Examining Post-Transcriptional Regulation of Skeletal Muscle Satellite Cell Homeostasis, Activation and Fate Determination

By

Crystal Dawn Pulliam

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written by Crystal Dawn Pulliam

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Dr. Leslie Leinwand

(Committee Chair)

_____

Dr. Bradley B. Olwin

Date_____

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Thesis directed by Professor Bradley Bruce Olwin

Abstract

Skeletal muscle is essential for respiration, mobility, reproduction and metabolism. Deficits in muscle function due to disease, injury or age reduce both quality of life and lifespan. Muscles are long-lived tissues that require maintenance to retain functional integrity throughout the life of an organism. Satellite cells are the adult stem cells responsible for muscle repair and maintenance. Upon myotrauma, satellite cells re-enter the cell cycle, proliferate, and terminally differentiate to repair the muscle. In uninjured tissue, satellite cells are quiescent and infrequently proceed through myogenesis for muscle maintenance. The molecular mechanisms that regulate satellite cell quiescence and activation are poorly defined. Additionally, no comprehensive studies have determined when satellite cells attain quiescence in the adult tissue or whether satellite cells in different muscle acquire quiescence at distinct times.

Here, I investigate when satellite cells attain quiescence. Using $Pax7^{ICreERT2}$; $R26R^{tdTomato}$ mice to fluorescently label satellite cells, I determine that hindlimb muscles establish quiescence by 12 weeks of age; whereas, extraocular muscles fail to establish quiescence by 27 weeks. Additionally, I find that satellite cell contribution to adult muscle is greater than was assumed based on the low number of cycling satellite cells in uninjured muscle. Furthermore, I show that satellite cells contribution varies by both age and muscle group.

By comparing genome-wide expression profiles of quiescent and activating satellite cells, I identify RNA post-transcriptional regulation via RNA binding proteins as a regulatory mechanism of satellite cell activation. Specifically, I investigate the CELF family of RNA binding proteins. I find that CELF1/2/4 targets are enriched in genes downregulated during satellite cell activation. microRNAs that regulate satellite cell fate share targets with CELF1/2/4 during satellite cell activation. Additionally, CELF1/2/4 targets transcripts in key regulatory pathways of satellite cell activation, including p38 MAPK. I further examined gene expression profiles of aged satellite cells and identify elevated expression of genes downregulated during satellite cell activation, particularly RNA splicing and processing genes, that likely contribute to the impaired activation, self renewal and proliferation of aged satellite cells. Together, these data suggest the RNA post-transcriptional regulation of gene expression as a key mechanism mediating satellite cell activation.

Dedication

I dedicate this thesis to my family for their loving and unwavering support.

John A. Pulliam

Nancy L. Pulliam

John A. Pulliam II

Jenny M. Pulliam

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Chapter 1: Introduction

Skeletal Muscle & Disease

Skeletal muscle is essential for respiration, mobility, reproduction, and metabolism. Deficits in muscle function due to disease, injury or age reduce both quality of life and lifespan. Skeletal muscles are long-lived tissues, surviving for an average of 15 years (Spalding et al. 2005). Such enduring tissues require maintenance from resident adult stem cells to retain functional integrity (Rando and Wyss-Coray 2014; Rando 2006). Loss of regenerative capacity results in progressive muscle wasting, which is a hallmark of muscular dystrophies and sarcopenia (Pagotto and Silveira 2014; Malatesta 2012). Muscular dystrophies are a collection of genetic myopathies with a combined prevalence of ~1/2700 (CDC 2014); however, sarcopenia affects the majority of elderly persons (Batsis et al. 2014; Pagotto and Silveira 2014). With a rapidly aging population, sarcopenia is increasingly a concern as the number of individuals at risk nears one billion (United Nations 2013). An understanding of muscle regeneration will provide the insight necessary to develop treatments for degenerative myopathies.

