochondrial membrane 70 homolog A (S. cerevisiae)</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>PABPC1</td>
<td>poly(A) binding protein, cytoplasmic 1</td>
<td>Cytoplasm</td>
<td>translation regulator</td>
</tr>
<tr>
<td>MAOA</td>
<td>monoamine oxidase A</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>CKAP4</td>
<td>cytoskeleton-associated protein 4</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>NEFL</td>
<td>neurofilament, light polypeptide</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>RPN1</td>
<td>ribophorin I</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>RPLP0 (includes EG:11837)*</td>
<td>ribosomal protein, large, P0</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td>Function</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------</td>
<td>------------------------</td>
</tr>
<tr>
<td>RPLP0*</td>
<td>ribosomal protein, large, P0</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>MOSC2</td>
<td>MOCO sulphurase C-terminal domain containing 2</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>VDAC1*</td>
<td>voltage-dependent anion channel 1</td>
<td>Cytoplasm</td>
<td>ion channel</td>
</tr>
<tr>
<td>SFXN3</td>
<td>sideroflexin 3</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>PHB2</td>
<td>prohibitin 2</td>
<td>Cytoplasm</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>VDAC2*</td>
<td>voltage-dependent anion channel 2</td>
<td>Cytoplasm</td>
<td>ion channel</td>
</tr>
<tr>
<td>CYB5R3*</td>
<td>cytochrome b5 reductase 3</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>RPS3*</td>
<td>ribosomal protein S3</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>RPL6</td>
<td>ribosomal protein L6</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>SLC25A3*</td>
<td>solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>SLC25A4</td>
<td>solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>ATP5C1</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, gamma</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td>Type</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>polypeptide 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDAC3</td>
<td>voltage-dependent anion channel 3</td>
<td>Cytoplasm</td>
<td>ion channel</td>
</tr>
<tr>
<td>RPS4X</td>
<td>ribosomal protein S4, X-linked</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>KRT14</td>
<td>keratin 14</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>GNB2L1</td>
<td>guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>RPL19*</td>
<td>ribosomal protein L19</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>SLC25A6</td>
<td>solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>CHCHD3*</td>
<td>coiled-coil-helix-coiled-coil-helix domain containing 3</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>RPL14</td>
<td>ribosomal protein L14</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>ATP5F1</td>
<td>ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>ATP5H (includes EG:10476)</td>
<td>ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d</td>
<td>Cytoplasm</td>
<td>transporter</td>
</tr>
<tr>
<td>RPL18</td>
<td>ribosomal protein L18</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>KRT10</td>
<td>keratin 10</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>RAB2A</td>
<td>RAB2A, member RAS oncogene family</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>CYB5B</td>
<td>cytochrome b5 type B (outer mitochondrial membrane)</td>
<td>Cytoplasm</td>
<td>enzyme</td>
</tr>
<tr>
<td>RPS9</td>
<td>ribosomal protein S9</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>RPL17</td>
<td>ribosomal protein L17</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>FGA</td>
<td>fibrinogen alpha chain</td>
<td>Extracellular Space</td>
<td>other</td>
</tr>
<tr>
<td>APOOL</td>
<td>apolipoprotein O-like</td>
<td>Extracellular Space</td>
<td>other</td>
</tr>
<tr>
<td>LEF1*</td>
<td>lymphoid enhancer-binding factor 1</td>
<td>Nucleus</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>SUN2</td>
<td>Sad1 and UNC84 domain containing 2</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>LMNA*</td>
<td>lamin A/C</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>LMNA*</td>
<td>lamin A/C</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>LMNA*</td>
<td>lamin A/C</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>LMNB2</td>
<td>lamin B2</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------</td>
<td>------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>LMNB1</td>
<td>lamin B1</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>NOP56</td>
<td>NOP56 ribonucleoprotein homolog (yeast)</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>NPM1</td>
<td>nucleophosmin 1</td>
<td>Nucleus</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>EMD</td>
<td>emerin</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>HIST1H1B*</td>
<td>histone cluster 1, H1b</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>HIST1H1C*</td>
<td>histone cluster 1, H1c</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>HIST1H1E</td>
<td>histone cluster 1, H1e</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>PHB</td>
<td>prohibitin</td>
<td>Nucleus</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>RPL10L</td>
<td>ribosomal protein L10-like</td>
<td>Nucleus</td>
<td>other</td>
</tr>
<tr>
<td>PRPH*</td>
<td>peripherin</td>
<td>Plasma Membrane</td>
<td>other</td>
</tr>
<tr>
<td>CACNB1*</td>
<td>calcium channel, voltage-dependent, beta 1 subunit</td>
<td>Plasma Membrane</td>
<td>ion channel</td>
</tr>
<tr>
<td>CACNB4</td>
<td>calcium channel, voltage-dependent, beta 4 subunit</td>
<td>Plasma Membrane</td>
<td>ion channel</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Location</td>
<td>Function</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>CACNB3</td>
<td>calcium channel, voltage-dependent, beta 3 subunit</td>
<td>Membrane ion</td>
<td></td>
</tr>
<tr>
<td>VAPA</td>
<td>VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa</td>
<td>Plasma Membrane</td>
<td>other</td>
</tr>
<tr>
<td>KRT73*</td>
<td>keratin 73</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>KRT78</td>
<td>keratin 78</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>NHSL1</td>
<td>NHS-like 1</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>MXRA7</td>
<td>matrix-remodelling associated 7</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>EG268795</td>
<td>ribosomal protein L7A, pseudogene 3</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>1700071K01RIK</td>
<td>RIKEN cDNA 1700071K01 gene</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>KRT77</td>
<td>keratin 77</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>GM5121</td>
<td>predicted gene 5121</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>LOC100047628</td>
<td>similar to Chain L, Structural Basis Of Antigen Mimicry In A Clinically Relevant Melanoma Antigen System</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>H2AFJ</td>
<td>H2A histone family, member J</td>
<td>unknown</td>
<td>other</td>
</tr>
<tr>
<td>EG240853</td>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d</td>
<td>unknown</td>
<td>other</td>
</tr>
</tbody>
</table>
For over 25 years Ca,β subunits have been viewed as increasingly important and complex mediators of Ca, function. Initially discovered as an intermediate-sized subunit which co-purified with the Ca, complex, Ca,β subunits were soon discovered to be required for normal Ca, function by regulating the expression, gating, and voltage dependence of these channels. Ca,s perform an essential role in many important physiological processes (neurotransmitter release, E-C coupling, intracellular signaling, etc), and Ca,β subunits are therefore also essential in many of these processes. Thus the importance of Ca,β subunits is well appreciated, and their functions well understood, in the context of Ca, regulation. Over the last decade, emerging research has hinted at novel, non-Ca, functions for Ca,β subunits, namely within the nucleus of different cell types. We further explored this non-classical role for Ca,β subunits by studying Ca,β1a nuclear localization in MPCs. Our findings not only define a specific nuclear signaling pathway for Ca,β1a in the regulation of MPC proliferation, but also provide general insight and strengthen the foundation for future study of all Ca,β subunits as Ca,-independent transcriptional regulators.

Ca,β1a in MPCs

Our initial finding that Ca,β1a mRNA and protein are expressed in MPCs (regardless of subcellular localization) was, in and of itself, surprising because these are non-excitable
cells which do not express Cαs. The importance of Caβ1a in differentiated skeletal muscle (myotubes and myofibers) is well known, because mutation of the Cacnb1 gene leads to absence of functional Ca1.1 expression and consequent failure of E-C coupling in skeletal muscle (Gregg et al., 1996). Ca1.1 expression itself is tightly regulated by MPC differentiation, as both the mRNA (Tomczak et al., 2004) and protein (Chapter 2, Figure 1) are silent until the onset of differentiation, at which time they are dramatically up regulated. This tight regulation of Ca1.1 makes the appearance of Caβ1a in MPCs seem more purposeful, and suggests a non-Cα role for Caβ1a. Although our study focuses on MPCs, an interesting question that we must now ask is when Caβ1a and other Caβ subunits, are first expressed in various organisms and cell types. In mice, we found the absence of Caβ1a to have an effect as early as E11.5, and it is possible Caβ1a and other Caβ subunits are expressed at even earlier times and in even less committed stem cell populations. In support of this idea, recent work has found that knockdown of Caβ4 (Ebert et al., 2008) and Caβ2 (Chernyavskaya et al., 2012) in very early stage zebrafish larvae caused significant defects in a Caα-independent manner.

**Caβ1a in the nucleus**

Protein size is an important determinant for nuclear entry of all proteins. Proteins with a diameter less than ≈10 nM (MW ≈40-50 kDa) can passively diffuse into the nucleus (Keminer and Peters, 1999), while those with larger diameter (corresponding to a MW >40-50 kDa) require active, GTP-dependent transport through nuclear import channels, made of α and β importins (Hicks and Raikhel, 1995). In order to travel through these nuclear import channels, an outside protein must generally have (or be associated with another protein that has) a NLS, which associates directly with importins. NLSs come in
several forms but are often basic stretches of amino acids rich in lysine and arginine, such as PKKKRKV in the SV-40 large T-antigen (Kalderon et al., 1984). Ca,β isoforms range in size from ≈50-75 kDa, and all are capable of nuclear import, even with sizable fluorescent tags, which increase the molecular weight substantially. Thus, Ca,β subunit nuclear import is almost certainly an active process mediated by nuclear import channels.

All four Ca,β isoforms have previously been found in the nuclei of multiple cell types. Examples of exogenous Ca,β subunits in the nucleus include: Ca,β1b, Ca,β2, and Ca,β4 in cardiomyocytes (Colecraft et al., 2002), Ca,β3 in HEK 293 cells (Beguin et al., 2006; Zhang et al., 2010), Ca,β1b and Ca,β2 in TsA201 cells (Leyris et al., 2009), Ca,β4 in C2C12 myoblasts and myotubes (Subramanyam et al., 2009), and Ca,β4 in primary hippocampal neurons (Subramanyam et al., 2009; Tadmouri et al., 2012). The only examples of a Ca,β subunit detected endogenously in the nucleus are limited to Ca,β4 in cochlear (Hibino et al., 2003a), cerebellar (Subramanyam et al., 2009), and hippocampal neurons (Tadmouri et al., 2012). Thus most of the speculation on Ca,β subunit nuclear function so far has focused on the Ca,β4 isoform. Ca,β4 indeed deserves this recognition, as Ca,β4 seems to be the only Ca,β isoform which exhibits constitutive nuclear localization. Nevertheless, we have now shown conclusive evidence that another Ca,β subunit, Ca,β1a, enters the nucleus under physiological conditions.

We detected both exogenous and endogenous Ca,β1a in the nucleus of MPCs by immunofluorescence and western blot, although under basal conditions only a very small amount of the total fraction was nuclear. However, after three hours of treatment with the CRM1 nuclear export channel-blocking drug LMB, Ca,β1a showed clear enrichment in the nucleus. Overall this suggests Ca,β1a dynamically traffics into and out of the nucleus.
Ca\textsubscript{\textit{v}}\textsubscript{1a} does not contain a canonical NLS, while it has been suggested Ca\textsubscript{\textit{v}}\textsubscript{4} does; this may explain the transient vs. constitutive nuclear localization of the two different isoforms. We did discover a potential NLS motif (KRKGRFKR, Hicks and Raikhel, 1995) in the N-terminal region of Ca\textsubscript{\textit{v}}\textsubscript{1a}, which was similar in location to the proposed NLS (RRS) of Ca\textsubscript{\textit{v}}\textsubscript{4} (Subramanyam et al., 2009). However, deletion of this sequence in Ca\textsubscript{\textit{v}}\textsubscript{1a} did not significantly affect its ability to enter the nucleus. The ability of Ca\textsubscript{\textit{v}}\textsubscript{1a} to enter the nucleus seems to reside within its SH3 domain, as Ca\textsubscript{\textit{v}}\textsubscript{1a}-YFP mutants truncated for this domain (Ca\textsubscript{\textit{v}}\textsubscript{1a}161-524) could not enter the nucleus, even after LMB treatment. Conversely, truncation mutants containing the SH3 and middle domains, but lacking GK and C-terminal domains (Ca\textsubscript{\textit{v}}\textsubscript{1a}-101-274), were constitutively nuclear. This agrees with previous reports that (1) the truncated splice variant Ca\textsubscript{\textit{v}}\textsubscript{4}c, which contains SH3 but not GK domains, is localized to the nucleus (Hibino et al., 2003a), and (2) split domain experiments in which a fluorescently labeled Ca\textsubscript{\textit{v}}\textsubscript{2}-SH3 construct constitutively localized in the nucleus of HEK 293 cells, while a Ca\textsubscript{\textit{v}}\textsubscript{2}-GK construct was completely absent from the nucleus (Takahashi et al., 2005). Together these data suggest that all Ca\textsubscript{\textit{v}}\textsubscript{\textit{\beta}} isoforms share a common profile of rapid nuclear-cytoplasmic trafficking; with nuclear localization facilitated by their SH3 domain, and cytoplasmic localization facilitated by their GK domain. Some Ca\textsubscript{\textit{v}}\textsubscript{4} splice variants appear to have an additional and more potent mechanism of nuclear entry, which may confer tissue-specific functions. Future experiments to examine whether Ca\textsubscript{\textit{v}}\textsubscript{1a} nuclear localization changes during myogenic differentiation may yield more insight into general mechanisms of Ca\textsubscript{\textit{v}}\textsubscript{\textit{\beta}} nuclear trafficking, and also into tissue specific function of Ca\textsubscript{\textit{v}}\textsubscript{1a} in skeletal muscle. Preliminary experiments in our laboratory (not shown) have found that Ca\textsubscript{\textit{v}}\textsubscript{1a} also enters
the nucleus of differentiated myotubes, suggesting a robust mechanism of nuclear entry not dependent on cellular differentiation state.

A major question remains whether Ca,β(1a) subunit nuclear entry is an inherent ability (mediated by a non-canonical NLS), or depends on binding partner(s). Evidence exists in favor of both scenarios, though the requirement of a binding partner seems more likely. All four Ca,β isoforms can enter the nucleus of a variety of cell types, suggesting a highly conserved mechanism. And although several nuclear Ca,β-protein binding partners (capable of localizing Ca,β to the nucleus) have been identified (Hibino et al., 2003a; Tadmouri et al., 2012; Zhang et al., 2010), these interactions lack consistency between different Ca,β subunits and cell types. Therefore, if Ca,β subunits enter the nucleus through a conserved interaction with another protein, this protein must be almost ubiquitously expressed. The finding that the SH3 domain of Ca,β1a and other Ca,β subunits is apparently necessary and sufficient for nuclear entry does not particularly help either argument, as this domain could contain an intrinsic, non-canonical NLS, or be responsible for interaction with nuclear chaperones. However the general conservation of this domain as a protein-protein interaction module (Mayer, 2001) points to the latter, and again suggests a conserved mechanism of nuclear entry for all Ca,β subunits.

The dynamic and transient nature of Ca,β1a in the nucleus may reflect an unstable interaction with another protein and presents two main scenarios (Figure 1): (1) Ca,β1a enters the nucleus by intrinsic NLS or transient chaperone interaction, associates with a binding partner present in overwhelming quantities, leading to their mutual export and final dissociation in the cytoplasm. In this model Ca,β1a may act as an inhibitor of other nuclear proteins such as transcription factors. Or (2), Ca,β1a enters the nucleus via
intrinsic NLS or transient chaperone interaction, and when possible binds to any number
of secondary, constitutively nuclear partners (protein or DNA) which are only available
in limited quantities but are required to maintain Ca_\_\_β_1_α in the nucleus. If a second,
“anchoring” option is not available, then Ca_\_\_β_1_α rapidly exits the nucleus. Our ChIP
experiments show that Ca_\_\_β_1_α binds to the promoter region of a large number of genes,
which supports the idea that Ca_\_\_β_1_α is able to form stable complexes in the nucleus, as
proposed in scenario #2. This model is also compatible with the multiplicity of Ca_\_\_β
subunit interactions with other proteins, as any number of nuclear binding partners could
function to anchor Ca_\_\_β in the nucleus – after it enters through a more conserved
mechanism. These models are also not mutually exclusive, and it is possible Ca_\_\_β_1_α and
other Ca_\_\_β subunits, act in both manners.

Ca_\_\_β_1_α subunit binding partners

Numerous non-Ca_\_\_ binding partners have been identified for Ca_\_\_β subunits (summarized
in Chapter 1, Table 1). Few of these interactions have been studied beyond the initial
publication in which they were identified, with the exception of RGK proteins, making it
difficult to draw overarching conclusions. One observation that can be made is that the
SH3 domain is often required for Ca_\_\_β interaction with other binding partners (when this
aspect was been explored). Ca_\_\_β subunit binding partners fall into four main categories so
far: non-Ca_\_\_ ion channels (Yu et al., 2008; Zou et al., 2008), enzymes (Catalucci et al.,
2009; Grueter et al., 2006; Tadmouri et al., 2012), scaffolding proteins (Beguin et al.,
2001; Gonzalez-Gutierrez et al., 2007; Hohaus et al., 2002; Kiyonaka et al., 2007; Vendel
et al., 2006), and transcription factors (Hibino et al., 2003a; Tadmouri et al., 2012; Zhang
et al., 2010). A recent publication by Michel de Waard’s laboratory found Ca_\_\_β interacts
with a protein complex containing the phosphatase PP2A, the transcription factor TRα, and the chromatin modifier HP1, to regulate gene transcription (Tadmouri et al., 2012). This finding further substantiates the promiscuous nature of Ca,β subunits in their interactions with other proteins, but also provides a coherent framework in which Ca,β subunits act as dynamic adaptor proteins for larger protein complexes. Importantly, we could not find an interaction between Ca,β1a and either HP1 or Rem, which further supports the idea that many of the Ca,β binding partners identified so far are tissue and isoform specific.

Because Ca,β1a nuclear import is likely facilitated by interaction with one or more additional proteins, we sought to identify which proteins Ca,β1a associates with specifically in the nucleus. Although our initial screening method succeeded in identifying a number of potential Ca,β1a nuclear interaction proteins, we were unable to validate any by secondary methods. Of the proteins identified, Histone H1, Actin, Lamins A/C and B, Syne-2, and Lef1 were the most attractive as bona fide Ca,β1a interacting partners because they are widely expressed. The lack of secondary validation does not entirely rule out any of these proteins as Ca,β1a interacting partners. Further screening employing similar methods will determine if any of these candidates are worth exploring in more depth.

**Ca,β1a global gene regulation**

Ca,β subunit nuclear localization suggests a possible role in gene regulation. Other studies have found gene regulation by Ca,β subunits in reporter assays (Hibino et al., 2003a; Zhang et al., 2010), and Ca,β4 was recently found to regulate tyrosine hydroxylase
expression in neurons (Tadmouri et al., 2012). To identify Ca$_\alpha$\beta gene targets at the genome-wide level, we examined Ca$_\alpha$\beta$_{1a}$ gene regulation by two separate, large scale methods: protein-DNA microarrays (ChIP-on-chip) and RNA microarrays.

ChIP-on-chip experiments reveal that Ca$_\alpha$\beta$_{1a}$ protein binds to the promoter regions of 952 separate genes in MPCs. Functional annotation of enriched peaks revealed Ca$_\alpha$\beta$_{1a}$ binds to the promoter regions of a broad set of genes, including many involved in signal transduction and stress response. We validated by standard ChIP that Ca$_\alpha$\beta$_{1a}$ binds to the promoter regions of a number of genes involved in development, such as Wnt3 (David et al., 2010), Tbx5 (Hatcher et al., 2001), Dlx2 (Suh et al., 2009), and Pax3 (Bober et al., 1994), as well as Cbx5 which codes for the ubiquitous chromatin silencing HP1 proteins.

Ca$_\alpha$\beta$_{1a}$ knockout (Cacnb1 $^{-/-}$) MPCs provide an elegant model to identify genes regulated by Ca$_\alpha$\beta$_{1a}$ in a Ca$_\alpha$-independent fashion. Because MPCs do not express Ca$_\alpha$s, then any alterations in gene expression found in Cacnb1 $^{-/-}$ MPCs can be dissociated from loss of Ca$_\alpha$ function. Compared to wild type (Cacnb1 $^{+/+}$) and heterozygous (Cacnb1 $^{+/-}$) MPCs, 1104 genes were up-regulated in Cacnb1 $^{-/-}$ MPCs (negatively regulated by Cacnb1) and 1888 genes were down-regulated in Cacnb1 $^{-/-}$ expression (positively regulated by Cacnb1). Further functional analysis highlighted many genes involved in cell cycle regulation and muscle development. It is important to note that although these genes are regulated by Ca$_\alpha$\beta$_{1a}$ in a Ca$_\alpha$-independent fashion, that does not necessarily mean they are regulated by Ca$_\alpha$\beta$_{1a}$ nuclear function either. Forty of the 1104 genes up-regulated in Cacnb1 $^{-/-}$ MPCs were also enriched for Ca$_\alpha$\beta$_{1a}$ binding in the ChIP-on-chip experiments, including Cbx5, while 75 of the 1888 down-regulated genes in Cacnb1 $^{-/-}$ MPCs were also enriched for Ca$_\alpha$\beta$_{1a}$ binding, including Pax3. Together these data suggest that Ca$_\alpha$\beta$_{1a}$
acts as both a positive and negative transcriptional regulator. Future experiments using similar paradigms with RNA-sequencing and ChIP-sequencing, on multiple Ca,β isoforms in multiple cell types, will be the next step towards truly defining conserved and isoform/tissue-specific gene-regulatory function of all Ca,β subunits.