Satellite Cells

Mammalian skeletal muscle is comprised of terminally differentiated myofibers, containing thousands of post-mitotic nuclei that are unable to contribute to muscle growth, repair or maintenance (Schmalbruch and Lewis 2000; Allbrook, Han, and Hellmuth 1971); these functions are performed by a population of resident stem cells known as satellite cells (Murphy et al. 2011; Lepper and Fan 2010; Lepper, Partridge, and Fan 2011; Shea et al. 2010; Lepper, Conway, and Fan 2009). Satellite cells reside adjacent to myofibers, above the myofiber membrane but beneath the basal lamina and comprise 2-11% of all myonuclei (Snow 1977; Schmalbruch and Hellhammer 1976; Allbrook, Han, and Hellmuth 1971; Cardasis and Cooper

1975; Megeney et al. 1996; Mauro 1961). In healthy, uninjured muscle, satellite cells are mitotically quiescent (Snow 1977; Schultz 1976). Upon myotrauma, extrinsic signals including HGF, nitric oxide, FGF2, and TNFα, trigger satellite cell activation and re-entry into the cell cycle (Do et al. 2012; Filippin et al. 2009; Chen, Jin, and Li 2007). Satellite cell activation requires p38α/β MAPK signaling (Jones et al 2005; Troy et al. 2012; Bernet et al. 2014), leading to expression of the myogenic regulatory factor MyoD and proliferation (Jones et al. 2005). Upon activation, satellite cells proceed through an initial division within 48h (D. D. W. Cornelison et al.; Troy et al. 2012). A subset will self-renew, replenishing the satellite cell pool for future regeneration (Troy et al. 2012). The majority, however, will form a rapidly proliferating population of myoblasts that will fuse with each other and with damaged myofibers, repairing the muscle (Webster and Fan 2013). The molecular mechanisms regulating this process are not well understood.

Satellite Cell Homeostasis

In addition to repairing injured muscle, satellite cells contribute to muscle growth and maintenance. During postnatal growth, satellite cells extensively contribute to the developing skeletal muscle (Bischoff 1990; Cardasis and Cooper 1975; Kelly and Zacks 1969; Kelly 1978; Ross, Duxson, and Harris 1987; Schultz 1976; Shea et al. 2010). In adult muscle, however, the majority of satellite cells have become mitotically quiescent and only 2-4% are cycling (Chakkalakal et al. 2012; Grounds and McGeachie 1987; McGeachie and Grounds 1987; Schultz 1976; Snow 1977). The limited prevalence of cycling satellite cells suggests low satellite cells turnover in healthy, adult skeletal muscle; however, the frequency of mitotic reentry is unknown. Additionally, the extent of satellite cell contribution to adult muscle for basal skeletal muscle

maintenance is unknown. Furthermore, no comprehensive studies have addressed when satellite cells acquire quiescence or whether satellite cells in different muscles acquire quiescence at distinct times.

Molecular Regulation of Satellite Cell Function

Satellite cells respond to extrinsic signals to both maintain quiescence and, upon exercise and injury, trigger cell cycle re-entry.

Signals Inhibiting Satellite Cell Activation

Myostatin is the best-characterized inhibitor of satellite cell activation and regeneration. Myostatin is a member of the TGF-β superfamily of signaling proteins (Sartori, Gregorevic, and Sandri 2014). Inactivation of myostatin results in substantial hypermuscularity and improved regeneration of sarcopenic muscle (Siriett et al. 2006, 2007; Stinckens, Georges, and Buys 2011). Myostatin signals through the ERK1/2 MAPK pathway to reduce Pax7 expression, independent of p38 MAPK signaling (McFarlane, Hennebry, et al. 2008). As Pax7 promotes satellite cell self-renewal, lack of Myostatin signaling increases self-renewal *in vitro* and the satellite cell population *in vivo* (McFarlane, Hennebry, et al. 2008; Siriett, Platt, et al. 2006). Further, Myostatin upregulates expression of the cyclin-dependent kinase inhibitor p21, decreasing Cdk2 and inhibiting satellite cell activation (Hauerslev, Vissing, and Krag 2014; McCroskery et al. 2003). Myostatin is expressed in both satellite cells and skeletal muscle and is subject to auto-regulation. Androgen Receptor, MyoD, FoxO1, SMAD transcription factors and TGF-β transcriptionally activate *Myostatin*. Myostatin then signals through SMAD3 to 1) repress *MyoD* transcription and 2) increase expression of miR-27a/b, which targets the *Myostatin* transcript, reducing expression (Allen and Unterman 2007; Carlson, Booth, and Gordon 1999; Dubois et al.

2014; McFarlane et al. 2014; Spiller et al. 2002). Myostatin interacts with multiple signaling pathways in satellite cells; however, the full molecular mechanisms have yet to be defined.