A consensus DNA binding motif (CCTTTAATCCAG ) was identified for Ca,β1a by our downstream analysis of ChIP-on-chip data. However, this does not prove that Ca,β1a acts as a *bona fide* transcription factor (direct binding to DNA), but rather may act as scaffold in part of a larger chromatin binding complex as suggested earlier. Experiments such as electrophoretic mobility shift assays (EMSA) are needed to test whether purified Ca,β1a binds directly to this and other DNA sequences.

**Ca,β1a regulation of Myog**

One gene we found of particular interest by RNA microarrays to be negatively regulated by Ca,β1a was myogenin. Myogenin, a member of the muscle regulatory family (MRF) of transcription factors, is well characterized as an essential protein for skeletal muscle development (Charge and Rudnicki, 2004; Hasty et al., 1993; Nabeshima et al., 1993). Although Ca,β1a was not found to bind to the *Myog* promoter in our ChIP-on-chip assays, standard ChIP confirmed Ca,β1a enrichment on the *Myog* promoter, and luciferase assays showed that Ca,β1a suppresses the *Myog* promoter. Additionally, we found significantly higher myogenin mRNA in the limb buds of *Cacnb1* −/− E11.5 embryos, demonstrating that Ca,β1a regulates myogenin *in vivo*. While myogenin is not expressed in the limb buds until E11.5, it is expressed in the myotome around E8.5 (Cheng et al., 1992). Thus an
interesting experiment would be to see if the number of myogenin + cells was increased in the somites of Cacnb1 -/- embryos after E8.5.

Cacnb1 -/- mice show increased myogenin expression at E11.5, and lower Pax7+ MPC numbers at E13.5. Thus, our model proposes that Ca,β1a acts as a negative regulator of myogenin in undifferentiated MPCs, allowing them ample time to proliferate before myogenin is turned on and they are signaled to differentiate. Loss of Ca,β1a in MPCs therefore causes precocious myogenin expression, which is associated with irreversible cell cycle exit and differentiation (Andres and Walsh, 1996). A similar function has been proposed for other proteins as temporally precise inhibitors of myogenin in MPCs of the embryonic mouse myotome and limb buds (Schuster-Gossler et al., 2007; Van Ho et al., 2011; Vasyutina et al., 2007). Additional experiments, such as quantifying myogenin expression in Ca,β1a knockdown and Cacnb1 +/- MPCs, and at multiple time points during Cacnb1 +/- and Cacnb1 -/- mouse embryonic development would strengthen our model.

As to the all important question of Ca,β(1a) binding partners; a number of other regulatory proteins are already known to bind to the Myog promoter, including MyoD (Gerber et al., 1997), Pbx1 (Berkes et al., 2004), Mef2 (Edmondson et al., 1992), and Six1(Spitz et al., 1998). Because we have provided strong evidence that Ca,β1a also binds to the Myog promoter, it would be worthwhile to test whether Ca,β1a interacts with any of these proteins.

It is worth noting that Ca,β1a could regulate myogenin in differentiated skeletal muscle, in addition to MPCs. Besides its important role in myogenic differentiation, myogenin also plays an important role in skeletal muscle response to denervation. While normally silent
in fully differentiated skeletal muscle, upon denervation myogenin is up-regulated where it in turn activates a number of downstream genes involved in neuromuscular junction remodeling (AChR and MuSK) (Tang et al., 2009) and muscle atrophy (MuRF1 and atrogin-1) (Moresi et al., 2010). In preliminary experiments (not shown), we found that Ca\(_{\beta_{1a}}\) can also traffic to the nuclei of differentiated myotubes (in vitro) and myofibers (in vivo), suggesting that Ca\(_{\beta_{1a}}\) has the ability to reach the Myog promoter in these systems as well. A recent work found increased AChR and MuSK mRNA expression in Cacnb1\(^{-/-}\) mouse skeletal muscle, which causes aberrant neuromuscular patterning (Chen et al., 2011). This phenotype was attributed to impaired Ca\(_{1.1}\) function, however it also resembles some characteristics of myogenin overexpression (Gundersen et al., 1995) and up-regulation in response to denervation (Tang et al., 2009). Although still very tenuous, together these data leave open the possibility that Ca\(_{\beta_{1a}}\) may also regulate in differentiated skeletal muscle.

**Concluding Remarks**

Ca\(_{\beta}\) subunit regulation of Ca\(_{\alpha}\)s is well understood, while Ca\(_{\alpha}\)\(_{\beta}\) subunit nuclear localization currently is not. We have described a novel role for Ca\(_{\alpha}β_{1a}\) in the nucleus of MPCs which is important for skeletal muscle development in vivo. These results further our understanding of Ca\(_{\alpha}β_{1a}\) function and skeletal muscle development, but also provide a foundation for future study of all Ca\(_{\alpha}\)\(_{\beta}\) subunits in their newly appreciate role as nuclear proteins capable of gene regulation. Our work, in combination with others, sets the stage for many years of exciting research on Ca\(_{\alpha}\)\(_{\beta}\) subunits, this time in a leading role.
Figure 1. Model for mechanism of Ca,β nuclear entry and function. Ca,β subunits enter the nucleus via intrinsic, non-canonical NLS (green barrel) or via interaction with an NLS-containing chaperone protein (green circle). Once inside the nucleus, Ca,β subunits either (1) bind to proteins present in high concentrations (yellow box) to facilitate their mutual export, or (2) bind to multiple protein (other shapes) and/or DNA targets which are constitutively nuclear, where they function to regulate gene expression such as the suppression of Myog for Ca,β₁a. If no constitutively nuclear targets are available, Ca,β subunits are quickly exported.
References


Block, B.A., T. Imagawa, K.P. Campbell, and C. Franzini-Armstrong. 1988. Structural evidence for direct interaction between the molecular components of the


Appendix

Increased CaVβ1a Expression in Skeletal Muscle with Aging Contributes Muscle Weakness

Jackson Taylor¹,², Zhenlin Zheng¹*, Zhong-Min Wang¹, Anthony Payne¹†, María L. Messi¹, and Osvaldo Delbono¹,²

*This work was published in Aging Cell in 2009, and was not directly part of my dissertation research, but is related.
Summary

Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) into the cytoplasm is a crucial part of excitation-contraction (E-C) coupling. E-C uncoupling, a deficit in Ca\textsuperscript{2+} release from the SR, is thought to be responsible for at least some of the loss in specific force observed in aging skeletal muscle.

E-C uncoupling may be caused by alterations in expression of the voltage-dependent calcium channel $\alpha_{1s}$ (Cav1.1) and $\beta_{1a}$ (Cav1.1a) subunits, both of which are necessary for E-C coupling to occur. While previous studies have found Cav1.1 expression declines in old rodents, Cav1.1a expression has not been previously examined in aging models. Western blot analysis shows a substantial increase of Cav1.1a expression over the full lifespan of FVB mice. To examine the specific effects of Cav1.1a overexpression, a Cav1.1a-YFP plasmid was electroporated \textit{in vivo} into young animals. The resulting increase in expression of Cav1.1a corresponded to decline of Cav1.1 over the same time period. YFP fluorescence, used as a measure of Cav1.1a-YFP expression in individual fibers, also showed an inverse relationship with charge movement, measured using the whole-cell patch-clamp technique. Specific force was significantly reduced in young Cav1.1a-YFP electroporated muscle fibers compared to sham-electroporated, age-matched controls. siRNA interference of Cav1.1a in young muscles reduced charge movement, while charge movement in old was restored to young control levels. These studies imply Cav1.1a serves as both a positive and negative regulator Cav1.1 expression, and that endogenous overexpression of Cav1.1a during old age may play a role in the loss of specific force.
INTRODUCTION

Depolarization of the sarcolemma leads to muscle fiber contraction and the generation of mechanical force in a process called excitation-contraction (E-C) coupling. Two key proteins are involved in E-C coupling: dihydropyridine receptor (DHPR) and ryanodine receptor (RyR). DHPR serves as a modest L-type Ca^{2+} channel but is primarily known for its function as a voltage sensor. DHPRs are located within invaginations of the sarcolemma called t-tubules, and are arranged into clusters of four known as tetrads. Each tetrad is positioned directly across from a single RyR, which are embedded within the membrane of the adjacent sarcoplasmic reticulum (SR). DHPRs contain four domains, each composed of six-transmembrane spanning segments. The S4 segment of each domain contains charged amino acid residues, and these residues respond to membrane depolarization by undergoing a conformational shift. This shift results in a proposed physical interaction of DHPR with RyR, causing RyR to open and release intracellular Ca^{2+} stores from the SR, allowing muscle contraction to occur (for review, see Melzer et al. 1995).

The decline in muscular strength with age, known as sarcopenia, is caused largely by a loss of total muscle mass - but also a disproportionate loss of strength. This loss of specific force (total force/cross sectional area) in old age (Brooks & Faulkner 1988; Gonzalez et al. 2000) is characterized in part by a deficit in Ca^{2+} release following depolarization (Delbono et al. 1995; Jimenez-Moreno et al. 2008), a phenomenon known as E-C uncoupling. E-C uncoupling is not a result of decreased Ca^{2+} stores or RyR
release function (Jimenez-Moreno et al. 2008), and therefore may be caused by alterations in the functionality and expression of DHPR and its subunits with aging. The primary DHPR subunit in skeletal muscle is Ca$_{\text{V}}$1.1, previously known as DHPR$\alpha_{1s}$ (Catterall et al. 2005). Ca$_{\text{V}}$1.1 is a large transmembrane protein which contains both the Ca$^{2+}$ conducting pore and the voltage sensing S4 domain. Four other auxiliary subunits bind Ca$_{\text{V}}$1.1 to make up DHPR (for review, see Flucher et al. 2005), with the most widely studied being the cytoplasmic Ca$_{\text{V}}$$\beta_{1a}$ subunit. Ca$_{\text{V}}$$\beta_{1a}$, a muscle specific member of the Ca$_{\text{V}}$$\beta$ family of proteins, binds to a region of the I-II intracellular loop of Ca$_{\text{V}}$1.1 known as the alpha interaction domain (AID) (Chen et al. 2004). Ca$_{\text{V}}$$\beta_{1a}$ is classically described by its role in chaperoning Ca$_{\text{V}}$1.1 to the plasma membrane and regulating L-type Ca$^{2+}$ current (Gregg et al. 1996; Strube et al. 1996; Beurg et al. 1997; Neuhuber et al. 1998). Most notably, E-C coupling cannot occur without Ca$_{\text{V}}$$\beta_{1a}$ (Gregg et al. 1996). Ca$_{\text{V}}$$\beta_{1a}$ binds to charged residues on RyR (Cheng et al. 2005) and neutralization of these residues reduces E-C coupling, suggesting a direct interaction with RyR. The correct organization of Ca$_{\text{V}}$1.1 into tetrads within the t-tubule membrane is also a specific function of the Ca$_{\text{V}}$$\beta_{1a}$ isoform (Schredelseker et al. 2005).

Although classically known for augmenting the expression and function of Ca$_{\text{V}}$1 subfamily of calcium channels, the Ca$_{\text{V}}$$\beta$ family of subunits may contribute to the down-regulation of Ca$_{\text{V}}$1 as well. A family of Ras-related G-proteins (RGKs) mediate the down-regulation of several Ca$_{\text{V}}$1 isoforms in a Ca$_{\text{V}}$$\beta$ dependent manner (Beguin et al. 2001). Additionally, the previously uncharacterized SH3 domain of Ca$_{\text{V}}$$\beta$ was shown to bind dynamin and mediate endocytosis of Ca$_{\text{V}}$1.2 (Gonzalez-Gutierrez et al. 2007).
As previous studies have shown that the Ca\textsubscript{V}1.1 subunit declines in old rodents (Renganathan \textit{et al.} 1997; Moreno \textit{et al.} 2006; O’Connell \textit{et al.} 2008) and this causes an impairment of E-C coupling (Renganathan \textit{et al.} 1997), we wanted to investigate what effects aging had on Ca\textsubscript{V}β\textsubscript{1a} expression, as this subunit is also critical for E-C coupling. We have found that Ca\textsubscript{V}β\textsubscript{1a} expression is highly increased in old mice, and that experimental overexpression of Ca\textsubscript{V}β\textsubscript{1a} reduces both the expression of Ca\textsubscript{V}1.1 and specific force in dissociated single fibers of young mice. Additionally, siRNA inhibition of Ca\textsubscript{V}β\textsubscript{1a} restores charge movement in aged muscle. These findings suggest that overexpression of Ca\textsubscript{V}β\textsubscript{1a} with aging contributes to E-C uncoupling by reducing the level of Ca\textsubscript{V}1.1.

RESULTS

\textbf{Ca\textsubscript{V}β\textsubscript{1a} subunit expression increases with age}

As our laboratory had earlier shown that Ca\textsubscript{V}1.1 expression declines in old animals (Renganathan \textit{et al.} 1997), we investigated whether Ca\textsubscript{V}β\textsubscript{1a} might also change with age. Hindlimb muscles from FVB mice were harvested at 2, 6, 14, and 24 months of age and subjected to a membrane extraction protocol (see Methods) for use in western blot analyses. Gluteus and hamstring muscles (designated “pool”) contain a mixture of type I (slow) and type II (fast) fiber types (Fig. 1A), while tibialis anterior (TA) and extensor digitorum longus (EDL) muscles contain predominantly type II fibers (Manttari and Jarvilehto, 2005; Burkholder \textit{et al.}, 1994) (Fig. 1B). Actin was used as a loading control
as it appears to remain relatively stable throughout the lifespan of FVB mice. In order to precisely compensate for any variations in loading or possible global alterations in protein expression with aging, the optical density of each Ca\textsubscript{v}1.1 band was divided by that of the corresponding actin band. The resulting values confirm a significant increase in Ca\textsubscript{v}\beta\textsubscript{1a} with age. Relative Ca\textsubscript{v}\beta\textsubscript{1a} expression increased continuously during aging, approximately doubling at each time point. This culminated in a substantial increase (~10-fold) between very young and very old age (Fig. 1C) in both muscle groups, though the effect was less pronounced in the TA/EDL muscle groups. The mean normalized intensity values (arbitrary units, AU) for each age group (n=4) are as follows: 0.20 ± 0.02 (2 months), 0.49 ± 0.06 (6 months), 1.14 ± 0.25 (14 months), and 2.34 ± 0.49 (24 months) for pool muscles and.

**Overexpression of Ca\textsubscript{v}\beta\textsubscript{1a} results in decreased Ca\textsubscript{v}1.1 expression**

We next sought to determine if the observed increase in Ca\textsubscript{v}\beta\textsubscript{1a} expression was directly involved in the age-related decline in Ca\textsubscript{v}1.1. In order to separate the effects of Ca\textsubscript{v}\beta\textsubscript{1a} overexpression from all other age-related changes, a Ca\textsubscript{v}\beta\textsubscript{1a}-YFP plasmid was electroporated into the TA muscle of young (4-5 month old) FVB mice in vivo. For these experiments we used TA because it is a superficial muscle suitable for electroporation in vivo, and large enough to provide tissue for protein analysis. Western blots of membrane fractions from electroporated muscles at 3 days (n=4), 7 days (n=3), and 2 weeks (n=4) confirm an increase in expression of endogenous Ca\textsubscript{v}\beta\textsubscript{1a}, which continued to rise during the two weeks following in vivo electroporation (Figure 2A). Conversely, Ca\textsubscript{v}1.1
expression levels from the same samples declined steadily over the two week time course. Quantification of Ca\textsubscript{V}1.1 band optical density shows an approximately 50% decrease compared to control at two weeks following Ca\textsubscript{V}\beta\textsubscript{1a}-YFP electroporation (Figure 2B). Sham electroporated muscles (n=4 for each time point) exhibited no significant changes in Ca\textsubscript{V}1.1 or Ca\textsubscript{V}\beta\textsubscript{1a} expression relative to non-electroporated controls. Additionally, quantitative real time RT-PCR was used to assess Ca\textsubscript{V}1.1 transcript levels at 2 weeks after Ca\textsubscript{V}\beta\textsubscript{1a}-YFP electroporation (Figure 2C). No difference was seen in Ca\textsubscript{V}\beta\textsubscript{1a}-YFP electroporated (n=4) vs. controls (n=3), suggesting that Ca\textsubscript{V}1.1 down regulation during Ca\textsubscript{V}\beta\textsubscript{1a} overexpression occurs only at the protein level. These results are significant for two reasons. First, they show use of the Ca\textsubscript{V}\beta\textsubscript{1a}-YFP plasmid to artificially overexpress Ca\textsubscript{V}\beta\textsubscript{1a} \textit{in vivo}. Second, Ca\textsubscript{V}\beta\textsubscript{1a} overexpression directly correlates to a decline in Ca\textsubscript{V}1.1 protein levels in young mice, thus providing evidence for Ca\textsubscript{V}\beta\textsubscript{1a} involvement in Ca\textsubscript{V}1.1 down-regulation; namely when present in high levels such as those observed during senescence.

**DHPR \(\beta_{1a}\)-YFP intensity corresponds to reduced charge movement**

The intensity of YFP fluorescence (F, arbitrary units) varied between individual FDB fibers following \textit{in vivo} electroporation with the Ca\textsubscript{V}\beta\textsubscript{1a}-YFP plasmid. Thus, the relative level of YFP intensity can be used to represent differences in individual fiber’s Ca\textsubscript{V}\beta\textsubscript{1a}-YFP expression level. Similarly, charge movement (Q) is a measure of single cell Ca\textsubscript{V}1.1 expression at the plasma membrane (Wang \textit{et al.} 2000; for review, see Rios & Pizarro 1991). Maximal charge movement (Q\textsubscript{max}) was plotted against the corresponding fiber’s
YFP intensity (Figure 3A). The resulting plot shows an inverse relationship between Cavnβ1α-YFP fluorescence level and Cavn1.1 membrane expression (r=0.91, n=10), where electroporated fibers with high YFP fluorescence (F > 40 AU) show increasingly impaired Qmax. Fibers with relatively low YFP fluorescence (F ≤ 40 AU) are presumed to exhibit minimal overexpression of Cavnβ1α-YFP, and subsequently showed no loss of Qmax. Fibers from muscles electroporated with a GFP plasmid (n=10) showed no difference in Qmax, regardless of relative F intensity. Figure 3B and Figure 4 display an example of two fibers with different YFP intensity, and the resulting difference in Q. Fiber D0912, a fiber with lower Cavnβ1α-YFP expression (F=42 AU) exhibited unimpaired maximal charge movement (Qmax =52.6 nC/μF). Conversely, fiber C0925 showed high levels of Cavnβ1α-YFP (F=71.3 AU) and substantially reduced maximal charge movement (Qmax =26.6 nC/μF). While the absolute Q-V relationship was reduced in fiber C0925 and others showing high AU (Fig. 4D), the relative Q-V relationship was unchanged (Fig. 4E). Therefore overexpression of Cavnβ1α-YFP does not shift the Q-V curve in the voltage axis, reaffirming the notion that the effects of Cavnβ1α-YFP are on Cavn1.1 membrane expression and not other alterations of the channel’s function.

DHPR β1a-YFP causes a decline in specific force

Loss of muscle strength with aging is thought to be partially caused by reduction of Cavn1.1, specifically those coupled to RyR in the T-tubule membrane, thereby resulting in an impairment of E-C coupling. As Cavnβ1α-YFP overexpression lead to a marked reduction Cavn1.1, shown by both western blot and charge movement studies, it seemed
likely that Cavβ1a-YFP overexpression would also result in a loss of specific force. Specific force (kPa) was measured 14 days after single fibers from FDB muscles were electroporated in vivo with either Cavβ1a-YFP (n=7), GFP (n=7), or non-electroporated (n=3) (Figure 5A). The electroporation process itself causes short-term damage to the muscle fiber in the days immediately following the procedure (Schertzer et al. 2006) and as such the GFP group showed a significant reduction in specific force compared to controls. However, the Cavβ1a-YFP group showed an even greater and statistically significant decline in specific force compared to the YFP electroporated fibers. Figure 5B shows representative tetanic contraction traces from Cavβ1a-YFP, GFP, and control groups. These results support the idea that overexpression of Cavβ1a-YFP impairs single fiber specific force by reducing the amount of Cav1.1 expressed at the plasma membrane.

**siRNA inhibition of β1a**

If overexpression of Cavβ1a is responsible for muscle impairment with aging, then inhibition of Cavβ1a may provide a way to reverse these effects. We created several siRNA sequences against Cavβ1a (Figure 6A). The effectiveness of these sequences was determined by transfecting each of them into separate C2C12 cell cultures and performing western blot analyses on the harvested cell lysates. Each sample was probed for Cavβ1a, and the optical density of each band was compared to that of a control, untransfected sample. Clones 69051 and 69052 caused the greatest decline in Cavβ1a expression (37% and 28% decline, respectively). Both of these clones were then electroporated in vivo into the FDB muscle of young and old FVB mice (Fig. 6B). In
young animals, siRNA against CaVβ1a caused a significant reduction of charge movement in dissociated FDB cells, recorded 7-11 days post electroporation. As demonstrated in Figure 3, electroporation of GFP alone does not result in a loss of charge movement. Old (24 month) mice naturally exhibit reduced charge movement, which was confirmed by our results. Interestingly, siRNA against CaVβ1a restored charge movement of old mice to young control levels. Sham electroporation of old muscles did not result in a further decline in charge movement, suggesting that the deleterious effect of electroporation on force reported above is due primarily to damage to contractile proteins and not due to reduced Cav1.1 expression.

DISCUSSION

Here we show that CaVβ1a is significantly overexpressed in aging muscle, and present a model by which this phenomenon may contribute to loss of specific force with aging. Exogenous overexpression of CaVβ1a corresponds to decreased CaV1.1 expression at the sarcolemma, as shown by western blot and charge movement studies. CaVβ1a overexpression also results in a loss maximal specific force, presumably by reducing the number of CaV1.1 channels in the t-tubule membrane coupled to RyRs. Because CaV1.1 subunits are critical for the transduction of sarcolemmal depolarizations into Ca\(^{2+}\) release from the SR, a decreased number of CaV1.1 subunits in the membrane would cause less Ca\(^{2+}\) to be released from intracellular stores, resulting in weakened contractions. To further implicate overexpression of CaVβ1a directly with decreased CaV1.1, we show that using siRNA to partially inhibit CaVβ1a in old muscle restores charge movement to young
control levels. Expectedly, inhibition of Ca\textsubscript{V}\beta\textsubscript{1a} in young muscle fibers significantly reduced charge movement. Thus, both the under and overexpression of Ca\textsubscript{V}\beta\textsubscript{1a} results in reduced expression of Ca\textsubscript{V}1.1.

**Ca\textsubscript{V}\beta\textsubscript{1a} Overexpression with Aging**

While the most striking increase in Ca\textsubscript{V}\beta\textsubscript{1a} expression appears to be between middle aged and very old animals, the rate of Ca\textsubscript{V}\beta\textsubscript{1a} increase is surprisingly uniform, with the normalized expression level roughly doubling at each time point. Relative Ca\textsubscript{V}\beta\textsubscript{1a} level in very young animals is substantially lower than the level seen even at 6 months of age (young adulthood). Thus, increasing Ca\textsubscript{V}\beta\textsubscript{1a} expression may also be a necessary component of muscle development. It is therefore tempting to speculate that the extreme levels of Ca\textsubscript{V}\beta\textsubscript{1a} seen in very old animals may reflect a developmental program hyperfunction, a common attribute of aging (Blagosklonny, 2006). Interestingly, the rate of increase of Ca\textsubscript{V}\beta\textsubscript{1a} expression did not seem to be as high in the TA/EDL muscle group compared to that of the Pool group. Type II muscle fibers are predominant in TA and EDL muscles, while there is a mixture of type I and type II fibers found in the gluteus and hamstring muscles used for our pool group. The apparent resistance to Ca\textsubscript{V}\beta\textsubscript{1a} overexpression seen in the predominantly type II muscle group is perplexing, as type I fibers are thought to be more resistant to age related changes such as denervation (Larsson, 1995).

The two most likely explanations of Ca\textsubscript{V}\beta\textsubscript{1a} overexpression with age are an increase in its transcription levels, or a failure in its ability to be properly degraded. In
regards to the latter, impairment of proteolysis is already known to be a hallmark of aging muscle (for review, see Combaret et al. 2009). Further experiments examining Ca\(\text{v}\)\(\beta\)\(_{1a}\) mRNA levels during aging should shed light on whether transcriptional upregulation plays any role. These explanations are not mutually exclusive and indeed it would seem probable that both contribute to the accumulation of Ca\(\text{v}\)\(\beta\)\(_{1a}\) in old muscle. Although we present a model by which Ca\(\text{v}\)\(\beta\)\(_{1a}\) overexpression causes deleterious effects by reducing the number of voltage sensing Ca\(\text{v}\)1.1 subunits in the t-tubule membrane, the relative expression of Ca\(\text{v}\)\(\beta\)\(_{1a}\) in old muscle is so high that it may also interfere with other key processes in a nonspecific manner. Ca\(\text{v}\)\(\beta\) subunits contain two conserved protein interaction domains: GK and SH3 (Chen et al., 2004) and have recently been shown to interact with several other proteins besides Ca\(\text{v}\)1 (for review, see Hidalgo and Neely, 2007). It is therefore possible that Ca\(\text{v}\)\(\beta\)\(_{1a}\) may interact with additional proteins in a currently uncharacterized manner.

**Potential mechanisms of Ca\(\text{v}\)\(\beta\)\(_{1a}\) involvement in Ca\(\text{v}\)1.1 down-regulation**

The notion that Ca\(\text{v}\)1.1 expression declines during old age is supported by several studies (Renganathan et al. 1997; Ryan et al. 2000; Moreno et al. 2006; Wang et al. 2007; Ohlendiek et al. 2008). Importantly, Ca\(\text{v}\)1.1 mRNA does not decrease significantly with age (Zheng et al. 2001), implicating some other mechanism responsible for the decline in its protein level. Here we demonstrate that Ca\(\text{v}\)1.1 expression also declines following exogenous overexpression of Ca\(\text{v}\)\(\beta\)\(_{1a}\). Thus, Ca\(\text{v}\)\(\beta\)\(_{1a}\) overexpression, both during natural aging and exogenous overexpression in young cells, coincides with a decline in Ca\(\text{v}\)1.1
expression. While we present evidence that $\text{Ca}_\text{V} \beta_{1a}$ overexpression causes a decline of $\text{Ca}_\text{V}1.1$ at the protein level, the precise mechanism behind this occurrence is not known. $\text{Ca}_\text{V}1.1$ mRNA does not decline in young mice following exogenous $\text{Ca}_\text{V} \beta_{1a}$ overexpression, suggesting $\text{Ca}_\text{V} \beta_{1a}$ does not regulate $\text{Ca}_\text{V}1.1$ at the transcriptional level, in agreement with the aforementioned aging studies on $\text{Ca}_\text{V}1.1$ mRNA. Due to the multiple regulatory functions that $\text{Ca}_\text{V} \beta_{1a}$ exerts on $\text{Ca}_\text{V}1.1$, both classically and those discovered more recently, there are several hypothetical mechanisms by which overexpression of $\text{Ca}_\text{V} \beta_{1a}$ could lead to a reduction of $\text{Ca}_\text{V}1.1$. The two most important classical functions of $\text{Ca}_\text{V} \beta_{1a}$ in skeletal muscle are increasing the trafficking of newly formed $\text{Ca}_\text{V}1.1$ subunit to the t-tubule membrane (Neuhuber et al. 1998; Bichet et al. 2000), and arranging DHPRs into orthogonal arrays, or tetrads (Schredelseker et al. 2005), which are presumed to be necessary for skeletal muscle E-C coupling. Interfering with tetrad formation is one possible way that $\text{Ca}_\text{V} \beta_{1a}$ overexpression could reduce $\text{Ca}_\text{V}1.1$ insertion into the membrane. Tetrads are critical for the precise alignment of DHPRs with RyR, which is required for proper transduction during E-C coupling. Because $\text{Ca}_\text{V} \beta_{1a}$ also binds to RyR (Cheng et al. 2005), it seems possible that overexpression of $\text{Ca}_\text{V} \beta_{1a}$ could interfere with the alignment and coupling of DHPR tetrads to RyR. Also, our group in collaboration with others has found that $\text{Ca}_\text{V} \beta_{1a}$ interacts with the junctional protein JP-45 (Anderson et al. 2006). Like $\text{Ca}_\text{V} \beta_{1a}$, JP-45 is localized to the t-tubule/SR junction (triad) and interacts with $\text{Ca}_\text{V}1.1$ via the AID. In co-immunoprecipitation experiments, treatment with exogenous purified $\text{Ca}_\text{V} \beta_{1a}$ reduces the ability of JP-45 to pull down $\text{Ca}_\text{V}1.1$, suggesting $\text{Ca}_\text{V} \beta_{1a}$ interferes with this interaction. As JP-45 KO mice have impaired muscle strength due to reduced levels of $\text{Ca}_\text{V}1.1$ (Delbono et al. 2007), disruption of the JP-45-$\text{Ca}_\text{V}1.1$
interaction associated with excess CaVβ1α is another possible mechanism by which CaVβ1α overexpression reduces the level of CaV1.1 at the membrane. Disruption of the DHPR tetrad – RyR complex, through any of the mechanisms mentioned above, could in turn make CaV1.1 more susceptible to endocytosis and proteolytic degradation.

Much attention has been given recently to the involvement of the RGK family of Ras-related GTP-binding proteins in CaV1 channel function and expression. CaVβ isoforms are necessary for the RGK (kir/Gem) mediated down-regulation of CaV1.2 (Beguin et al. 2001), and CaVβ2α forms a trimeric complex with the RGK Rem and the CaV1 AID (Finlin et al., 2006). In skeletal muscle, overexpression of Rem leads to a decline in the number of CaV1.1 channels in the membrane (Bannister et al. 2008). Thus overexpression of CaVβ1α may contribute to CaV1.1 down-regulation via interaction with RGKs, although the necessity for up-regulation of CaVβ1α itself is unclear. Interestingly, Beguin and colleagues (2006) have recently shown that the RGKs Rad and Rem may sequester CaVβ3 subunits into the nucleus in a process regulated by calmodulin and 14-3-3. A previous study by Colecraft et al. (2002) also showed the presence of CaVβ1b, CaVβ2α, and CaVβ4 isoforms in the nucleus of cardiomyocytes. In additional to nuclear localization, Hibino et al. (2003) demonstrated that the CaVβ4 splice variant CaVβ4c acts as a transcriptional regulator by binding to and inhibiting the gene silencing ability of the nuclear protein CHCB2/HP1γ. Although we found no influence of CaVβ1α on CaV1.1 mRNA levels, based on these observations it is worth speculating on the possibility of CaVβ1α acting as transcription factor, perhaps even in a self regulating fashion.
The GK domain of Cavβ subunits binds with high affinity to the AID of Cav1 channels (Chen et al. 2004). While this is the consensus binding site of the classically defined Cav1- Cavβ complex, there is evidence that an additional Cavβ subunit can bind to a region on the C-terminus of Cav1 channels (in addition to the high affinity AID) and that this secondary binding modulates the functional properties of Cav1 (Qin et al. 1996; Birnbaumer et al. 1998; Gerster et al. 1999; Canti et al. 2001; García et al. 2005). The question of Cav1 containing multiple Cavβ binding sites is particularly relevant to our present results. Colecraft’s group present model (Dalton et al. 2005) in which Cavβ initially traffics Cav1 to the plasma membrane, then either remains bound to the AID or dissociates, producing two populations of Cav1 with either high (Cavβ associated) or low (Cavβ-less) gating activity. However Colecraft’s model does not exclusively rule out the possibility of one or more additional binding sites on Cav1. The possibility of Cav1 containing multiple Cavβ binding sites of differing affinities offers a logical explanation for the pleiotropic effect of Cavβ1a shown here. If Cavβ1a is present in high concentrations (eg; exogenously applied or due to overexpression with aging), it may then be able to bind the proposed secondary, low affinity binding site with increasing frequency. In additional support of this hypothesis, the SH3 domain of Cavβ2 has recently been shown to interact with dynamin to promote endocytosis of Cav1.2 (Gonzalez-Gutierrez et al. 2007). Interestingly, this group found that disruption of the AID was necessary for the Cavβ2-dynamin mediated endocytosis, further supporting the possibility that the presence of Cavβ at a secondary binding site results in Cav1 endocytosis. Alternatively, rather than
directly mediating channel endocytosis via protein-protein interactions, Cavβ overexpression may alter Cav1 expression indirectly via calcium dependent mechanisms.

Garcia et al. (2005) showed that pressure injection of purified Cavβ1a into dissociated muscle fibers produces a rapid increase in both L-type Ca\(^{2+}\) current and intracellular Ca\(^{2+}\) release, without altering charge movement. Endogenous overexpression of Cavβ1a in the short term may produce similar physiological effects. In turn, a chronic increase in intracellular Ca\(^{2+}\) may activate some form of negative feedback, perhaps culminating in Cav1.1 endocytosis and proteolysis. Indeed, Sanchez’s group has also shown that long-term increase of L-type calcium current in skeletal muscle leads to proteolytic down-regulation of Cav1.1, likely via a local Ca\(^{2+}\)-dependent protease such as calpain (Carrillo et al. 2004).

Sarcopenia is a major cause of loss of independence in the elderly and presents a substantial public health cost (Janssen et al. 2004). As sarcopenia is caused in part by muscle dysfunction beyond the obvious loss in mass, E-C uncoupling may be a significant contributor to sarcopenia in aging humans. E-C uncoupling appears to be primarily caused by a decline in functional Cav1.1 subunits, which leads to an impairment of the mechanical coupling between neural signals and Ca\(^{2+}\) release necessary for contraction. Here we have shown that another protein, Cavβ1a, with several regulatory influences on Cav1.1, also exhibits significant changes in expression level with aging. Combined with evidence that Cavβ1a overexpression causes Cav1.1 decline in young muscle, our findings present a potentially novel and physiologically significant contributing factor to the loss of skeletal muscle strength with aging.
Experimental Procedures

Animals

Muscles were dissected from FVB (Friend Virus B, our colony) mice between 1.5 and 24 months of age. FVB mice have a maximum lifespan of 25 months and have been used previously as a model of aging skeletal muscle in our laboratory (Renganathan et al. 1998; Payne et al. 2004). Animals were housed at Wake Forest University School of Medicine (WFUSM). Mice were killed by cervical dislocation. Animal handling and procedures were approved by the Animal Care and Use Committee of WFUSM.

Microsome preparation

Isolation of the t-tubule membrane was performed using a modified version of the protocol by (Knudson et al. 1989). Briefly, whole muscles were dissected and pulverized in liquid nitrogen and then homogenized in ice-cold Buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monobasic, 1 mM MgCl₂, 0.5 mM EDTA, 303 mM sucrose with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) using a handheld tissue tearor. Homogenate was centrifuged at 12,000 RPM for 20 min at 4°C, and the pellet discarded. Supernatant was filtered through four layers of cheesecloth and centrifuged at 40,000 RPM for 90 min at 4°C in a Beckman Type Ti.70i rotor. The pellet was rinsed with ice cold PBS and resuspended with a glass homogenizer in fresh Digitonin buffer (1% digitonin (w/v), 185 mM KCl, 1.5 mM CaCl₂, 10 mM HEPES pH 7.4 with complete protease inhibitor cocktail). Samples were left on ice for 1h
and then vortexed. Protein concentration was measured using bicinchoninic protein assay using BSA digitonin standards.

**Antibodies**

Primary antibodies used for immunoblots were monoclonal VD2 to Ca\(\nu\)\(\beta\)\(1a\) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), monoclonal IIF7 to Ca\(\nu\)1.1; a generous gift from Dr. Kevin P. Campbell of the University of Iowa, and actin (Chemicon International, Temecula, CA, USA). NA931V goat anti-mouse (Amersham Health, Little Chalfont, Buckinghamshire, UK) was used as a secondary antibody.

**Western blots**

For Ca\(\nu\)\(\beta\)\(1a\) subunit, microsomes were mixed with equal volume \(\beta\)-mercaptoethanol buffer and boiled for 5 minutes. For Ca\(\nu\)1.1, microsomes were mixed with equal volume double strength Urea buffer and incubated at room temperature for 30 min (Murray & Ohlendieck 1998). SDS-PAGE was conducted using a 4.5% stacking gel with a 10% resolving gel in a Mini-Protean gel system (BioRad Laboratories, Hemel-Hempstead, Herts., UK). Gels were transferred to PVDF membranes (Amersham Health, Little Chalfont, Buckinghamshire, UK) overnight at 4°C. Blots were blocked in 5% non-fat dry milk with 0.1% Tween in TBS for Ca\(\nu\)\(\beta\)\(1a\) and PBS for all other antibodies. Primary antibody concentrations were as follows: 1:1000 (Ca\(\nu\)\(\beta\)\(1a\)), 1:5000 (Ca\(\nu\)1.1) and
1:250,000 for actin. Secondary antibodies were used at a 1:5000 dilution. Band intensity was measured using Kodak Gel Doc imaging system.

**Quantitative Real Time RT-PCR**

Total RNA was isolated using TriReagent according to the manufacturers protocol (Molecular Research Center, Cincinnati, OH), and reverse transcribed to cDNA using random hexamers. Following reverse transcription, cDNA expression was assessed by quantitative RT-PCR (Taqman Gene Expression Master Mix kit, Applied Biosystems) on a Stratagene MX3000P. Primers and probes for Ca\textsubscript{V}1.1 were purchased from Applied Biosystems.

**Muscle Electroporation**

Intramuscular plasmid injection and electroporation were performed according to Schertzer *et al.* (2006) and DiFranco *et al.* (2006). Briefly, FDB or TA muscles were injected with 30 Al of 0.5 U/Al hyaluronidase and injected 1 hr later with 20 μg Ca\textsubscript{V}\textsubscript{β1a–YFP} or 20 μg Ca\textsubscript{V}\textsubscript{β1a} siRNA equal volume saline solution. A pair of platinum plate electrodes was placed under the skin on adjacent sides of the muscle. Eight, 150 V, 20-ms square-wave pulses of 1-Hz frequency were generated using a Grass stimulator (Grass S48; W. Warwick, RI, USA) and delivered to the muscle. The polarity was then reversed and a further 8 pulses were delivered to the muscle. For sham electroporations, the same electroporation protocol was followed with a saline-only injection.

**siRNA sequences**
C2C12 cells were transfected with five siRNA sequences (Open Biosystems, Huntsville, AL, USA) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN, USA). The source of shRNA has accession # NM_031173. The following five siRNA sequences were used:

Seq. 69048:
CCGGCCAGTGTAATGAAATGA2CTACTCGAGTAGTCATTTCCATTACCAC2CTGGTTTTTG

Seq. 69049:
CCGGCCAGCAAACCATCATCTATTATCGAAGATGATGATGTGTTTGCTGGGGTTTTTG

Seq. 69050:
CCGGCCAGGGAAGCTCTCAATCCAAACTCGAGTTTGGATTGAGACTTCCCTCGTTTTTG

Seq. 69051:
CCGGCCCTCGGATACAACATCCAACACTCGA2GTGGATGTTGTATCCGAGGTTTTTG

Seq. 69052:
CCGGCCAGGGAAGAATCTGAGCTTCTGGATTGAGATTGCAGTATCCGAGGTTTTTG

Charge movement recordings
Enzymatically dissociated flexor digitorum brevis (FDB) fibers were transferred to a small, flow-through Lucite chamber positioned on a microscope stage. Fibers were continuously perfused with the external solution using a push-pull syringe pump (WPI). Only fibers exhibiting a clean surface and no contracture were used for electrophysiological recordings. Muscle fibers were voltage-clamped using an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981; Wang et al. 1999). Patch pipettes were pulled from borosilicate glass (Boralex, WPI, Sarasota, FL, USA) using a Flaming Brown micropipette puller (P97, Sutter Instrument Co., Novato, CA, USA) and then fire-polished to obtain electrode resistances ranging from 450 to 650 kΩ. In the cell-attached configuration, the seal resistance was in the range of 1-4.5 GΩ, and in the whole-cell configuration, values ranged between 75 and 120 MΩ (Wang et al. 1999). The pipette was filled with the following solution: 140 mM Cs-aspartate, 5 mM Mg-aspartate, 20 mM Cs₂EGTA (ethylene glycol-bis(â-aminoethyl ether)-N,N,N′,N′'-tetraacetic acid), and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), and pH was adjusted to 7.4 with CsOH (Adams et al., 1990; Wang et al., 1999). The external solution contained: 150 mM TEA(tetraethylammonium hydroxide)-CH₃SO₃, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Na-HEPES, 0.05 mM BTS, and 0.001 mM tetrodotoxin (Delbono 1992; Delbono et al. 1997). Solution pH was adjusted to 7.4 with CsOH. All the experiments were conducted at room temperature (21-22°C).

Confocal microscopy
FDB fibers fluorescence was analyzed using a Radiance 2100 confocal microscope (Bio-Rad/Zeiss). YFP fluorescence was detected at 488nm. Confocal microscope fluorescence acquisition parameters were maintained constant across recordings are described below.