Signals Promoting Satellite Cell Activation and Proliferation

Multiple factors promote satellite cell activation, including HGF, nitric oxide, MMP-2, FGF-2, TNFα, Testosterone, IFG, Wnt7a and MGF (Chen, Zajac, and MacLean 2005; DO et al. 2012; Filippin et al. 2009; Ge, Zhang, and Jiang 2013; Goldspink 2004; Le Grand et al. 2009; von Maltzahn, Bentzinger, and Rudnicki 2012; Yamada et al. 2006, 2008). Some satellite cell activating factors, such as Testosterone, are systemic (Joubert and Tobin 1995; Chen, Zajac, and MacLean 2005). Other satellite cell activating factors, such as FGF-2, are released upon myotrauma by the extracellular matrix, damaged muscle fibers, and infiltrating immune cells (Clarke, Khakee, and McNeil 1993). Still other factors, including HGF and MMP-2, are synthesized and secreted by satellite cells to regulate their own activation (Tatsumi, Hattori, et al. 2002; Yamada, Sankoda, et al. 2008). Multiple satellite cell activating factors are released by several cell type; nitric oxide, for example, is secreted by both myofibers and infiltrating macrophages in injured muscle (Butterfield, Best, and Merrick 2006).

Satellite cells detect extracellular activation signals through trans-membrane receptors and heparan sulfate proteoglycans (HSPG). HSPGs can facilitate interactions between growth factors and their receptors (Rapraeger). The satellite cell activation signals FGF-2 and HGF, for example, interact with two HSPGs expressed on satellite cells, Syndecan-3 (Sdc3) and Syndecan-4 (Sdc4) (D. D. Cornelison et al. 2001). In satellite cells, Sdc3 and Sdc4 facilitate both FGF-2 and HGF signaling (Pisconti, 2012). The FGF-2 receptors FGFR1 and FGFR4 (Johnson and Allen 1995) as well as the HGF receptor, c-MET, (Webster and Fan 2013) are expressed on

satellite cells. Activation of FGFR1, 4 or c-MET by ligand binding induces signaling through the ERK1/2 and p38α/β MAPK pathways (Pisconti, 2012).

The paired-box transcript factor Pax7 plays dual roles in regulating satellite cell quiescence and activation. Pax7 is expressed in quiescent satellite cells and promotes expression of Id2 and Id3, repressors of MyoD (Kumar et al. 2009). In activated satellite cells, however, Pax7 interacts with histone methyltransferases to induce chromatin remodeling and promote transcription of the myogenic gene MYF5 (McKinnell et al. 2008; Hu et al. 2008). In activated satellite cells, Pax7 is indirectly downregulated through MyoD. Activated satellite cells signal through p38α/β MAPK to upregulate MyoD expression. MyoD promotes expression of miR-206 and miR-486, which repress Pax7 expression (Dey, Gagan, and Dutta 2011). MyoD additionally induces expression of a cohort of myogenic genes, committing activated cells to the myogenic lineage. Once activated, satellite cells proceed through several rounds of proliferation, as transient amplifying myoblasts (Jones et al. 2005).

Cell Cycle Exit

A subset of myoblasts will downregulate MyoD, re-express Pax7 and return to quiescence, replenishing the satellite cell pool for future rounds of regeneration (Troy et al. 2012). The majority of myoblasts will exit the cell cycle and commit to terminal differentiation, becoming myocytes. MyoD promotes differentiation by inducing expression of cell cycle inhibitors, including p21 and Rb (Guo et al. 1995; Martelli et al. 1994). Additionally, MyoD induces expression of myogenin, which is required for terminal differentiation. Myocytes fuse with each other, forming *de novo* myofibers, and fuse with existing myofibers, thereby repairing the muscle.

Review: Alteration of activation pathways in aged satellite cells

Note to thesis committee: In Chapter 3, I explore the role of the CELF family in regulating satellite cell activation. Data from Chapter 3 is incorporated into this review (to be submitted for publication in Fall, 2014) in the following sections:

- *Post-transcriptional regulation of adult satellite cell activation: RNA Binding Proteins*
- CELF1/2/4 *targets are downregulated during satellite cell activation*
- CELF1/2/4 *targets downregulated during satellite cell activation are enriched for microRNA regulators of satellite cell fate*
- CELF1/2/4 *targets downregulated during satellite cell activation are enriched for the p38 MAPK signaling pathway*
- *Dysregulation of satellite cell activation pathways with age*
- *Dysregulated expression of p38 MAPK pathway members in aged satellite cells*
- CELF1/2/4 *targets p38 MAPK pathway members that are dysregulated with age*