**Single Intact Muscle Fiber Contraction**

At time of sacrifice, FDB muscles were carefully dissected and pinned into a Petri dish lined with Sylgard (Dow Corning, Auburn, MI, USA) in a Ca\(^{2+}\)-containing physiological solution (see below). All contraction experiments were carried out at room temperature (21-22°C). Single intact fiber dissection followed procedures previously published (Lannergren & Westerblad 1987; Gonzalez *et al.* 2000). Following dissection, tendons of single intact fibers were placed in custom-made micro-clips, and these clips were connected to a force transducer and a micropositioner for length control. Fibers were adjusted to optimum length (L\(_O\)) by using single twitches, elicited by 0.5-ms square wave pulses at 10 V. Once at L\(_O\), fibers were stimulated with 350-ms trains of pulses, using frequencies varying from 50 to 100 Hz. The stimulation frequency that elicited maximum force was used for the remainder of the experiment (Payne *et al.* 2004).

**Statistical analysis**

All data are presented as means ± s.e.m. Data were analyzed with *Student* t-test or one-way repeated measures ANOVA, with Tukey’s multiple comparisons test applied *post hoc* when appropriate. An alpha value of *P* < 0.05 was considered significant.
ACKNOWLEDGMENTS

The present study was supported by grants to Osvaldo Delbono from the National Institutes of Health/National Institute on Aging (AG13934, AG033385, and AG15820), the Muscular Dystrophy Association (MDA #33149), and the Wake Forest University Claude D. Pepper Older Americans Independence Center (P30-AG21332). The authors would like to thank Dr. Kurt G. Beam (University of Colorado Health Sciences, Department of Physiology and Biophysics) for providing the CaVβ1a–YFP plasmid, Dr. Franz Hofmann (University of Saarland, Pharmacology and Toxicology) for the initial cDNA sequence of CaVβ1a, and Dr. Hang Shi and Dr. Juan Codina for their expert assistance with the qRT-PCR experiments.
References


FIGURE LEGENDS

**Fig. 1 Relative Ca\textsubscript{v}β\textsubscript{1a} expression across the lifespan of FVB mice.** (A) Representative immunoblot of Ca\textsubscript{v}β\textsubscript{1a} (52 kDa) expression in P100 fractions collected from gluteus and hamstring muscles (pool) at 2, 6, 14, and 24 months of age (24 months represents the very old age of this strain). Below is the mean Ca\textsubscript{v}β\textsubscript{1a} band intensity for each age group (n=4), normalized to the intensity of the corresponding actin band. Each age group reached a statistically significant difference from all of the others, with the exception of 6 and 14 month age groups. Data represent mean ± SE. *P \leq 0.01 (one-way ANOVA). (B) The same as A, except representative of TA and EDL muscle groups (n=4). The 24 month age group reached significant difference from all other ages. *P < 0.01(one-way ANOVA). (C) Mean fold increase of pool and TA/EDL samples by age. Values represent normalized sample values from each age group divided by the corresponding 2 month normalized value.

**Fig. 2 Ca\textsubscript{v}1.1 and Ca\textsubscript{v}β\textsubscript{1a} expression in TA muscles following *in vivo* electroporation of a Ca\textsubscript{v}β\textsubscript{1a}-YFP plasmid.** (A) Representative immunoblots from control muscle (lane 1) and from muscles harvested 3 days (lane 2), 1 week (lane 3), and 2 weeks (lane 4) post-electroporation. (B) Ca\textsubscript{v}1.1 and endogenous Ca\textsubscript{v}β\textsubscript{1a} protein expression in muscle following Ca\textsubscript{v}β\textsubscript{1a}-YFP electroporation (n=4 for 3 days and 2 weeks, n=3 at 1 week), normalized to control (non-electroporated) muscle. Sham electroporation (n=4 at each time point) produced no significant difference in relative Ca\textsubscript{v}1.1 or Ca\textsubscript{v}β\textsubscript{1a} expression. Data represent mean ± SE. *P < 0.001 (one-way ANOVA). (C) Ca\textsubscript{v}1.1 mRNA levels
measured using qPCR in control (non electroporated, n=3) and Cavβ1a-YFP electroporated (2 weeks, n=4) animals.

Fig. 3  Cav1.1 charge movement (Q) and YFP (Cavβ1a-YFP) fluorescence (F) in electroporated FDB fibers.  (A) Qmax-F relationship of single fibers electroporated with Cavβ1a-YFP (●)(n=10) or GFP (○)(n=10).  Detection of F above ~40 AU correlates with decreased charge movement (r²=0.91).  (B) Two representative fibers from A.  Fiber D0912 exhibits F=42 AU, Q=52.6 nC/μF.  Fiber C0925 exhibits F=71.3 AU, Q=26.6 nC/μF.  (Also see Fig. 4 for more detail on these two fibers).  Fluorescence acquisition parameters on confocal microscope: 20x objective, zoom = 3.5x, Iris = 3.8mm, Gain = 80, box size = 512x512 pixels (172.6x172.6 μm), pixel dwell time 37.76 μs.  Excitation wavelength = 488 nm, Emission = 528 nm.

Fig. 4  Charge movement from FDB fibers expressing Cavβ1a-YFP.  (A – B) Charge movement recordings from fiber D0912 (A), representing a fiber expressing low levels of Cavβ1a-YFP (indicated by F intensity in Figure 3), and from fiber C0925 (B), representing a fiber expressing high levels of Cavβ1a-YFP (indicated by F intensity in Figure 3).  (C) The voltage step protocol.  A 2-sec pre-pulse at -30 mV was followed by a 15-ms repolarization to -50 mV, followed by a 25-ms test pulse of varying membrane voltage and repolarization.  (D) Absolute Q-V relationship of fibers D0912 (●) and C0925 (○).  Note that the fiber which expresses higher levels of Cavβ1a-YFP displays lower charge movement.  (E) Relative Q-V relationship of the same fibers in D.  Q values were
normalized to $Q_{\text{max}}$ for each fiber. Overexpression of Cavβ1a-YFP does not shift the $Q$-$V$ curve in the voltage axis or modify the steepness of the curve. Data points were fitted to a Boltzmann equation of the form: $Q = \frac{Q_{\text{max}}}{1 + \exp(V_{1/2} - V_m)/k}$, where $Q_{\text{max}}$ is the maximal charge; $V_{1/2}$ is the charge half-activation potential; $V_m$ is the membrane potential; and $k$ is the steepness of the curve. $Q_{\text{max}}$, $V_{1/2}$, and $K$ values were: 56.9 nC/μF, 12.3 mV and 3.37 for fiber D0912, and 30.2 nC/μF, 17.1 mV, and 14.9 for fiber C0925.

**Fig. 5** Specific force of single intact FDB fibers from young mice electroporated and expressing Cavβ1a-YFP or GFP. (A) Specific force (kPa) of single intact FDB fibers from Cavβ1a-YFP (n=7), GFP (n=7) and non-electroporated controls (n=3). Cavβ1a-YFP expressing fibers display significantly lower specific force compared to GFP expressing fibers. Data represent mean ± SE. *P<0.05 (one-way ANOVA) (B) Representative tetanic contraction traces from fibers expressing Cavβ1a-YFP, GFP, and control groups.

**Fig. 6** siRNA restoration of charge movement *in vivo* (A) Effect of five different siRNA sequences on Cavβ1a expression in C2C12 myotubes. Data are presented as percent change in immunoblot band intensity versus control. Clones 69051 and 69052 cause the greatest decline in endogenous Cavβ1a expression (37% and 28% decline, respectively). (B) Charge movement from FDB muscle fibers 7-11 days post-electroporation. FDB muscles were electroporated with 20 μg of siRNA sequence 69051 and 10 μg of siRNA sequence 69052. Fibers were dissociated, patch-clamped, and subjected to the same protocol as in Figure 4. The number of fibers examined for young control, sham GFP-electroporated and Cavβ1a siRNA electroporated was 13, 10, and 17,
respectively, and for old control, Cav\(\beta_{1a}\) siRNA and sham electroporated was 21, 16 and 11, respectively. Data represent mean ± SE. *P <0.05 (one-way ANOVA).

Table 1. Contractile Properties of Control and Electroporated Fibers.

Figure List

Figure 1
FIGURE 2
Figure 3
FIGURE 4
FIGURE 5
FIGURE 6
Supplementary Tables for Chapter 2

Supplementary Table 1 - Primer sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward (5’-3’), OR sequence (shRNA), OR product ID (Taqman probes)</th>
<th>Reverse 5’-3’</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavβ1a</td>
<td>GTCCAGAAGAGCGGCATGTC</td>
<td>GAAGGGGATGC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>B-actin</td>
<td>TGAGCTCGGTTTTACACCCCTTTCT</td>
<td>ACTCAGGGCATG</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Cavβ1a-58-524</td>
<td>GCTAGAAATTCATGTCCGACGGGA GCACCT</td>
<td>GCTAGTCGACAT GGCATGTCCTGG C</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>Cavβ1a-101-524</td>
<td>GCTAGAAATTCATGAGGCTTTTGTC TGTCGGACAAAT</td>
<td>GCTAGTCGACAT GGCATGTCCTGG C</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>Cavβ1a-161-524</td>
<td>GCTAGAAATTCATGGAGAGGCCATCA TCCTGTTG</td>
<td>GCTAGTCGACTG GTTTGTCTTGG CTTT</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>Cavβ1a-101-274</td>
<td>GCTAGAAATTCATGCCCCCCCAACTGGACAGC</td>
<td>GCTAGTCGACGG AAGGCACCACGT C</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>Cavβ1a-1-99</td>
<td>GCTAGAAATTCATGCGCAAGAGA GCGCATGTCCC</td>
<td>GCTAGGATCTTT TGGTCTTGGCTT TCTCGAG</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>Cavβ1a-161-274</td>
<td>GCTAGAAATTCAT GTGGCTTTTGCTGTCGGACAAAT</td>
<td>GCTAGTCGACGG AAGGCACCACGT C</td>
<td>Cavβ1a-YFP truncations</td>
</tr>
<tr>
<td>69048 (Open Biosystems)</td>
<td>CCGGCCCAGCTGGTAAAGAAATGACT ACTCGAGTAGCTTTTCATTACCAC TGGTTTTTG</td>
<td></td>
<td>shRNA</td>
</tr>
<tr>
<td>69049 (Open Biosystems)</td>
<td>CCGGCCCAGCAACACACATCATCAT TCTCGAGATGATGATGTGTTGCT GGGTTTTTG</td>
<td></td>
<td>shRNA</td>
</tr>
<tr>
<td>69050 (Open Biosystems)</td>
<td>CCGGCGAGGGAAGTCTCAATCCAA ACTCGAGTGGATTGAGACTTCCC TCGTTTTTG</td>
<td></td>
<td>shRNA</td>
</tr>
<tr>
<td></td>
<td>Sequence 1</td>
<td>Sequence 2</td>
<td>Method</td>
</tr>
<tr>
<td>----</td>
<td>------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>69051 (Open Biosystems)</td>
<td>CCGGCCCTCGGATACAACATCCAAC ACTCGAGTGGTAGGATGTTGATCAGG AGGTTTTTG</td>
<td>shRNA</td>
<td></td>
</tr>
<tr>
<td>69052 (Open Biosystems)</td>
<td>CCGGGGCTCAGGAGAAATCTCAGCT TCTCGAGAAGCTGAGATTTCTCCTG AGCTTTTTTG</td>
<td>shRNA</td>
<td></td>
</tr>
<tr>
<td>SHC002 (Sigma)</td>
<td>CCGGCAACAAGATGAAGACACCA ACTCGAGTGGGCTTCCATCTTG TTGTTTTT</td>
<td>shRNA</td>
<td></td>
</tr>
<tr>
<td>Casp2</td>
<td>TGCAGCCCGGAAAAGCTGG</td>
<td>AGGCGAGAAGT AGCGCGGGA</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Dlx2</td>
<td>GCCGGCACCCTCACCCTAGCA</td>
<td>GTCTCATCGG GCCATCCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Cbx5</td>
<td>ACGGATCTCTCCTGCGCCT</td>
<td>ACGATTTCCGC CTGCTGCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Cbx5</td>
<td>GCCAGCGACGCGGGAACTCGT</td>
<td>ACGGCATCCGGT TGGCGTT</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Cbx5</td>
<td>GCCGCCCTTCCATCTGTCGT</td>
<td>ATCCGGTTTGGGC GGGCGTT</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Pax3#1</td>
<td>GGCGGAGGACTTGGGTGCGA</td>
<td>GCACGCGGTCGC ACGACGGG</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Pax3#2</td>
<td>CCCGTCGTCGACACCAGACTGC</td>
<td>GCCCTGGGGACCG TCAGGGAT</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Pax3#3</td>
<td>AGGCAGCCAGATTGGGTGCTG</td>
<td>GCCGTTGGGCTG GGTCTGCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Tbx5</td>
<td>GACGCTCGTCTTGCTCCGTG</td>
<td>AGAGGTAGGGGTG GGTCTGCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Tnnt3</td>
<td>CCCACCAGCACCACACACGAC</td>
<td>GCCCATGAGCAG ACCTCGCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Tnnt3</td>
<td>GCCTGCTTGATGGGACACCCTC</td>
<td>GGCTGGGGGCTC CTTGCGA</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Wnt3</td>
<td>GCCCAAGCCCGACCCTTCAC</td>
<td>GGGCAGGCTGATA AGGTAAG</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Myog#1</td>
<td>GACCCCTTCCAGCTCGTCGA</td>
<td>GTGGGGGTGGGT GCATTCCC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Myog#2</td>
<td>GTTGGTGTAGGGGCTTCGG</td>
<td>CCCTCGGCTGTT CGGGCTTA</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Myog#3</td>
<td>CCCACCTCCCTGCCCCACA</td>
<td>CCGCAGCCCCTC ACACCAAC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
<td>-------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>BDNF I</td>
<td>CCTGCATCTGTTGGGAGAC</td>
<td>GCCCTTGCTCGTG GACGTTTA</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Caenb1</td>
<td>GAGAGACATGACAGACTCAGCTC</td>
<td>GAGA</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>Myog (Applied Bio)</td>
<td>Mm00446194_m1</td>
<td>ACACCCCTGCCC AGTGTAAGAGC</td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>GAPDH (Applied Bio)</td>
<td>Mm03302249_g1</td>
<td></td>
<td>ChIP-PCR</td>
</tr>
<tr>
<td>EMSA Probe #1</td>
<td>5’-TGGTGGCGCATGCCTTTAATCCAG CACTCGGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMSA Probe #2</td>
<td>5’-CGTCTTGATGTGCAGCAACAGCTTA GA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Supplementary Table 2 - Antibodies**

<table>
<thead>
<tr>
<th>Name</th>
<th>Clone</th>
<th>Dilution</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavβ1α (Santa Cruz)</td>
<td>H50</td>
<td>1:500</td>
<td>WB, ICC</td>
</tr>
<tr>
<td>Cavβ1α (GeneTex)</td>
<td>CAB1</td>
<td>1:500</td>
<td>WB</td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td>1:500,000</td>
<td>WB</td>
</tr>
<tr>
<td>Cavβ1.1 (Developmental Studies Hybridoma Bank)</td>
<td>IID5E1</td>
<td>1:10,000</td>
<td>WB</td>
</tr>
<tr>
<td>Troponin T</td>
<td>RV-C2</td>
<td>1:500</td>
<td>WB</td>
</tr>
<tr>
<td>Normal Mouse IgG (Santa Cruz)</td>
<td>SC-2025</td>
<td>1:500</td>
<td>WB, ICC</td>
</tr>
<tr>
<td>Normal Rabbit IgG (Santa Cruz)</td>
<td>SC-2027</td>
<td>1:500</td>
<td>WB, ICC, ChIP-chip</td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>GAPDH (Imgenex)</td>
<td>1D4</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>HP1 (Santa Cruz)</td>
<td>FL-191</td>
<td>1:500</td>
<td>WB</td>
</tr>
<tr>
<td>GFP (Invitrogen)</td>
<td>3E6</td>
<td></td>
<td>IP, ChIP</td>
</tr>
<tr>
<td>α7 Integrin-APC (Ablab)</td>
<td>R2F2</td>
<td>1:50</td>
<td>FACS</td>
</tr>
<tr>
<td>CD31-FITC (eBioscience)</td>
<td>390</td>
<td>1:50</td>
<td>FACS</td>
</tr>
<tr>
<td>CD45-FITC (eBioscience)</td>
<td>30-F11</td>
<td>1:50</td>
<td>FACS</td>
</tr>
<tr>
<td>Antibody</td>
<td>dilution</td>
<td>method</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>----------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>Ki67 (Novus)</td>
<td>SP6</td>
<td>1:500</td>
<td>ICC</td>
</tr>
<tr>
<td>Pax7 (Developmental Studies Hybridoma Bank)</td>
<td>PAX7</td>
<td>1:100</td>
<td>ICC</td>
</tr>
<tr>
<td>Pax7 (Pro Sci Inc)</td>
<td>27-687</td>
<td>1:50</td>
<td>IHC</td>
</tr>
<tr>
<td>Myogenin (Developmental Studies Hybridoma Bank)</td>
<td>F5D</td>
<td>1:100</td>
<td>ICC</td>
</tr>
<tr>
<td>Sheep anti mouse HRP (Santa Cruz)</td>
<td>NA931VS</td>
<td>1:25000</td>
<td>WB</td>
</tr>
<tr>
<td>Donkey anti rabbit HRP (Amersham)</td>
<td>NA9340V</td>
<td>1:25000</td>
<td>WB</td>
</tr>
<tr>
<td>Alexafluor 488 (Invitrogen)</td>
<td></td>
<td>1:1000</td>
<td>ICC, IHC</td>
</tr>
<tr>
<td>Alexafluor 568 (Invitrogen)</td>
<td></td>
<td>1:1000</td>
<td>ICC</td>
</tr>
</tbody>
</table>

**Supplementary Table 3 - ChIP buffers**

**Pre-IP Dilution Buffer (Store at RT)**

- 10 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5)
- 10 mM NaCl
- 3 mM MgCl2
- 1 mM CaCl2
- 4% IGEPAL
- 1 mM PMSF (add fresh)

**IP Dilution Buffer (Store at RT without protease inhibitors)**

- 20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8)
- 2 mM EDTA
1% Triton X-100

150 mM NaCl

Protease Inhibitor Stock (add fresh)

**Protease Inhibitor Stock**

Prepare a 25X stock by dissolving 1 protease inhibitor tablet in 2 mL of nuclease-free water

**ChIP Wash 1 (Store at RT)**

20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8)

2 mM EDTA

1% Triton X-100

150 mM NaCl

1 mM PMSF (add fresh)

**ChIP Wash 2 (Store at RT)**

20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8)

2 mM EDTA

1% Triton X-100

0.1% SDS

500 mM NaCl

1 mM PMSF (add fresh)

**ChIP Wash 3 (Store at RT)**

10 mM Tris-HCl (made from stock 1M Tris-HCl pH 8)
1 mM EDTA
0.25M LiCl
0.5% IGEPAL
0.5% Deoxycholate (sodium salt)

**Elution Buffer**

25 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5)
10 mM EDTA
0.5% SDS

**Supplementary Table 4. Ca\(_{\beta1a}\)-ChIP-on-chip enriched gene promoters**

<table>
<thead>
<tr>
<th>REFSEQ_MRN</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_213730</td>
<td>keratin 39</td>
</tr>
<tr>
<td>NM_010604</td>
<td>potassium inwardly-rectifying channel, subfamily J, member 16</td>
</tr>
<tr>
<td>NM_177588</td>
<td>threonine synthase-like 1 (bacterial)</td>
</tr>
<tr>
<td>NM_012032</td>
<td>serine incorporator 3</td>
</tr>
<tr>
<td>NM_011206</td>
<td>protein tyrosine phosphatase, non-receptor type 18</td>
</tr>
<tr>
<td>NM_199150</td>
<td>cDNA sequence BC049730</td>
</tr>
<tr>
<td>NM_145839</td>
<td>RasGEF domain family, member 1B; hypothetical protein</td>
</tr>
<tr>
<td></td>
<td>LOC100044232</td>
</tr>
<tr>
<td>NM_007414</td>
<td>ADP-ribosylarginine hydrolase</td>
</tr>
<tr>
<td>NM_029348</td>
<td>zinc finger and BTB domain containing 4</td>
</tr>
<tr>
<td>NM_011879</td>
<td>IK cytokine</td>
</tr>
<tr>
<td>NM_027121</td>
<td>vitamin K epoxide reductase complex, subunit 1-like 1</td>
</tr>
<tr>
<td>NM_026480</td>
<td>oocyte expressed protein homolog (dog)</td>
</tr>
<tr>
<td>NM_027478</td>
<td>RIKEN cDNA 5730494N06 gene; predicted gene 7368</td>
</tr>
<tr>
<td>NM_011709</td>
<td>whey acidic protein</td>
</tr>
<tr>
<td>NM_026577</td>
<td>ADP-ribosylation factor-like 13B</td>
</tr>
<tr>
<td>NM_001045522</td>
<td>cDNA sequence BC030499</td>
</tr>
<tr>
<td>NM_028076</td>
<td>transmembrane and ubiquitin-like domain containing 2</td>
</tr>
<tr>
<td>NM_172836</td>
<td>RIKEN cDNA 9930021J03 gene</td>
</tr>
<tr>
<td>NM_203489</td>
<td>vomeronasal 1 receptor, D15</td>
</tr>
<tr>
<td>NM_011184</td>
<td>proteasome (prosome, macropain) subunit, alpha type 3;</td>
</tr>
</tbody>
</table>
NM_054071 predicted gene 5406
NM_080843 fibroblast growth factor receptor-like 1; similar to fibroblast growth factor receptor 5 beta
NM_010640 suppressor of cytokine signaling 4
NM_146729 kallikrein 1-related peptidase b11
NM_172823 olfactory receptor 784
NM_178714 leishmanolysin-like (metallopeptidase M8 family)
NM_022428 leucine rich repeat and fibronectin type III domain containing 5
NM_010516 Iroquois related homeobox 6 (Drosophila)
NM_011063 cysteine rich protein 61
NM_029045 suppressor of cytokine signaling 4
NM_181682 desmoglein 1 beta
NM_153521 leucine rich repeat containing 41
NM_145439 transmembrane channel-like gene family 6
NM_023564 phospholipid scramblase 3; similar to phospholipid scramblase 3
NM_172595 ADP-ribosylation factor-like 15
NM_010054 distal-less homeobox 2
NM_008137 guanine nucleotide binding protein, alpha 14
NM_199301 mitochondrial GTPase 1 homolog (S. cerevisiae)
NM_207152 olfactory receptor 1524, pseudogene 1; olfactory receptor 1338
NM_011099 predicted gene 6560; predicted gene 2124; predicted gene 6992; pyruvate kinase, muscle; similar to M2-type pyruvate kinase
NM_010279 glial cell line derived neurotrophic factor family receptor alpha 1
NM_007738 collagen, type VII, alpha 1
NM_025972 N-acylethanolamine acid amidase
NM_026137 WD repeat domain 13
NM_009452 tumor necrosis factor (ligand) superfamily, member 4
NM_020579 UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3
NM_181075 RIKEN cDNA 2610524H06 gene
NM_011461 Spi-C transcription factor (Spi-1/PU.1 related)
NM_001037927 WD repeat domain 93
NM_008006 fibroblast growth factor 2
NM_00103267 glutamine rich 2
NM_010937 similar to neuroblastoma ras oncogene; neuroblastoma ras oncogene
NM_026917 zinc finger, DHHC domain containing 3
NM_146458 olfactory receptor 1199
NM_011787 autocrine motility factor receptor
NM_025487 RIKEN cDNA 1700011A15 gene
NM_139140 spermatogenesis associated, serine-rich 2
NM_026403 RIKEN cDNA 2610027L16 gene
NM_173004 contactin 4
NM_028141 zinc finger protein 661
NM_144914  transmembrane protein 184a
NM_172473  HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1
NM_026380  regulator of G-protein signaling 8
NM_016961  mitogen-activated protein kinase 9
NM_025331  guanine nucleotide binding protein (G protein), gamma 11
NM_026301  ring finger protein 125
NM_027423  polymerase (RNA) III (DNA directed) polypeptide B
NM_027852  retinoic acid receptor responder (tazarotene induced) 2
NM_028043  DNA segment, Chr 1, Brigham & Women's Genetics 0212 expressed
NM_023186  chitinase, acidic
NM_009820  runt related transcription factor 2
NM_172913  TOX high mobility group box family member 3
NM_178657  predicted gene 2042; predicted gene 10436; oogenesin 1; expressed sequence C87977
NM_008768  orosomucoid 1
NM_007386  aconitase 1
NM_177190  t-complex 11 like 1
NM_026724  ribosomal protein L34; predicted gene 10154; similar to ribosomal protein L34; predicted gene 7800; predicted gene 4705
NM_011173  protein S (alpha)
NM_139807  vaserin
NM_023544  regulatory solute carrier protein, family 1, member 1; DNA-damage inducible protein 2
NM_001045514  AT-hook transcription factor
NM_001005847  aspartylglucosaminidase
NM_172589  lipoma HMGIC fusion partner-like 2
NM_198092  ubiquitin specific peptidase 2
NM_178683  DEP domain containing 1B
NM_172405  family with sequence similarity 175, member A
NM_175266  EPM2A (laforin) interacting protein 1
NM_133350  microtubule-associated protein, RP/EB family, member 3
NM_172786  interleukin 20 receptor, alpha
NM_138664  DnaJ (Hsp40) homolog, subfamily C, member 28
NM_175150  thioredoxin domain containing 15
NM_019456  amyloid beta (A4) precursor protein-binding, family B, member 1
NM_011537  T-box 5
NM_008216  hyaluronan synthase 2
NM_172796  similar to putative protein; schlafen 9; similar to schlafen 9
NM_001039552  RIKEN cDNA 9030025P20 gene; predicted gene 3435; RIKEN cDNA 2210404J11 gene
NM_001034891  RIKEN cDNA 9030025P20 gene; predicted gene 3435; RIKEN
<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_146432</td>
<td>olfactory receptor 1512</td>
</tr>
<tr>
<td>NM_001024147</td>
<td>predicted gene 5868</td>
</tr>
<tr>
<td>NM_019868</td>
<td>heterogeneous nuclear ribonucleoprotein H2</td>
</tr>
<tr>
<td>NM_016661</td>
<td>similar to Adenosylhomocysteinase (AdoHcyase)</td>
</tr>
<tr>
<td></td>
<td>(Liver copper-binding protein) (CUBP); S-adenosylhomocysteine hydrolase</td>
</tr>
<tr>
<td>NM_009946</td>
<td>complexin 2</td>
</tr>
<tr>
<td>NM_018863</td>
<td>prodynorphin</td>
</tr>
<tr>
<td>NM_001033451</td>
<td>zinc finger protein 408</td>
</tr>
<tr>
<td>NM_029943</td>
<td>apurinic/apyrimidin endonuclease 2</td>
</tr>
<tr>
<td>NM_016888</td>
<td>UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2</td>
</tr>
<tr>
<td>NM_009489</td>
<td>vomeronasal 2, receptor pseudogene 44; vomeronasal 2, receptor 34;</td>
</tr>
<tr>
<td>NM_028894</td>
<td>LON peptidase N-terminal domain and ring finger 3</td>
</tr>
<tr>
<td>NM_009413</td>
<td>tumor protein D52-like 1</td>
</tr>
<tr>
<td>NM_007610</td>
<td>caspase 2</td>
</tr>
<tr>
<td>NM_008689</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
</tr>
<tr>
<td>NM_144553</td>
<td>discs, large (Drosophila) homolog-associated protein 5</td>
</tr>
<tr>
<td>NM_027427</td>
<td>TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor</td>
</tr>
<tr>
<td>NM_010216</td>
<td>c-fos induced growth factor</td>
</tr>
<tr>
<td>NM_026435</td>
<td>ubiquitin-fold modifier 1</td>
</tr>
<tr>
<td>NM_025450</td>
<td>mitochondrial ribosomal protein S17</td>
</tr>
<tr>
<td>NM_153099</td>
<td>testis serine protease 2</td>
</tr>
<tr>
<td>NM_020017</td>
<td>melanoma antigen, family A, 3</td>
</tr>
<tr>
<td>NM_207565</td>
<td>olfactory receptor 1113</td>
</tr>
<tr>
<td>NM_013754</td>
<td>insulin-like 6</td>
</tr>
<tr>
<td>NM_017463</td>
<td>pre B-cell leukemia transcription factor 2</td>
</tr>
<tr>
<td>NM_009985</td>
<td>cathepsin W</td>
</tr>
<tr>
<td>NM_010716</td>
<td>ligase III, DNA, ATP-dependent</td>
</tr>
<tr>
<td>NM_199058</td>
<td>G protein-coupled receptor 6</td>
</tr>
<tr>
<td>NM_008878</td>
<td>serine (or cysteine) peptidase inhibitor, clade F, member 2</td>
</tr>
<tr>
<td>NM_001034910</td>
<td>defensin beta 42</td>
</tr>
<tr>
<td>NM_021517</td>
<td>PDZ domain containing 1</td>
</tr>
<tr>
<td>NM_199311</td>
<td>C-type lectin domain family 4, member a1</td>
</tr>
<tr>
<td>NM_019702</td>
<td>similar to Hbs11 protein; predicted gene 9923; Hbs1-like (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_019553</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 21</td>
</tr>
<tr>
<td>NM_016892</td>
<td>copper chaperone for superoxide dismutase</td>
</tr>
<tr>
<td>NM_028784</td>
<td>coagulation factor XIII, A1 subunit</td>
</tr>
<tr>
<td>NM_009033</td>
<td>RNA binding motif protein, X chromosome retrogene</td>
</tr>
<tr>
<td>NM_175031</td>
<td>serine/threonine kinase 36 (fused homolog, Drosophila)</td>
</tr>
</tbody>
</table>
NM_153167  WD repeat domain 32
NM_008065  GA repeat binding protein, alpha
NM_199157  interferon kappa
NM_146649  olfactory receptor 1160
NM_013469  annexin A11; predicted gene 2260; predicted gene 2274
NM_008152  G-protein coupled receptor 65
NM_011606  C-type lectin domain family 3, member b
NM_001024714 hypothetical protein LOC100044175; chymase 2, mast cell
NM_010269  ganglioside-induced differentiation-associated-protein 2
NM_198862  neuregulin 2
NM_146801  olfactory receptor 904
NM_147079  hypothetical protein LOC100044258; olfactory receptor 547
NM_009376  intraflagellar transport 88 homolog (Chlamydomonas)
NM_010256  phosphoribosylglycinamidase formyltransferase
NM_207574  olfactory receptor 1383
NM_009044  reticulendotheliosis oncogene
NM_027772  prenyl (solanesyl) diphosphate synthase, subunit 2
NM_033322  leucine zipper transcription factor-like 1; predicted gene 6776
NM_028066  coagulation factor XI
NM_025345  RIKEN cDNA 0610037P05 gene
NM_010655  similar to ribosomal protein L38; predicted gene 13020; ribosomal protein L38; predicted gene 4991; karyopherin (importin) alpha 2; predicted gene 9028; predicted gene 8129; predicted gene 7123; predicted gene 5832; p predicted gene 10184; predicted gene 7379; predicted gene 10259
NM_146908  olfactory receptor 1280
NM_178920  mal, T-cell differentiation protein 2
NM_025816  Tax1 (human T-cell leukemia virus type I) binding protein 1
NM_009141  similar to LPS-induced CXC chemokine; chemokine (C-X-C motif) ligand 5
NM_172961  4-aminobutyrate aminotransferase
NM_026817  RAB, member of RAS oncogene family-like 2A
NM_030735  vomeronasal 1 receptor, D9; vomeronasal 1 receptor, D22; predicted gene 5892
NM_010670  keratin associated protein 12-1
NM_027357  proteasome (prosome, macropain) 26S subunit, non-ATPase, 1
NM_133971  ankkyrin repeat domain 10
NM_010226  forkhead box S1
NM_030718  ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase, transferase B, alpha 1-3-galactosyltransferase)
NM_010372  granzyme D
NM_030696  solute carrier family 16 (monocarboxylic acid transporters), member 3
NM_199422  S100 calcium binding protein A7A
NM_001003911  a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 7
NM_054049  odd-skipped related 2 (Drosophila)
NM_175032  RIKEN cDNA 4930431L04 gene
NM_028815  centrosomal protein 97
NM_010952  predicted gene 9329; predicted gene 7543; ornithine decarboxylase antizyme 2
NM_024188  3-oxoacid CoA transferase 1
NM_134127  cytochrome P450, family 4, subfamily f, polypeptide 15
NM_001045542  predicted gene 12253
NM_009804  catalase
NM_009843  indolethylamine N-methyltransferase
NM_021331  glucose-6-phosphatase, catalytic, 2
NM_001007572  transient receptor potential cation channel, subfamily V, member 5
NM_175003  expressed sequence AU040829
NM_011455  serine (or cysteine) peptidase inhibitor, clade B, member 9g
NM_008950  protease (prosome, macropain) 26S subunit, ATPase 5
NM_009803  nuclear receptor subfamily 1, group I, member 3
NM_198415  creatine kinase, mitochondrial 2
NM_028370  protection of telomeres 1B
NM_027838  SUMO/sentrin specific peptidase 8
NM_007663  cadherin 16
NM_032394  myosin VIIB
NM_009647  predicted gene 13639; similar to adenylate kinase 4; adenylate kinase 3-like 1
NM_001076789  chromobox homolog 5 (Drosophila HP1a)
NM_007542  biglycan
NM_001005860  C-type lectin domain family 4, member a4
NM_018830  N-acylsphingosine amidohydrolase 2
NM_001025574  RIKEN cDNA 5430413K10 gene; RIKEN cDNA 4833413D08 gene
NM_146622  olfactory receptor 360
NM_021380  interleukin 20
NM_026637  gamma-glutamyl cyclotransferase
NM_008366  interleukin 2; similar to Interleukin-2 precursor (IL-2) (T-cell growth factor) (TCGF)
NM_134184  vomeronasal 1 receptor, C29
NM_173053  LIM motif-containing protein kinase 2
NM_001003672  similar to protocadherin; protocadherin alpha 10; protocadherin alpha 9; protocadherin alpha 6; protocadherin alpha 12; similar to protocadherin alpha 11; protocadherin alpha 7; protocadherin alpha 11; protocadherin alpha 1; protocadherin alpha 5; protocadherin alpha 4; protocadherin alpha 2; protocadherin alpha subfamily C, 1; protocadherin alpha subfamily C, 2; protocadherin alpha cluster
cortexin 1
NM_183315  capping protein (actin filament) muscle Z-line, alpha 1; similar to capping protein (actin filament) muscle Z-line, alpha 1; predicted gene 3608; predicted gene 5920
NM_175015  ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3
NM_019939  membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)
NM_173420  RIKEN cDNA D930020E02 gene
NM_028127  predicted gene 5780; FERM domain containing 6
NM_011867  solute carrier family 26, member 4
NM_178888  GTPase activating RANGAP domain-like 3
NM_207302  zinc finger, RAN-binding domain containing 1
NM_207708  synaptogyrin 1
NM_028550  RIKEN cDNA 1700074P13 gene
NM_010829  mutS homolog 3 (E. coli)
NM_011824  gremlin 1
NM_018792  histone H1-like protein in spermatids 1
NM_008353  interleukin 12 receptor, beta 1
NM_146330  olfactory receptor 958
NM_133755  tubulin, gamma complex associated protein 2
NM_147045  olfactory receptor 683
NM_025708  transmembrane protein 186
NM_175092  ras homolog gene family, member f
NM_183115  coiled-coil domain containing 125
NM_145542  S-adenosylhomocysteine hydrolase-like 1
NM_028876  transmembrane emp24 protein transport domain containing 5;
similar to Transmembrane emp24 protein transport domain containing 5
NM_028910  olfactory receptor 701
NM_053147  protocadherin beta 22
NM_026270  AKT1 substrate 1 (proline-rich)
NM_008944  proteasome (prosome, macropain) subunit, alpha type 2
NM_011199  parathyroid hormone 1 receptor
NM_008901  POU domain, class 3, transcription factor 4
NM_134232  predicted gene 9807; similar to vomeronasal receptor V1RG10;
vomeronasal 1 receptor, G10; vomeronasal 1 receptor, G10-like
NM_025724  RIKEN cDNA 4921510H08 gene
NM_009711  artemin
NM_178067  myosin binding protein C, fast-type
NM_009559  zinc finger protein 57
NM_026188  hypothetical protein LOC100044197; RIKEN cDNA 1700028P14 gene
NM_028242  HIV TAT specific factor 1
NM_134089  scribbled homolog (Drosophila)
NM_007802  cathepsin K
NM_144917  ELMO/CED-12 domain containing 3
NM_178397  Fas associated factor family member 2
NM_027439  ATPase, H+ transporting, lysosomal accessory protein 2
NM_029367  sperm acrosome associated 3
NM_175401  F-box and WD-40 domain protein 17
NM_001011774 olfactory receptor 1028
NM_009102  retinal pigment epithelium derived rhodopsin homolog
NM_134059  DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
NM_015798  F-box protein 15
NM_021345  protein tyrosine phosphatase-like A domain containing 1
NM_026404  predicted gene 5866; solute carrier family 35, member A4
NM_027399  six transmembrane epithelial antigen of the prostate 1
NM_013526  growth differentiation factor 6
NM_029286  RIKEN cDNA 1700041C02 gene
NM_01037742 RIKEN cDNA 1110034B05 gene
NM_018747  A kinase (PRKA) anchor protein 7
NM_011332  chemokine (C-C motif) ligand 17
NM_176976  similar to KIAA1731 protein; RIKEN cDNA 5830418K08 gene
NM_019472  myosin X
NM_194343  tripartite motif-containing 45
NM_008077       glutamic acid decarboxylase 1  
NM_011508       similar to translation initiation factor SUI1; predicted gene 6428;  
                 similar to Eukaryotic translation initiation factor 1  
                 (eIF1) (Protein translation factor SUI1 homolog); predicted gene,  
                 EG434356; similar to isolog of yeast sui1 and rice gos2;  
                 putative; eukaryotic translation initiation factor 1  
NM_009661       arachidonate 8-lipoxygenase  
NM_019641       stathmin 1; predicted gene 11223; predicted gene 6393  
NM_028460       platelet endothelial aggregation receptor 1  
NM_175771       transmembrane protein 47  
NM_016866       serine/threonine kinase 39, STE20/SPS1 homolog (yeast)  
NM_008945       proteasome (prosome, macropain) subunit, beta type 4  
NM_001024706    predicted gene 3494; predicted gene 3099; predicted gene 6676;  
NM_146554       olfactory receptor 803  
NM_146074       transcription factor B1, mitochondrial  
NM_198598       reproductive homeobox 11  
NM_009343       PHD finger protein 1  
NM_031197       solute carrier family 2 (facilitated glucose transporter), member 2  
NM_133940       F-box and leucine-rich repeat protein 14  
NM_011941       mitogen-activated protein kinase binding protein 1  
NM_175200       amyotrophic lateral sclerosis 2 (juvenile) chromosome region,  
                 candidate 11 (human)  
NM_028611       RIKEN cDNA 2410091C18 gene  
NM_009027       RAS protein-specific guanine nucleotide-releasing factor 2  
NM_010745       lymphocyte antigen 86  
NM_146066       G1 to S phase transition 1  
NM_146750       olfactory receptor 689  
NM_205823       toll-like receptor 12  
NM_177431       a disintegrin-like and metallopeptidase (reprolysin type) with  
                 thrombospondin type 1 motif, 20  
NM_172885       similar to hypothetical protein; transmembrane protein 132D  
NM_023556       mevalonate kinase; similar to mevalonate kinase  
NM_175280       RIKEN cDNA 4930529M08 gene  
NM_010029       DEAD (Asp-Glu-Ala-Asp) box polypeptide 4  
NM_028037       acyl-Coenzyme A dehydrogenase family, member 10  
NM_010648       killer cell lectin-like receptor, subfamily A, member 3  
NM_010576       integrin alpha 4  
NM_183015       cyclin B3  
NM_027725       WD repeat domain 69  
NM_033324       DiGeorge syndrome critical region gene 8  
NM_001039521    RRN3 RNA polymerase I transcription factor homolog (yeast)  
NM_001033128    Bardet-Biedl syndrome 1 (human)  
NM_029281       hypothetical protein LOC100044276; zinc finger protein 820
<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_172698</td>
<td>RIKEN cDNA 4732418C07 gene</td>
</tr>
<tr>
<td>NM_025887</td>
<td>RAB5A, member RAS oncogene family; similar to small GTP-binding protein rab5</td>
</tr>
<tr>
<td>NM_026906</td>
<td>cathepsin 3</td>
</tr>
<tr>
<td>NM_013736</td>
<td>transcription elongation factor B (SIII), polypeptide 3</td>
</tr>
<tr>
<td>NM_026643</td>
<td>RIKEN cDNA 2410017P07 gene</td>
</tr>
<tr>
<td>NM_021507</td>
<td>sulfide quinone reductase-like (yeast)</td>
</tr>
<tr>
<td>NM_173759</td>
<td>RIKEN cDNA A730017C20 gene</td>
</tr>
<tr>
<td>NM_029098</td>
<td>limb region 1 like</td>
</tr>
<tr>
<td>NM_008372</td>
<td>interleukin 7 receptor</td>
</tr>
<tr>
<td>NM_001025261</td>
<td>similar to Tpd52 protein; tumor protein D52</td>
</tr>
<tr>
<td>NM_008338</td>
<td>interferon gamma receptor 2</td>
</tr>
<tr>
<td>NM_146119</td>
<td>family with sequence similarity 129, member B</td>
</tr>
<tr>
<td>NM_028481</td>
<td>coiled-coil domain containing 18</td>
</tr>
<tr>
<td>NM_172788</td>
<td>SH3 domain containing ring finger 3</td>
</tr>
<tr>
<td>NM_138660</td>
<td>cancer susceptibility candidate 3</td>
</tr>
<tr>
<td>NM_144795</td>
<td>pyrroline-5-carboxylate reductase 1</td>
</tr>
<tr>
<td>NM_013491</td>
<td>chloride channel 1</td>
</tr>
<tr>
<td>NM_008650</td>
<td>methylmalonyl-Coenzyme A mutase</td>
</tr>
<tr>
<td>NM_172497</td>
<td>EF hand domain family, member B</td>
</tr>
<tr>
<td>NM_033321</td>
<td>purinergic receptor P2X, ligand-gated ion channel, 5</td>
</tr>
<tr>
<td>NM_026269</td>
<td>RIKEN cDNA 1110007L15 gene; predicted gene 3606</td>
</tr>
<tr>
<td>NM_009285</td>
<td>stanniocalcin 1</td>
</tr>
<tr>
<td>NM_027987</td>
<td>CD300 antigen like family member G</td>
</tr>
<tr>
<td>NM_023058</td>
<td>protein kinase, membrane associated tyrosine/threonine 1</td>
</tr>
<tr>
<td>NM_178609</td>
<td>E2F transcription factor 7</td>
</tr>
<tr>
<td>NM_194336</td>
<td>macrophage activation 2 like</td>
</tr>
<tr>
<td>NM_021392</td>
<td>adaptor-related protein complex AP-4, mu 1</td>
</tr>
<tr>
<td>NM_152804</td>
<td>polo-like kinase 2 (Drosophila)</td>
</tr>
<tr>
<td>NM_183171</td>
<td>fasciculation and elongation protein zeta 1 (zygin I)</td>
</tr>
<tr>
<td>NM_011342</td>
<td>SEC22 vesicle trafficking protein homolog B (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_010317</td>
<td>guanine nucleotide binding protein (G protein), gamma 4</td>
</tr>
<tr>
<td>NM_026842</td>
<td>ubiquilin 1</td>
</tr>
<tr>
<td>NM_029681</td>
<td>Williams-Beuren syndrome chromosome region 28 (human)</td>
</tr>
<tr>
<td>NM_001025351</td>
<td>hypothetical protein LOC100044285; defensin beta 46</td>
</tr>
<tr>
<td>NM_146187</td>
<td>free fatty acid receptor 2</td>
</tr>
<tr>
<td>NM_175751</td>
<td>zinc finger protein 608</td>
</tr>
<tr>
<td>NM_007949</td>
<td>excision repair cross-complementing rodent repair deficiency, complementation group 2</td>
</tr>
<tr>
<td>NM_172613</td>
<td>ATPase type 13A4</td>
</tr>
<tr>
<td>NM_026989</td>
<td>splicing factor, arginine/serine-rich 11</td>
</tr>
<tr>
<td>NM_146711</td>
<td>olfactory receptor 43</td>
</tr>
<tr>
<td>NM_027873</td>
<td>UbiA prenyltransferase domain containing 1</td>
</tr>
</tbody>
</table>
NM_025699  RIKEN cDNA 3230401D17 gene
NM_021385  RAD18 homolog (S. cerevisiae)
NM_027699  RIKEN cDNA 1700108M19 gene
NM_177155  killer cell lectin-like receptor family I member 2; similar to killer cell
           lectin-like receptor family I member 2
NM_028672  family with sequence similarity 161, member A
NM_026523  neuromedin B
NM_172478  TBC1 domain family, member 25
NM_023418  phosphoglycerate mutase 1
NM_198649  actin binding LIM protein family, member 3
NM_008427  potassium inwardly-rectifying channel, subfamily J, member 4
NM_146945  olfactory receptor 345
NM_146907  olfactory receptor 1282
NM_009021  retinoic acid induced 1
NM_178774  similar to RIKEN cDNA 9630019K15 gene; proline rich region 18
NM_011227  RAB20, member RAS oncogene family
NM_001011851  olfactory receptor 412
NM_019737  UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6; similar to Beta-1,4-galactosyltransferase 6
           (Beta-1,4-GalTase 6) (Beta4Gal-T6) (b4Gal-T6)
           (UDP-galactose:beta-N-acetylgalcosamine beta-1, 4-galactosyltransferase 6)
           (UDP-Gal:beta-GlcNAc beta-1,4-galactosyltransferase 6)
NM_138670  mercaptopyruvate sulfurtransferase
NM_031880  tyrosine kinase, non-receptor, 1
NM_022023  glia maturation factor, beta
NM_007387  acid phosphatase 2, lysosomal
NM_029669  DnaJ (Hsp40) homolog, subfamily C, member 18
NM_177725  leucine rich repeat containing 8A
NM_008742  neurotrophin 3
NM_033552  solute carrier family 4, sodium bicarbonate cotransporter-like, member 10
NM_030060  basic leucine zipper transcription factor, ATF-like 3
NM_028404  DNA topoisomerase 1, mitochondrial
NM_026116  Bardet-Biedl syndrome 2 (human)
NM_001001932  early endosome antigen 1
NM_007540  brain derived neurotrophic factor
NM_019450  interleukin 1 family, member 6
NM_001024726  zinc finger protein 607
NM_146344  olfactory receptor 1495
NM_183094  X-linked lymphocyte-regulated 4D; X-linked lymphocyte-regulated 4E, pseudogene; X-linked
           lymphocyte-regulated 4B; X-linked lymphocyte-regulated 4C;
           hypothetical protein LOC100044049