Introduction

Advances in health care and nutrition over the past century have resulted in increased life expectancy and global population aging. Recent reports predict 20% of the world population will be elderly by 2050, an estimated 2 billion people (United Nations 2013). With a rapidly growing aged population, incidences of age-associated diseases are expected to increase. The majority of elderly will develop the muscle wasting disease sarcopenia (Batsis et al. 2014; Pagotto and Silveira 2014). Sarcopenic individuals experience loss of muscle mass and strength resulting in increased frailty and risk of injury and death (Cawthon et al. 2007; Cruz-Jentoft et al. 2010;

Lauretani et al. 2003; Narici and Maffulli 2010). While the pathogenic mechanisms of sarcopenia are not fully understood, it clearly involves impaired skeletal muscle regeneration.

Aged skeletal muscle regeneration by satellite cells is functionally compromised at every step of myogenesis. Aged satellite cells have delayed activation, reduced proliferation and differentiation (Bernet et al. 2014; Yablonka-Reuveni, Seger, and Rivera 1999; Zwetsloot and Childs 2013). Although Notch, Wnt, TGF-β and TNF-α pathway dysregulation is implicated in the proliferation and differentiation defects, the molecular mechanisms underlying aged satellite cell activation defects have not been thoroughly investigated (Degens 2010; Chakkalakal and Brack 2012). We recently demonstrated that impaired FGF signaling and elevated p38 α/β MAPK activity results in reduced activation and self-renewal of aged satellite cells (Bernet et al. 2014). The cause of FGF and p38 α/β MAPK pathway dysregulation has yet to be determined.

My work and several studies suggest that post-transcriptional mechanisms regulate satellite cell activation (Farina et al. 2012, Hausburg et al., in revision). Post-transcriptional and post-translational, as opposed to transcriptional, regulation allows cells to rapidly respond to changes in their environment. A well-understood example is the cellular response to hypoxia. In oxygen rich environments, the transcription factor HIF1α is continuously produced and immediately degraded. Under hypoxic conditions, HIF1α is stabalized and promotes the hypoxic response (Pawlus and Hu 2013). Similar response mechanisms regulate myogenic and cell cycle gene expression in quiescent satellite cells, priming satellite cells to quickly re-enter the cell cycle upon myotrauma (Farina et al. 2012, Hausburg et al., in revision).

In this review, I will discuss our current knowledge of satellite cell activation pathways and their dysregulation with age, incorporating novel insights from transcriptome analysis of adult satellite cells during activation compared to aged satellite cells. Specifically, I will focus on

post-transcriptional regulation of select signaling pathways by micro-RNAs and RNA binding proteins.

Signaling pathways of adult satellite cell activation

To identify genes involved with satellite cell activation *in vivo*, we performed unbiased, global gene expression analysis comparing quiescent satellite cells to satellite cells isolated 12 hours post-injury (Farina et al. 2012). To eliminate genes not directly related to activation, we disregarded transcripts that changed expression in activation-deficient *Sdc4-/-* satellite cells. *Sdc4-/-* satellite cells fail to activate, proliferate and express MyoD within 48 hours in culture and fail to regenerate muscle (D. D. W. Cornelison et al. 2004; Farina et al. 2012). This resulted in a transcriptional profile of 4093 genes up- or downregulated during satellite cell activation ("activation dataset", Farina et al. 2012).

A majority of genes are downregulated during activation (56%), suggesting that satellite cell quiescence is actively maintained by a set of 'quiescence genes' that must be downregulated for cell cycle re-entry (Farina et al. 2012). This hypothesis is consistent with expression profiling studies comparing quiescent satellite cells with proliferating myoblasts and non-myogenic cells (S. Fukada et al. 2007) or with neonatal and dystrophic satellite cells (Pallafacchina et al. 2010; S.-I. Fukada et al. 2013). Recently, Rodgers, *et. al.* discovered that satellite cells can exist is two quiescent states with distinct transcriptomes, supporting the hypothesis that quiescence is actively maintained (Rodgers et al. 2014).