205
NM_176987  RIKEN cDNA 4732471D19 gene
NM_007762  corticotropin releasing hormone receptor 1
NM_010749  mannos-6-phosphate receptor, cation dependent
NM_026208  RIKEN cDNA 1700019N19 gene
NM_172855  UDP-N-acetyl-alpha-D-galactosamine:polypeptide
            N-acetylgalactosaminyltransferase 5
NM_181543  G protein-coupled receptor 151
NM_013512  erythrocyte protein band 4.1-like 4a
NM_182928  adrenomedullin 2
NM_009855  CD80 antigen
NM_029963  predicted gene 13328; mitochondrial ribosomal protein S5
NM_010298  glycine receptor, beta subunit
NM_010833  moesin
NM_145413  similar to RIKEN cDNA C530043G21 gene; family with
            sequence similarity 20, member B
NM_01001809 olfactory receptor 218
NM_019976  proline/serine-rich coiled-coil 1
NM_007605  capping protein (actin filament) muscle Z-line, alpha 3
NM_016745  ATPase, Ca++ transporting, ubiquitous
NM_177750  FERM and PDZ domain containing 3
NM_010715  ligase I, DNA, ATP-dependent
NM_181392  general transcription factor IIH, polypeptide 5
NM_011086  phosphoinositide kinase, FYVE finger containing
NM_007476  predicted gene 5823; ADP-ribosylation factor 1; predicted gene 8230
NM_010050  deiodinase, iodothyronine, type II
NM_029956  methylmalonic aciduria (cobalamin deficiency) type B
            homolog (human)
NM_172600  RIKEN cDNA 6720456H20 gene
NM_177033  von Willebrand factor C domain containing 2
NM_009433  testis-specific protein, Y-encoded-like 1
NM_183024  ribonucleoprotein, PTB-binding 2
NM_153508  calssyntenin 3
NM_146733  olfactory receptor 482
NM_181548  ES cell-expressed Ras
NM_134094  neurocalcin delta
NM_013853  ATP-binding cassette, sub-family F (GCN20), member 2
NM_029706  carboxypeptidase B1 (tissue)
NM_027213  mediator of RNA polymerase II transcription, subunit 6
            homolog (yeast)
NM_194257  protein phosphatase 4, regulatory subunit 1-like; family with
            sequence similarity 118, member B
NM_009253  serine (or cysteine) peptidase inhibitor, clade A, member 3M
NM_021470  ring finger protein 32
NM_013777  aldo-keto reductase family 1, member C12
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_178750</td>
<td>synovial sarcoma translocation gene on chromosome 18-like 1</td>
</tr>
<tr>
<td>NM_178608</td>
<td>receptor accessory protein 1</td>
</tr>
<tr>
<td>NM_134179</td>
<td>vomeronasal 1 receptor, C24</td>
</tr>
<tr>
<td>NM_028717</td>
<td>amyotrophic lateral sclerosis 2 (juvenile) homolog (human)</td>
</tr>
<tr>
<td>NM_001039515</td>
<td>ADP-ribosylation factor-like 4A</td>
</tr>
<tr>
<td>NM_009443</td>
<td>trans-golgi network protein 2; trans-golgi network protein</td>
</tr>
<tr>
<td>NM_016918</td>
<td>nudix (nucleoside diphosphate linked moiety X)-type motif 5</td>
</tr>
<tr>
<td>NM_024221</td>
<td>predicted gene 6123; pyruvate dehydrogenase (lipoamide) beta</td>
</tr>
<tr>
<td>NM_172402</td>
<td>solute carrier family 25, member 32</td>
</tr>
<tr>
<td>NM_020009</td>
<td>mechanistic target of rapamycin (serine/threonine kinase)</td>
</tr>
<tr>
<td>NM_026999</td>
<td>zinc finger protein 688</td>
</tr>
<tr>
<td>NM_134040</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 1</td>
</tr>
<tr>
<td>NM_026464</td>
<td>WD repeat domain 55</td>
</tr>
<tr>
<td>NM_007454</td>
<td>adaptor protein complex AP-1, beta 1 subunit</td>
</tr>
<tr>
<td>NM_028227</td>
<td>BRCA1 associated protein</td>
</tr>
<tr>
<td>NM_053099</td>
<td>SET binding protein 1</td>
</tr>
<tr>
<td>NM_152894</td>
<td>processing of precursor 1, ribonuclease P/MRP family, (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_146315</td>
<td>olfactory receptor 62</td>
</tr>
<tr>
<td>NM_001037916</td>
<td>coiled-coil domain containing 17</td>
</tr>
<tr>
<td>NM_001003824</td>
<td>potassium voltage-gated channel, subfamily Q, member 2</td>
</tr>
<tr>
<td>NM_029499</td>
<td>membrane-spanning 4-domains, subfamily A, member 4C</td>
</tr>
<tr>
<td>NM_028375</td>
<td>CAAX box 1 homolog C (human)</td>
</tr>
<tr>
<td>NM_181278</td>
<td>RIKEN cDNA B230219D22 gene</td>
</tr>
<tr>
<td>NM_178694</td>
<td>zer-1 homolog (C. elegans)</td>
</tr>
<tr>
<td>NM_177280</td>
<td>RIKEN cDNA B230206H07 gene</td>
</tr>
<tr>
<td>NM_134067</td>
<td>expressed sequence AW209491</td>
</tr>
<tr>
<td>NM_029238</td>
<td>solute carrier family 35, member F4</td>
</tr>
<tr>
<td>NM_010203</td>
<td>fibroblast growth factor 5</td>
</tr>
<tr>
<td>NM_178056</td>
<td>TM2 domain containing 3</td>
</tr>
<tr>
<td>NM_008478</td>
<td>L1 cell adhesion molecule</td>
</tr>
<tr>
<td>NM_026967</td>
<td>Ras homolog enriched in brain like 1</td>
</tr>
<tr>
<td>NM_001025605</td>
<td>predicted gene 527</td>
</tr>
<tr>
<td>NM_029803</td>
<td>interferon, alpha-inducible protein 27 like 2A</td>
</tr>
<tr>
<td>NM_146124</td>
<td>Rho GTPase activating protein 1; predicted gene 8514</td>
</tr>
<tr>
<td>NM_023146</td>
<td>RAN binding protein 13</td>
</tr>
<tr>
<td>NM_021347</td>
<td>gasdermin A</td>
</tr>
<tr>
<td>NM_172809</td>
<td>sacsin</td>
</tr>
<tr>
<td>NM_033560</td>
<td>vacuolar protein sorting 37A (yeast); similar to Vps37a protein</td>
</tr>
<tr>
<td>NM_198603</td>
<td>cDNA sequence BC060267</td>
</tr>
<tr>
<td>NM_008645</td>
<td>murinoglobulin 1; predicted gene 7298</td>
</tr>
<tr>
<td>NM_172588</td>
<td>serine incorporator 5</td>
</tr>
<tr>
<td>NM_007551</td>
<td>chemochine (C-X-C motif) receptor 5</td>
</tr>
<tr>
<td>NM_008252</td>
<td>predicted gene 13160; predicted gene 8681; predicted gene 13237;</td>
</tr>
</tbody>
</table>
predicted gene 4169; predicted gene 8284; similar to High mobility group box 2; predicted gene 13167; high mobility group box 2; predicted gene 13232

NM_153548  RIKEN cDNA E430025E21 gene
NM_146590  hypothetical protein LOC100044199; olfactory receptor 1083; olfactory receptor 1085
NM_009556  zinc finger protein 42
NM_009163  sphingosine phosphate lyase 1
NM_013712  integrin beta 1 binding protein 2
NM_175530  F-box protein 46
NM_020288  similar to olfactory receptor Olfr747; olfactory receptor 749; olfactory receptor 747
NM_011620  troponin T3, skeletal, fast
NM_146243  ARP2 actin-related protein 2 homolog (yeast); predicted gene 6828
NM_024291  kyphoscoliosis peptidase
NM_172598  WD repeat and HMG-box DNA binding protein 1
NM_153398  zinc finger and BTB domain containing 24
NM_026045  PRP18 pre-mRNA processing factor 18 homolog (yeast)
NM_009242  secreted acidic cysteine rich glycoprotein; similar to Secreted acidic cysteine rich glycoprotein
NM_178185  histone cluster 1, H2ad; histone cluster 1, H2ae; histone cluster 1, H2ag; histone cluster 1, H2ah; histone cluster 1, H2ai; similar to histone 2a; histone cluster 1, H2an; histone cluster 1, H2ao; histone cluster 1, H2ac; histone cluster 1, H2ab
NM_199468  zinc finger, CCHC domain containing 5
NM_173371  hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
NM_146936  olfactory receptor 1417
NM_173732  transmembrane protein 194
NM_023663  receptor-interacting serine-threonine kinase 4
NM_026492  predicted gene 14459; similar to Ssxb3 protein; synovial sarcoma, X member B, breakpoint 1; synovial sarcoma, X member B, breakpoint 2; synovial sarcoma, X member B, breakpoint 3; synovial sarcoma, X member B, breakpoint 11; predicted gene 6797; synovial sarcoma, X member B, breakpoint 12; synovial sarcoma, X member B, breakpoint 6; similar to synovial sarcoma, X member B, breakpoint 10; synovial sarcoma, X member B, breakpoint 7; synovial sarcoma, X member B, breakpoint 9
NM_008995  peroxisomal biogenesis factor 5
NM_177322  angiotensin II receptor, type 1a
NM_009676  aldehyde oxidase 1
NM_026198  transmembrane protein 167B
NM_176930  neuron-glial-CAM-related cell adhesion molecule
NM_025989  glycoprotein 2 (zymogen granule membrane)
<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_177919</td>
<td>transcription elongation factor A (SII)-like 5</td>
</tr>
<tr>
<td>NM_028898</td>
<td>regulatory associated protein of MTOR, complex 1</td>
</tr>
<tr>
<td>NM_028355</td>
<td>transmembrane protein 48</td>
</tr>
<tr>
<td>NM_134017</td>
<td>methionine adenosyltransferase II, beta</td>
</tr>
<tr>
<td>NM_023742</td>
<td>deltex 2 homolog (Drosophila)</td>
</tr>
<tr>
<td>NM_011394</td>
<td>hypothetical protein LOC100045882; solute carrier family 20, member 2</td>
</tr>
<tr>
<td>NM_027100</td>
<td>RWD domain containing 2A</td>
</tr>
<tr>
<td>NM_008034</td>
<td>folate receptor 1 (adult)</td>
</tr>
<tr>
<td>NM_145947</td>
<td>solute carrier family 26, member 7</td>
</tr>
<tr>
<td>NM_175460</td>
<td>nicotinamide nucleotide adenylyltransferase 2</td>
</tr>
<tr>
<td>NM_053111</td>
<td>similar to ribonuclease 7; eosinophil-associated, ribonuclease A family, member 7; eosinophil-associated, ribonuclease A family, er 6</td>
</tr>
<tr>
<td>NM_008527</td>
<td>similar to natural killer cell receptor-P1; predicted gene 4697; killer cell lectin-like receptor subfamily B member 1C</td>
</tr>
<tr>
<td>NM_026183</td>
<td>solute carrier family 47, member 1</td>
</tr>
<tr>
<td>NM_172479</td>
<td>solute carrier family 38, member 5</td>
</tr>
<tr>
<td>NM_025936</td>
<td>arginyl-tRNA synthetase</td>
</tr>
<tr>
<td>NM_207573</td>
<td>olfactory receptor 1380</td>
</tr>
<tr>
<td>NM_008622</td>
<td>general transcription factor IIIIC, polypeptide 2, beta; Mpv17 transgene, kidney disease mutant</td>
</tr>
<tr>
<td>NM_007570</td>
<td>B-cell translocation gene 2, anti-proliferative</td>
</tr>
<tr>
<td>NM_028938</td>
<td>leucine-rich repeats and IQ motif containing 3</td>
</tr>
<tr>
<td>NM_009255</td>
<td>serine (or cysteine) peptidase inhibitor, clade E, member 2</td>
</tr>
<tr>
<td>NM_009516</td>
<td>WEE 1 homolog 1 (S. pombe)</td>
</tr>
<tr>
<td>NM_001010839</td>
<td>trace amine-associated receptor 7F</td>
</tr>
<tr>
<td>NM_146104</td>
<td>predicted gene 15429; anterior pharynx defective 1a homolog (C. elegans)</td>
</tr>
<tr>
<td>NM_019663</td>
<td>protein inhibitor of activated STAT 1</td>
</tr>
<tr>
<td>NM_001077202</td>
<td>heparan sulfate 6-O-sulfotransferase 2</td>
</tr>
<tr>
<td>NM_011263</td>
<td>RE1-silencing transcription factor</td>
</tr>
<tr>
<td>NM_011100</td>
<td>protein kinase, cAMP dependent, catalytic, beta</td>
</tr>
<tr>
<td>NM_197989</td>
<td>predicted gene 9731; RIKEN cDNA 1810009O10 gene</td>
</tr>
<tr>
<td>NM_024217</td>
<td>CKLF-like MARVEL transmembrane domain containing 3</td>
</tr>
<tr>
<td>NM_007541</td>
<td>bone gamma carboxyglutamate protein 1</td>
</tr>
<tr>
<td>NM_145970</td>
<td>coiled-coil and C2 domain containing 1A</td>
</tr>
<tr>
<td>NM_010289</td>
<td>gap junction protein, alpha 10</td>
</tr>
<tr>
<td>NM_001011831</td>
<td>olfactory receptor 1500</td>
</tr>
<tr>
<td>NM_172861</td>
<td>solute carrier family 7 (cationic amino acid transporter, y+ system), member 14</td>
</tr>
<tr>
<td>NM_024439</td>
<td>histocompatibility 47</td>
</tr>
<tr>
<td>NM_175116</td>
<td>purinergic receptor P2Y, G-protein coupled, 5</td>
</tr>
<tr>
<td>NM_146678</td>
<td>olfactory receptor 1428</td>
</tr>
<tr>
<td>NM_026938</td>
<td>transmembrane protein 160</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NM_001004142</td>
<td>NLR family, pyrin domain containing 1A</td>
</tr>
<tr>
<td>NM_026239</td>
<td>transmembrane protein 35</td>
</tr>
<tr>
<td>NM_133825</td>
<td>DNA segment, Chr 1, ERATO Doi 622, expressed</td>
</tr>
<tr>
<td>NM_029205</td>
<td>RIKEN cDNA 4930546H06 gene</td>
</tr>
<tr>
<td>NM_021434</td>
<td>G protein-coupled receptor 180</td>
</tr>
<tr>
<td>NM_146417</td>
<td>olfactory receptor 877</td>
</tr>
<tr>
<td>NM_008778</td>
<td>p21 protein (Cdc42/Rac)-activated kinase 3</td>
</tr>
<tr>
<td>NM_001037099</td>
<td>calcium channel, voltage-dependent, beta 4 subunit</td>
</tr>
<tr>
<td>NM_146123</td>
<td>calcium channel, voltage-dependent, beta 4 subunit</td>
</tr>
<tr>
<td>NM_027698</td>
<td>exonuclease domain containing 1</td>
</tr>
<tr>
<td>NM_030749</td>
<td>endoplasmic reticulum chaperone SIL1 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_199322</td>
<td>DOT1-like, histone H3 methyltransferase (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_010340</td>
<td>G-protein-coupled receptor 50</td>
</tr>
<tr>
<td>NM_080849</td>
<td>NIMA (never in mitosis gene a)-related expressed kinase 8</td>
</tr>
<tr>
<td>NM_178685</td>
<td>protocadherin 20</td>
</tr>
<tr>
<td>NM_146206</td>
<td>two pore segment channel 2</td>
</tr>
<tr>
<td>NM_029578</td>
<td>TDP-glucose 4,6-dehydratase</td>
</tr>
<tr>
<td>NM_028568</td>
<td>dual specificity phosphatase 21</td>
</tr>
<tr>
<td>NM_027973</td>
<td>myeloid leukemia factor 1 interacting protein</td>
</tr>
<tr>
<td>NM_008096</td>
<td>group specific component</td>
</tr>
<tr>
<td>NM_008008</td>
<td>fibroblast growth factor 7</td>
</tr>
<tr>
<td>NM_001038613</td>
<td>olfactomedin 1</td>
</tr>
<tr>
<td>NM_019735</td>
<td>APAF1 interacting protein; similar to MMRP19</td>
</tr>
<tr>
<td>NM_180958</td>
<td>coiled-coil domain containing 79</td>
</tr>
<tr>
<td>NM_030138</td>
<td>ArfGAP with coiled-coil, ankyrin repeat and PH domains 2</td>
</tr>
<tr>
<td>NM_133661</td>
<td>solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12</td>
</tr>
<tr>
<td>NM_138303</td>
<td>Yip1 domain family, member 2</td>
</tr>
<tr>
<td>NM_027768</td>
<td>RIKEN cDNA 5430402E10 gene; predicted gene 14750</td>
</tr>
<tr>
<td>NM_134091</td>
<td>small G protein signaling modulator 3</td>
</tr>
<tr>
<td>NM_133345</td>
<td>inhibitor of growth family, member 4</td>
</tr>
<tr>
<td>NM_009801</td>
<td>carbonic anhydrase 2</td>
</tr>
<tr>
<td>NM_134096</td>
<td>family with sequence similarity 19, member A5</td>
</tr>
<tr>
<td>NM_175358</td>
<td>zinc finger, DHHC domain containing 15</td>
</tr>
<tr>
<td>NM_018858</td>
<td>phosphatidylethanolamine binding protein 1</td>
</tr>
<tr>
<td>NM_172794</td>
<td>zinc finger protein 454</td>
</tr>
<tr>
<td>NM_011480</td>
<td>sterol regulatory element binding transcription factor 1</td>
</tr>
<tr>
<td>NM_021887</td>
<td>interleukin 21 receptor</td>
</tr>
<tr>
<td>NM_011204</td>
<td>protein tyrosine phosphatase, non-receptor type 13</td>
</tr>
<tr>
<td>NM_010938</td>
<td>nuclear respiratory factor 1</td>
</tr>
<tr>
<td>NM_146656</td>
<td>olfactory receptor 444</td>
</tr>
<tr>
<td>NM_013552</td>
<td>hyaluronan mediated motility receptor (RHAMM)</td>
</tr>
<tr>
<td>NM_146600</td>
<td>olfactory receptor 700</td>
</tr>
</tbody>
</table>

211
NM_146726   olfactory receptor 514
NM_010390   histocompatibility 2, Q region locus 1; histocompatibility 2, Q region locus 9; similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor; histocompatibility 2, Q region locus 8; histocompatibility 2, Q region locus 2; similar to MHC class Ib antigen; histocompatibility 2, Q region locus 7; histocompatibility 2, Q region locus 6; hypothetical protein LOC100044307; similar to H2 class I histocompatibility antigen, Q7 alpha chain precursor (QA-2 antigen); RIKEN cDNA 0610037M15 gene
NM_009896   suppressor of cytokine signaling 1
NM_026793   myc target 1
NM_001001882 regulator of telomere elongation helicase 1
NM_010819   C-type lectin domain family 4, member d
NM_207523   predicted gene 3174; predicted gene 8112; predicted gene 8158; ribosomal protein L23a; predicted gene 5384; predicted gene 8264; predicted gene 6233; similar to 60S ribosomal protein L23a; predicted gene 7413; predicted gene 5351; predicted gene 6177; similar to ribosomal protein L23a; predicted gene 7065; predicted gene 7755; predicted gene 10353; predicted gene 8137; predicted gene 3362; predicted gene 10132; predicted gene 6118
NM_028224   Fas apoptotic inhibitory molecule 2
NM_133683   transmembrane protein 19
NM_025291   steroid receptor RNA activator 1
NM_027406   similar to Aldehyde dehydrogenase 1 family, member L1; aldehyde dehydrogenase 1 family, member L1
NM_029427   patatin-like phospholipase domain containing 5
NM_019861   cathepsin F
NM_007768   C-reactive protein, pentraxin-related
NM_011648   thyroid stimulating hormone receptor
NM_009369   transforming growth factor, beta induced
NM_172766   nuclear factor related to kappa B binding protein
NM_024201   coiled-coil domain containing 127
NM_001011841 olfactory receptor 1465
NM_133365   dynein, axonemal, heavy chain 5
NM_028725   short chain dehydrogenase/reductase family 42E, member 1
NM_176835   DnaJ (Hsp40) homolog, subfamily C, member 22
NM_001011517 olfactory receptor 1194, pseudogene 1; olfactory receptor 1193
NM_020487   protease, serine, 21
NM_013697   transthyretin
NM_008287   heat-responsive protein 12
NM_181545   similar to schlafen 8; schlafen 8
NM_001011776 olfactory receptor 527
NM_172416   osteopetrosis associated transmembrane protein 1

212
<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_146434</td>
<td>olfactory receptor 995</td>
</tr>
<tr>
<td>NM_008744</td>
<td>similar to Netrin-1 precursor; netrin 1</td>
</tr>
<tr>
<td>NM_198161</td>
<td>basic helix-loop-helix domain containing, class B9</td>
</tr>
<tr>
<td>NM_145585</td>
<td>THUMP domain containing 1; similar to THUMP domain containing 1</td>
</tr>
<tr>
<td>NM_173778</td>
<td>SH2 domain containing 7</td>
</tr>
<tr>
<td>NM_001033448</td>
<td>predicted gene 962</td>
</tr>
<tr>
<td>NM_019935</td>
<td>OVO homolog-like 1 (Drosophila)</td>
</tr>
<tr>
<td>NM_030252</td>
<td>cDNA sequence BC003266</td>
</tr>
<tr>
<td>NM_028807</td>
<td>RIKEN cDNA 1200009I06 gene</td>
</tr>
<tr>
<td>NM_024240</td>
<td>GINS complex subunit 4 (Sld5 homolog)</td>
</tr>
<tr>
<td>NM_026533</td>
<td>similar to ribosomal protein S13; predicted gene 12270; predicted gene 6834;</td>
</tr>
<tr>
<td></td>
<td>predicted gene 15483; predicted gene 6573; ribosomal protein S13; predicted</td>
</tr>
<tr>
<td></td>
<td>gene 10159</td>
</tr>
<tr>
<td>NM_028295</td>
<td>protein disulfide isomerase associated 5</td>
</tr>
<tr>
<td>NM_133780</td>
<td>similar to nuclear pore complex-associated intranuclear coiled-coil protein TPR; translocated promoter region</td>
</tr>
<tr>
<td>NM_001011814</td>
<td>olfactory receptor 524</td>
</tr>
<tr>
<td>NM_009093</td>
<td>predicted gene 7079; predicted gene 7618; predicted gene 10126; predicted</td>
</tr>
<tr>
<td></td>
<td>gene 10059; predicted gene 5735; similar to 40S ribosomal protein S29;</td>
</tr>
<tr>
<td></td>
<td>predicted gene 2581; predicted gene 6134; ribosomal protein S29; predicted</td>
</tr>
<tr>
<td></td>
<td>gene 14303</td>
</tr>
<tr>
<td>NM_015775</td>
<td>transmembrane protease, serine 2</td>
</tr>
<tr>
<td>NM_031376</td>
<td>phosphoinositide-3-kinase adaptor protein 1</td>
</tr>
<tr>
<td>NM_011561</td>
<td>thymine DNA glycosylase; predicted gene 5597; predicted gene 9855; predicted</td>
</tr>
<tr>
<td></td>
<td>gene 5806</td>
</tr>
<tr>
<td>NM_144544</td>
<td>RIKEN cDNA 2210407C18 gene</td>
</tr>
<tr>
<td>NM_029352</td>
<td>dual specificity phosphatase 9</td>
</tr>
<tr>
<td>NM_026132</td>
<td>thioredoxin domain containing 8</td>
</tr>
<tr>
<td>NM_134157</td>
<td>ATPase, H+ transporting, lysosomal V1 subunit B1</td>
</tr>
<tr>
<td>NM_028040</td>
<td>RNA pseudouridylate synthase domain containing 4</td>
</tr>
<tr>
<td>NM_010726</td>
<td>phytanoyl-CoA hydroxylase</td>
</tr>
<tr>
<td>NM_172053</td>
<td>a disintegrin-like and metallopeptidase (reprolysin type) with thrombospo</td>
</tr>
<tr>
<td></td>
<td>ndin type 1 motif, 16</td>
</tr>
<tr>
<td>NM_146864</td>
<td>olfactory receptor 768</td>
</tr>
<tr>
<td>NM_008648</td>
<td>major urinary protein 4</td>
</tr>
<tr>
<td>NM_172400</td>
<td>DnaJ (Hsp40) homolog, subfamily C, member 8; predicted gene 4928</td>
</tr>
<tr>
<td>NM_146988</td>
<td>olfactory receptor 447</td>
</tr>
<tr>
<td>NM_175382</td>
<td>RIKEN cDNA 2700049P18 gene</td>
</tr>
<tr>
<td>NM_001004170</td>
<td>predicted gene 5478</td>
</tr>
<tr>
<td>NM_011149</td>
<td>peptidylprolyl isomerase B</td>
</tr>
<tr>
<td>NM_026186</td>
<td>coiled-coil domain containing 49</td>
</tr>
<tr>
<td>NM_029741</td>
<td>protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF),</td>
</tr>
<tr>
<td></td>
<td>interacting protein (liprin), alpha 3</td>
</tr>
<tr>
<td>NM_027402</td>
<td>fibronectin type III domain containing 5</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NM_177016</td>
<td>solute carrier family 17 (sodium phosphate), member 4</td>
</tr>
<tr>
<td>NM_207262</td>
<td>androgen binding protein epsilon</td>
</tr>
<tr>
<td>NM_011780</td>
<td>a disintegrin and metallopeptidase domain 23; similar to ADAM23</td>
</tr>
<tr>
<td>NM_016744</td>
<td>phosphodiesterase 1A, calmodulin-dependent</td>
</tr>
<tr>
<td>NM_011401</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 3</td>
</tr>
<tr>
<td>NM_172825</td>
<td>G protein-coupled receptor 128</td>
</tr>
<tr>
<td>NM_207222</td>
<td>LIM domain only 3</td>
</tr>
<tr>
<td>NM_029613</td>
<td>keratin associated protein 4-7</td>
</tr>
<tr>
<td>NM_145576</td>
<td>Zinc finger protein 212</td>
</tr>
<tr>
<td>NM_025929</td>
<td>RIKEN cDNA 2010109I03 gene</td>
</tr>
<tr>
<td>NM_198661</td>
<td>oogenesin 2</td>
</tr>
<tr>
<td>NM_009626</td>
<td>alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide</td>
</tr>
<tr>
<td>NM_001025192</td>
<td>coxsackie virus and adenovirus receptor</td>
</tr>
<tr>
<td>NM_144802</td>
<td>heterogeneous nuclear ribonucleoprotein L-like; glutathione peroxidase 4</td>
</tr>
<tr>
<td>NM_176968</td>
<td>5'-nucleotidase domain containing 1</td>
</tr>
<tr>
<td>NM_177693</td>
<td>lens intrinsic membrane protein 2</td>
</tr>
<tr>
<td>NM_178890</td>
<td>ankyrin repeat and BTB (POZ) domain containing 2</td>
</tr>
<tr>
<td>NM_146415</td>
<td>olfactory receptor 291</td>
</tr>
<tr>
<td>NM_172704</td>
<td>DnaJ (Hsp40) homolog, subfamily C, member 11</td>
</tr>
<tr>
<td>NM_133809</td>
<td>kynurenine 3-monoxygenase (kynurenine 3-hydroxylase)</td>
</tr>
<tr>
<td>NM_021431</td>
<td>nudix (nucleoside diphosphate linked moiety X)-type motif 11; nudix (nucleoside diphosphate linked moiety X)-type motif 10</td>
</tr>
<tr>
<td>NM_145513</td>
<td>TIP41, TOR signalling pathway regulator-like (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_026907</td>
<td>secreted and transmembrane 1B</td>
</tr>
<tr>
<td>NM_172870</td>
<td>basonuclin 2</td>
</tr>
<tr>
<td>NM_026862</td>
<td>CD177 antigen</td>
</tr>
<tr>
<td>NM_019447</td>
<td>hepatocyte growth factor activator</td>
</tr>
<tr>
<td>NM_001033302</td>
<td>predicted gene 129</td>
</tr>
<tr>
<td>NM_144537</td>
<td>carboxypeptidase A5</td>
</tr>
<tr>
<td>NM_146054</td>
<td>fermitin family homolog 2 (Drosophila)</td>
</tr>
<tr>
<td>NM_133762</td>
<td>non-SMC condensin II complex, subunit G2</td>
</tr>
<tr>
<td>NM_175360</td>
<td>oligonucleotide/oligosaccharide-binding fold containing 1</td>
</tr>
<tr>
<td>NM_054048</td>
<td>REST corepressor 2</td>
</tr>
<tr>
<td>NM_213616</td>
<td>ATPase, Ca++ transporting, plasma membrane 4</td>
</tr>
<tr>
<td>NM_010101</td>
<td>sphingosine-1-phosphate receptor 3</td>
</tr>
<tr>
<td>NM_001037743</td>
<td>RIKEN cDNA 4921506M07 gene</td>
</tr>
<tr>
<td>NM_007545</td>
<td>harakiri, BCL2 interacting protein (contains only BH3 domain)</td>
</tr>
<tr>
<td>NM_172922</td>
<td>ankyrin repeat and kinase domain containing 1</td>
</tr>
<tr>
<td>NM_001008547</td>
<td>RIKEN cDNA 1700001J03 gene</td>
</tr>
<tr>
<td>NM_026708</td>
<td>TLC domain containing 1</td>
</tr>
<tr>
<td>NM_010667</td>
<td>keratin 86</td>
</tr>
<tr>
<td>NM_009422</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>Gene Accession</td>
<td>Description</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NM_032003</td>
<td>ectonucleotide pyrophosphatase/phosphodiesterase 5</td>
</tr>
<tr>
<td>NM_146568</td>
<td>olfactory receptor 1012</td>
</tr>
<tr>
<td>NM_015741</td>
<td>keratin associated protein 9-1</td>
</tr>
<tr>
<td>NM_011393</td>
<td>solute carrier family 1 (glial high affinity glutamate transporter), member 2</td>
</tr>
<tr>
<td>NM_019936</td>
<td>cysteine-rich PDZ-binding protein</td>
</tr>
<tr>
<td>NM_007883</td>
<td>desmoglein 2; similar to Dsg2 protein</td>
</tr>
<tr>
<td>NM_001013368</td>
<td>E2F transcription factor 8</td>
</tr>
<tr>
<td>NM_134229</td>
<td>vomeronasal 1 receptor, E10</td>
</tr>
<tr>
<td>NM_011351</td>
<td>sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C</td>
</tr>
<tr>
<td>NM_001039094</td>
<td>neuronal growth regulator 1</td>
</tr>
<tr>
<td>NM_198000</td>
<td>RIKEN cDNA 1700001O22 gene</td>
</tr>
<tr>
<td>NM_175001</td>
<td>predicted gene 6997; mitochondrial ribosomal protein L22</td>
</tr>
<tr>
<td>NM_010196</td>
<td>fibrinogen alpha chain</td>
</tr>
<tr>
<td>NM_053080</td>
<td>aldehyde dehydrogenase family 1, subfamily A3</td>
</tr>
<tr>
<td>NM_025520</td>
<td>LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae); similar to Lsm5 protein; similar to LSM5 homolog, U6 small nuclear RNA associated; predicted gene 7846</td>
</tr>
<tr>
<td>NM_147064</td>
<td>olfactory receptor 449</td>
</tr>
<tr>
<td>NM_001039554</td>
<td>angiopoietin-like 7</td>
</tr>
<tr>
<td>NM_013613</td>
<td>nuclear receptor subfamily 4, group A, member 2</td>
</tr>
<tr>
<td>NM_207223</td>
<td>ArfGAP with coiled-coil, ankyrin repeat and PH domains 3</td>
</tr>
<tr>
<td>NM_026630</td>
<td>zinc finger protein 850; gametocyte specific factor 1-like</td>
</tr>
<tr>
<td>NM_016779</td>
<td>dentin matrix protein 1</td>
</tr>
<tr>
<td>NM_145499</td>
<td>cytochrome P450, family 2, subfamily c, polypeptide 70</td>
</tr>
<tr>
<td>NM_177133</td>
<td>RIKEN cDNA E330018D03 gene</td>
</tr>
<tr>
<td>NM_146944</td>
<td>olfactory receptor 348</td>
</tr>
<tr>
<td>NM_207242</td>
<td>NPC1-like 1</td>
</tr>
<tr>
<td>NM_178413</td>
<td>threonine synthase-like 2 (bacterial)</td>
</tr>
<tr>
<td>NM_177132</td>
<td>RIKEN cDNA 1520401A03 gene</td>
</tr>
<tr>
<td>NM_153540</td>
<td>expressed sequence C85492</td>
</tr>
<tr>
<td>NM_007998</td>
<td>ferrochelatase</td>
</tr>
<tr>
<td>NM_146965</td>
<td>olfactory receptor 1250</td>
</tr>
<tr>
<td>NM_146007</td>
<td>collagen, type VI, alpha 2</td>
</tr>
<tr>
<td>NM_007694</td>
<td>chromogranin B</td>
</tr>
<tr>
<td>NM_173866</td>
<td>glutamic pyruvate transaminase (alanine aminotransferase) 2</td>
</tr>
<tr>
<td>NM_008549</td>
<td>mannosidase 2, alpha 1</td>
</tr>
<tr>
<td>NM_028221</td>
<td>RIKEN cDNA 2310065K24 gene</td>
</tr>
<tr>
<td>NM_178883</td>
<td>golgin, RAB6-interacting</td>
</tr>
<tr>
<td>NM_147088</td>
<td>olfactory receptor 569</td>
</tr>
<tr>
<td>NM_011612</td>
<td>tumor necrosis factor receptor superfamily, member 9</td>
</tr>
</tbody>
</table>
NM_145938  ribonuclease P 40 subunit (human)
NM_172718  small G protein signaling modulator 1
NM_001033550  leucine rich repeat containing 8 family, member B
NM_008968  prostaglandin I2 (prostacyclin) synthase
NM_010336  lysophosphatidic acid receptor 1
NM_177093  leucine rich repeat containing 58; predicted gene, OTTMUSG00000025724
NM_173411  cDNA sequence BC030500
NM_01024720  hemicentin 1
NM_017368  CUG triplet repeat, RNA binding protein 1
NM_172121  hypothetical protein LOC100044250; zinc finger CCCH type containing 3; predicted gene 7353
NM_146896  olfactory receptor 1203
NM_175256  HEG homolog 1 (zebrafish)
NM_008056  frizzled homolog 6 (Drosophila)
NM_146710  olfactory receptor 398
NM_178686  centrosomal protein 120
NM_028243  prolylcarboxypeptidase (angiotensinase C)
NM_007627  cholecystokinin B receptor
NM_029761  docking protein 5
NM_153542  leucine rich repeat containing 20
NM_054051  phosphatidylinositol-5-phosphate 4-kinase, type II, beta
NM_145573  mitochondrial ribosomal protein S35
NM_172491  RIKEN cDNA D130040H23 gene
NM_029037  RIKEN cDNA 4930444A02 gene
NM_184052  insulin-like growth factor 1
NM_008534  lymphocyte antigen 9
NM_029804  heterogeneous nuclear ribonucleoprotein M
NM_008088  growth arrest specific 7
NM_016750  H2A histone family, member Z; predicted gene 6722; predicted gene 8203
NM_011507  succinate-Coenzyme A ligase, GDP-forming, beta subunit
NM_153501  pantothenate kinase 2 (Hallervorden-Spatz syndrome)
NM_029297  dynein light chain roadblock-type 2
NM_153539  family with sequence similarity 5, member C
NM_153416  achalasia, adrenocortical insufficiency, alacrimia
NM_175228  RIKEN cDNA 4930578C19 gene
NM_172504  predicted gene 318; predicted gene 4776
NM_010114  kallikrein 1-related peptidase b22
NM_025912  RIKEN cDNA 2010011I20 gene
NM_146063  keratin 79
NM_146207  cullin 4A
NM_009746  B-cell CLL/lymphoma 7C
<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_029418</td>
<td>RIKEN cDNA 9130401M01 gene</td>
</tr>
<tr>
<td>NM_009974</td>
<td>casein kinase 2, alpha prime polypeptide; similar to casein kinase II, alpha prime subunit</td>
</tr>
<tr>
<td>NM_009528</td>
<td>wingless-related MMTV integration site 7B</td>
</tr>
<tr>
<td>NM_007840</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 5; predicted gene 12183</td>
</tr>
<tr>
<td>NM_146596</td>
<td>olfactory receptor 703</td>
</tr>
<tr>
<td>NM_029736</td>
<td>solute carrier family 10 (sodium/bile acid cotransporter family), member 7</td>
</tr>
<tr>
<td>NM_011068</td>
<td>peroxisomal biogenesis factor 11 alpha</td>
</tr>
<tr>
<td>NM_00103308</td>
<td>cDNA sequence BC013712</td>
</tr>
<tr>
<td>NM_021551</td>
<td>solute carrier family 22 (organic cation transporter), member 17</td>
</tr>
<tr>
<td>NM_146505</td>
<td>olfactory receptor 148</td>
</tr>
<tr>
<td>NM_145519</td>
<td>FERM, RhoGEF and pleckstrin domain protein 2</td>
</tr>
<tr>
<td>NM_207239</td>
<td>general transcription factor III C 1</td>
</tr>
<tr>
<td>NM_201227</td>
<td>DAN domain family, member 5</td>
</tr>
<tr>
<td>NM_199302</td>
<td>RIKEN cDNA 4930555K19 gene; leucine rich repeat and sterile alpha motif containing 1</td>
</tr>
<tr>
<td>NM_146841</td>
<td>olfactory receptor 617</td>
</tr>
<tr>
<td>NM_009133</td>
<td>stathmin-like 3</td>
</tr>
<tr>
<td>NM_011538</td>
<td>T-box 6</td>
</tr>
<tr>
<td>NM_194341</td>
<td>API gamma subunit binding protein 1</td>
</tr>
<tr>
<td>NM_025721</td>
<td>sperm equatorial segment protein 1</td>
</tr>
<tr>
<td>NM_008421</td>
<td>potassium voltage gated channel, Shaw-related subfamily, member 1</td>
</tr>
<tr>
<td>NM_009949</td>
<td>carnitine palmitoyltransferase 2</td>
</tr>
<tr>
<td>NM_183087</td>
<td>RIKEN cDNA 5730507A09 gene</td>
</tr>
<tr>
<td>NM_026243</td>
<td>mannosyl (alpha-1,3-)glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme C (putative)</td>
</tr>
<tr>
<td>NM_021318</td>
<td>four and a half LIM domains 5</td>
</tr>
<tr>
<td>NM_001029841</td>
<td>src-like adaptor</td>
</tr>
<tr>
<td>NM_026521</td>
<td>zinc finger protein 706; predicted gene 4002; similar to PNAS-106; predicted gene 10193</td>
</tr>
<tr>
<td>NM_080555</td>
<td>phosphatidic acid phosphatase type 2B</td>
</tr>
<tr>
<td>NM_177278</td>
<td>l(3)mbt-like 4 (Drosophila)</td>
</tr>
<tr>
<td>NM_007451</td>
<td>solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5; similar to ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (Solute carrier family 25 member 5); predicted gene 5529; predicted gene 8429; predicted gene 5256</td>
</tr>
<tr>
<td>NM_011581</td>
<td>thrombospondin 2</td>
</tr>
<tr>
<td>NM_028922</td>
<td>phosphatidic acid phosphatase type 2 domain containing 2</td>
</tr>
<tr>
<td>NM_134002</td>
<td>casein kinase 1, gamma 2</td>
</tr>
<tr>
<td>NM_009969</td>
<td>colony stimulating factor 2 (granulocyte-macrophage)</td>
</tr>
<tr>
<td>NM_030145</td>
<td>LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae);</td>
</tr>
</tbody>
</table>
predicted gene 12571
NM_172733 2-deoxyribose-5-phosphate aldolase homolog (C. elegans)
NM_054066 phospholipase C, zeta 1
NM_021566 junctophilin 2
NM_013892 propoprotein convertase subtilisin/kexin type 1 inhibitor
NM_009277 tripartite motif-containing 21; similar to Tripartite motif protein 21
NM_181589 cytoskeleton associated protein 2-like
NM_025613 EP300 interacting inhibitor of differentiation 1
NM_001005230 olfactory receptor 1024
NM_028115 TruB pseudouridine (psi) synthase homolog 1 (E. coli)
NM_019878 sulfotransferase family 1B, member 1
NM_007432 alkaline phosphatase 3, intestine, not Mn requiring
NM_025760 protein tyrosine phosphatase-like A domain containing 2
NM_030074 zinc finger protein 687
NM_134250 hepatitis A virus cellular receptor 2
NM_010628 kinesin family member 9
NM_025910 myc induced nuclear antigen
NM_183405 cytochrome c oxidase subunit VIb polypeptide 2
NM_177702 RIKEN cDNA 4833427G06 gene
NM_213728 keratin 72
NM_011875 proteasome (prosome, macropain) 26S subunit, non-ATPase, 13
NM_026728 enoyl Coenzyme A hydratase domain containing 2
NM_153796 progressive external ophthalmoplegia 1 (human)
NM_177825 predicted gene 5094
NM_028913 zinc finger protein 819
NM_008209 major histocompatibility complex, class I-related
NM_009791 asp (abnormal spindle)-like, microcephaly associated (Drosophila)
NM_147115 olfactory receptor 578
NM_009713 arylsulfatase A
NM_013881 Unc-51 like kinase 2 (C. elegans)
NM_172547 RIKEN cDNA 9130014G24 gene
NM_020036 calmodulin 4
NM_016967 oligodendrocyte transcription factor 2
NM_028236 carcinoembryonic antigen-related cell adhesion molecule 18
NM_145383 rhodopsin
NM_172126 a disintegrin and metallopeptidase domain 1a
NM_010323 gonadotropin releasing hormone receptor
NM_008002 fibroblast growth factor 10
NM_011151 similar to serine/threonine phosphatase; protein phosphatase 1B, magnesium dependent, beta isoform
NM_001010834 solute carrier family 10 (sodium/bile acid cotransporter family), member 5
NM_139063 muted
<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_001025573</td>
<td>RIKEN cDNA 2010107G12 gene</td>
</tr>
<tr>
<td>NM_207544</td>
<td>vomeronasal 1 receptor, D11</td>
</tr>
<tr>
<td>NM_009437</td>
<td>thiosulfate sulfurtransferase, mitochondrial</td>
</tr>
<tr>
<td>NM_013862</td>
<td>RAB GTPase activating protein 1-like</td>
</tr>
<tr>
<td>NM_008341</td>
<td>insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>NM_052993</td>
<td>core 1 synthase, glycoprotein-N-acetylglucosamine 3-beta-galactosyltransferase, 1</td>
</tr>
<tr>
<td>NM_013547</td>
<td>homogentisate 1, 2-dioxygenase</td>
</tr>
<tr>
<td>NM_183320</td>
<td>predicted gene 7903; predicted gene 5128</td>
</tr>
<tr>
<td>NM_008781</td>
<td>paired box gene 3</td>
</tr>
<tr>
<td>NM_009608</td>
<td>actin, alpha, cardiac muscle 1; similar to alpha-actin (AA 27-375)</td>
</tr>
<tr>
<td>NM_144936</td>
<td>transmembrane protein 45b</td>
</tr>
<tr>
<td>NM_027828</td>
<td>family with sequence similarity 110, member C</td>
</tr>
<tr>
<td>NM_010407</td>
<td>hemopoietic cell kinase</td>
</tr>
<tr>
<td>NM_007997</td>
<td>ferrodoxin reductase</td>
</tr>
<tr>
<td>NM_183023</td>
<td>regulating synaptic membrane exocytosis 4</td>
</tr>
<tr>
<td>NM_013886</td>
<td>hepatoma-derived growth factor, related protein 3</td>
</tr>
<tr>
<td>NM_029865</td>
<td>ocludin/ELL domain containing 1</td>
</tr>
<tr>
<td>NM_026002</td>
<td>metadherin</td>
</tr>
<tr>
<td>NM_020587</td>
<td>splicing factor, arginine/serine-rich 4 (SRp75)</td>
</tr>
<tr>
<td>NM_008906</td>
<td>cathepsin A</td>
</tr>
<tr>
<td>NM_008043</td>
<td>frequently rearranged in advanced T-cell lymphomas</td>
</tr>
<tr>
<td>NM_053097</td>
<td>predicted gene 4477; camello-like 3</td>
</tr>
<tr>
<td>NM_146252</td>
<td>TBC1 domain family, member 13</td>
</tr>
<tr>
<td>NM_147023</td>
<td>olfactory receptor 385</td>
</tr>
<tr>
<td>NM_010915</td>
<td>kallikrein 1-related peptidase b4</td>
</tr>
<tr>
<td>NM_001002008</td>
<td>cDNA sequence BC049807</td>
</tr>
<tr>
<td>NM_172500</td>
<td>RIKEN cDNA 4831426I19 gene</td>
</tr>
<tr>
<td>NM_175554</td>
<td>claspin homolog (Xenopus laevis)</td>
</tr>
<tr>
<td>NM_010099</td>
<td>similar to EDA-A1; ectodysplasin-A</td>
</tr>
<tr>
<td>NM_027769</td>
<td>copine III</td>
</tr>
<tr>
<td>NM_175514</td>
<td>family with sequence similarity 171, member B</td>
</tr>
<tr>
<td>NM_026296</td>
<td>RIKEN cDNA 4930548H24 gene</td>
</tr>
<tr>
<td>NM_001038592</td>
<td>glutaredoxin 2 (thioltransferase)</td>
</tr>
<tr>
<td>NM_153116</td>
<td>GTP-binding protein 10 (putative); predicted gene 6877</td>
</tr>
<tr>
<td>NM_133671</td>
<td>U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 2</td>
</tr>
<tr>
<td>NM_029646</td>
<td>interleukin 34</td>
</tr>
<tr>
<td>NM_022309</td>
<td>core binding factor beta</td>
</tr>
<tr>
<td>NM_026810</td>
<td>mutL homolog 1 (E. coli)</td>
</tr>
<tr>
<td>NM_001033139</td>
<td>expressed sequence AI846148</td>
</tr>
<tr>
<td>NM_173029</td>
<td>adenylate cyclase 10</td>
</tr>
<tr>
<td>NM_146358</td>
<td>olfactory receptor 677</td>
</tr>
<tr>
<td>NM_001002272</td>
<td>trophinin</td>
</tr>
<tr>
<td>NM_146278</td>
<td>olfactory receptor 729</td>
</tr>
<tr>
<td>NM_133212</td>
<td>toll-like receptor 8</td>
</tr>
<tr>
<td>NM_027865</td>
<td>transmembrane protein 25</td>
</tr>
<tr>
<td>NM_019423</td>
<td>elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2</td>
</tr>
<tr>
<td>NM_025381</td>
<td>predicted gene 9349; ATPase, H+ transporting, lysosomal V1 subunit F</td>
</tr>
<tr>
<td>NM_019480</td>
<td>estrogen receptor-binding fragment-associated gene 9</td>
</tr>
<tr>
<td>NM_019709</td>
<td>membrane-bound transcription factor peptidase, site 1</td>
</tr>
<tr>
<td>NM_025310</td>
<td>FtsJ homolog 3 (E. coli)</td>
</tr>
<tr>
<td>NM_001017362</td>
<td>AT rich interactive domain 3C (BRIGHT-like)</td>
</tr>
<tr>
<td>NM_173024</td>
<td>serine (or cysteine) peptidase inhibitor, clade A, member 3B</td>
</tr>
<tr>
<td>NM_177234</td>
<td>RIKEN cDNA B230340J04 gene</td>
</tr>
<tr>
<td>NM_019639</td>
<td>predicted gene 5928; predicted gene 12617; predicted gene 4802; similar to ribosomal protein S27a; predicted gene 13215; predicted gene 6111; predicted gene 7808; predicted gene 6014; predicted gene 8317; ubiquitin C; ubiquitin B; similar to fusion protein: ubiquitin (bases 43_513); ribosomal protein S27a (bases 217_532); similar to ubiquitin B; predicted gene 8649; ribosomal protein S27A; predicted gene 11517; predicted gene 11808; predicted gene 8430; RIKEN cDNA 2810422J05 gene; similar to Ubc protein; predicted gene 13815; ubiquitin A-52 residue ribosomal protein fusion product 1; predicted gene 8797; predicted gene 1821; predicted gene 11759; predicted gene 5239; predicted gene 6438</td>
</tr>
<tr>
<td>NM_027290</td>
<td>minichromosome maintenance deficient 10 (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_177378</td>
<td>ring finger protein 150; similar to ring finger protein 150</td>
</tr>
<tr>
<td>NM_173876</td>
<td>chloride channel 3</td>
</tr>
<tr>
<td>NM_025591</td>
<td>RIKEN cDNA 2010309E21 gene; similar to CG5323-PA; predicted gene 6396; predicted gene 6624</td>
</tr>
<tr>
<td>NM_010497</td>
<td>isocitrate dehydrogenase 1 (NADP+), soluble</td>
</tr>
<tr>
<td>NM_134238</td>
<td>vomeronasal 1 receptor, H13</td>
</tr>
<tr>
<td>NM_013812</td>
<td>CDK2 (cyclin-dependent kinase 2)-associated protein 1; predicted gene 12184</td>
</tr>
<tr>
<td>NM_183165</td>
<td>pyridine nucleotide-disulphide oxidoreductase domain 1</td>
</tr>
<tr>
<td>NM_147054</td>
<td>olfactory receptor 584</td>
</tr>
<tr>
<td>NM_029360</td>
<td>transmembrane 4 superfamily member 5</td>
</tr>
<tr>
<td>NM_010170</td>
<td>coagulation factor II (thrombin) receptor-like 2</td>
</tr>
<tr>
<td>NM_008423</td>
<td>potassium voltage-gated channel, Shal-related family, member 1</td>
</tr>
<tr>
<td>NM_028491</td>
<td>RIKEN cDNA 1700040L02 gene</td>
</tr>
<tr>
<td>NM_010251</td>
<td>gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4</td>
</tr>
<tr>
<td>NM_172785</td>
<td>zinc finger CCCH type containing 12D</td>
</tr>
<tr>
<td>NM_019453</td>
<td>Mediterranean fever</td>
</tr>
<tr>
<td>NM_009688</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
NM_173436  cysteine-rich perinuclear theca 2

Supplementary Table 5

\textbf{Ca,\textbeta_{1a}}-\text{ChIP-on-chip enriched genes that are up-regulated in microarrays from cacnb1 -/- MPCs:}

Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
cathepsin W
chromobox homolog 5 (\textit{Drosophila HP1a})
FERM, RhoGEF and pleckstrin domain protein 2
G1 to S phase transition 1
heparan sulfate 6-O-sulfotransferase 2
metadherin
phosphoribosylglycinamido formyltransferase
processing of precursor 1, ribonuclease P/MRP family, (\textit{S. cerevisiae})
RIKEN cDNA 9130401M01 gene
serine (or cysteine) peptidase inhibitor, clade B, member 9g
serine/threonine kinase receptor associated protein
WD repeat and HMG-box DNA binding protein 1
WD repeat domain 55
X-linked inhibitor of apoptosis
aconitase 1
asp (abnormal spindle)-like, microcephaly associated (\textit{Drosophila})
cDNA sequence BC067068
complexin 2
G protein-coupled receptor 180
glia maturation factor, beta
HIV TAT specific factor 1
protein disulfide isomerase associated 5
sacsin
apurinic/apyrimidinic endonuclease 2
clasp homolog (Xenopus laevis)
DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
dual specificity phosphatase 9
E2F transcription factor 7
E2F transcription factor 8
fibronectin type III domain containing 5
glial cell line derived neurotrophic factor family receptor alpha 1
hyaluronan synthase 2
ligase I, DNA, ATP-dependent
minichromosome maintenance deficient 10 (S. cerevisiae)
olfactomedin 1
oligonucleotide/oligosaccharide-binding fold containing 1
solute carrier family 25, member 32
stanniocalcin 1

Supplementary Table 5 continued

Ca,β1a-ChIP-on-chip enriched genes that are down-regulated in microarrays from cacnb1 -/- MPCs:
aldehyde oxidase 1
biglycan
CD80 antigen
c-fos induced growth factor
C-type lectin domain family 4, member d
dentin matrix protein 1
gamma-glutamyl cyclotransferase
growth arrest specific 7
guanine nucleotide binding protein (G protein), gamma 11
myosin X
paired box gene 3
phosphatidic acid phosphatase type 2B
prostaglandin I2 (prostacyclin) synthase
RAB20, member RAS oncogene family
thiosulfate sulfurtransferase, mitochondrial
activating transcription factor 3
amyloid beta (A4) precursor protein-binding, family B, member 1
tartemin
basonuclin 2
cathepsin F
ey early endosome antigen 1
ectonucleotide
pyrophosphatase/phosphodiesterase 5
kinesin family member 1B
lipoma HMGIC fusion partner-like 2
lysophosphatidic acid receptor 1
major histocompatibility complex, class I-related
peroxisomal biogenesis factor 11 alpha
phytanoyl-CoA hydroxylase
protein tyrosine phosphatase-like A
domain containing 2
RIKEN cDNA 2610524H06 gene
serine incorporator 3
solute carrier family 16 (monocarboxylic acid transporters), member 3
transforming growth factor, beta induced transmembrane protein 25
transmembrane protein 35
zinc finger and BTB domain containing 4
catalase
cthepsin A
cDNA sequence BC049807
creatine kinase, mitochondrial 2
cytochrome c oxidase subunit VIb polypeptide 2
deiodinase, iodothyronine, type II
enoyl Coenzyme A hydratase domain containing 2
family with sequence similarity 110, member C
family with sequence similarity 5, member C
F-box and WD-40 domain protein 17
fibroblast growth factor 7
hepatitis A virus cellular receptor 2
hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
interferon, alpha-inducible protein 27 like
2A
LON peptidase N-terminal domain and ring finger 3
macrophage activation 2 like
N-acylethanolamine acid amidase
neuroligin 2
phosphodiesterase 1A, calmodulin-dependent
protein tyrosine phosphatase, non-receptor type 13
REST corepressor 2
RIKEN cDNA 6330406I15 gene
ring finger protein 125
serine (or cysteine) peptidase inhibitor, clade E, member 2
solute carrier family 2 (facilitated glucose transporter), member 3
Solute carrier family 26, member 7
sphingosine-1-phosphate receptor 3
thrombospondin 2
tumor necrosis factor receptor superfamily, member 9
tumor protein D52-like 1
ubiquitin specific peptidase 2
vasorin
zinc finger protein 608
CURRICULUM VITAE

Jackson Richard Taylor

Department of Internal Medicine/Gerontology
3121 Grey Building
Office Phone: 336-716-9820
Mobile Phone: 919-357-6275
E-mail: jtaylor@wakehealth.edu

Education and Professional Training:

Ph.D. Neuroscience Wake Forest University Health Sciences 2007-2013

- Thesis Project: Studying the non-classical role of a calcium channel auxiliary subunit (Cavβ1a) as a novel transcription factor and regulator of muscle development. Advisor: Osvaldo Delbono M.D., Ph.D.
- Introduction to Neuroscience Course Review Committee. 08/09-12/09 Reviewed full academic years worth of course material for redundancy and relevance. Advisor: Ron Oppenheim, Ph.D.

B.S. Biology (Minor in English) East Carolina University 2003-2007

- Research Assistant, Microbiology Department. 10/06-06/07 Examined mechanisms of drug resistance in breast cancer cell lines using biochemical techniques. Advisor: James A. McCubrey, Ph.D.
- Research Assistant, English Department. 09/06-05/07 Conducted biographical and historical research on the Paul Greene Collection at UNC Chapel Hill. Advisor: Margaret Bauer, Ph.D.

Awards and Honors:

Ruth L Kirschstein Predoctoral Individual National Research Service Grant Award (NRSA) recipient from the National Institute on Aging - F31AG039934. 07/2011-12/2012

Platform Speaker “Cavβ1a Nuclear Translocation: Potential Mechanisms and Functions” The 55th Annual Meeting of the Biophysical Society. 03/07/2011

Wake Forest Health Sciences Translational Science Institute (TSI) Ignition Funds award recipient ($5,000). 02/2011-02/2012
Professional Memberships:
2010-present Biophysical Society
2007-present Western North Carolina Chapter of the Society for Neuroscience
2007 Society for Neuroscience

Bibliography

Journal Articles:


In preparation:

Zhang T, Taylor JR, Delbono O. Fast Skeletal Muscle Troponin T is a Novel Interacting Partner of Voltage Gated Calcium Channel β1a Subunits.

Book Chapters:


Abstracts:


References:

Osvaldo Delbono, M.D., Ph.D.
Professor, Department of Internal Medicine/Gerontology
Wake Forest University Health Sciences
Winston-Salem, NC 27157
336.717.9802 telephone
336.758.4733 fax
odelbono@wakehealth.edu

James A. McCubrey, Ph.D.
Professor, Department of Microbiology and Immunology
Brody School of Medicine
East Carolina University
Greenville, NC 27834
252.744.2704 telephone
252.744.3104 fax
mccubreyj@ecu.edu

Ronald Oppenheim, Ph.D.
Professor, Department of Neurobiology and Anatomy
Wake Forest University Health Sciences
Winston-Salem, NC 27157
336.716.4405 telephone
roppenheim@wakehealth.edu