Gene set enrichment analysis was performed to identify potential regulator pathways of satellite cell activation. The activation dataset was analyzed using three independent enrichment algorithms: DAVID, GSEA, and ToppFun (Chen et al. 2009; Huang, Sherman, and Lempicki

2009; Subramanian et al. 2005). The top eight enriched KEGG pathways identified by at least two of the three algorithms are listed in Table 1. The activation dataset is enriched for multiple pathways with known myogenic roles (Table1, Table 2, Table 3). The Wnt, TGF-β, Notch, and FGF pathways are dysregulated in aged satellite cells, impairing proliferation and differentiation. These pathways have been covered in a recent review and will only be discussed here in relation to activation (Chakkalakal and Brack 2012). Here, I will focus on the FGF, p38 and ERK1/2 MAPK signaling pathways, their roles in satellite cell activaiton, and altered expression in aged satellite cells.

Pathway	# Genes in Pathway	# Genes in Activation Dataset	DAVID p-value	GSEA p-value	ToppFun p-value
Cell cycle	128	32	9.40E-03	2.66E-12	2.84E-03
Wnt	151	38	9.90E-03	1.98E-14	5.36E-03
MAPK	267	55	2.06E-02	4.44E-16	
TGF-beta	87	22	3.02E-02	1.17E-10	1.14E-02
Insulin	138	31	4.34E-02	3.70E-12	
Jak/ Stat	155	31	5.59E-02	2.32E-09	
Toll-like receptor	102	23	6.18E-02	2.50E-08	
Notch	48	13	7.53E-02	2.34E-06	3.25E-02

Table 1 Pathways Enriched During Satellite Cell Activation. KEGG Pathways and associated p-values enriched in the activation database as determined by at least two of three independent analyses by DAVID, GSEA and ToppFun.

Database	Pathway	# Genes in Pathway	# Genes in Activation Dataset	Enrichment Detected By	p-value
Biocarta	CDC42/Rac	16	8	GSEA	1.29E-06
Biocarta	ERK	28	10	GSEA	2.64E-06
KEGG	PI3K-Akt	22	7	ToppFun	7.43E-05
Biocarta	Akt	22	7	GSEA	2.01E-04
Wiki Pathways	Androgen receptor signaling pathway	88	27	ToppFun	3.55E-04
Biocarta	p38 MAPK	40	9	GSEA	4.65E-04
Biocarta	IGF1	21	6	GSEA	1.10E-03
Biocarta	IGFR1	23	6	GSEA	1.84E-03
Wiki Pathways	mRNA processing	136	34	ToppFun	3.72E-03
Wiki Pathways	TNF-alpha/NF-kB Signaling Pathway	196	45	ToppFun	5.50E-03
Wiki Pathways	EGFR1 Signaling Pathway	177	41	ToppFun	6.66E-03
Wiki Pathways	Hedgehog Signaling Pathway	22	8	ToppFun	1.56E-02
Wiki Pathways	TOR signaling	34	10	ToppFun	3.37E-02
Panther	EGF	141	37	DAVID	4.21E-02
Wiki Pathways	AGE/RAGE pathway	66	16	ToppFun	4.99E-02
KEGG	Estrogen signaling pathway	100	22	ToppFun	6.45E-02
Panther	FGF	569	33	DAVID	8.19E-02

Table 2 Myogenic Pathways Enriched During Satellite Cell Activation. Myogenic pathways and associated p-values enriched in the activation database as determined by at least one of three independent analyses by DAVID, GSEA and ToppFun.

mRNA Processing	FGF Signaling Pathway	ERK Signaling Pathway	p38 MAPK Signaling Pathway
CDC40	Akt3	EGFR	ATF2
CELF1	Fgf14	GNAS	CDC42
CLK1	Fgf4	GNB1	HMGN1
CLK4	Fgfr3	GNGT1	MAP3K1
CPSF3	Grap	ITGB1	MEF2A
DDX1	Kras	MAPK3	RAC1
DHX15	Map2k7	MKNK2	RAPGEF2
DHX38	Map3k1	NGFR	RIPK1
DNAJC8	Map3k6	PPP2CA	TGFB3
HNRNPC	Mapk10	SOS1	
HNRNPH2	Mapk12		
HNRNPK	Mapk3		
HNRNPR	Nras		
NCBP2	Pebp4		
NONO	Pik3c2a		
NXF1	Ppp2ca		
PABPN1	Ppp2r5b		
POLR2A	Ppp2r5e		
PRPF4B	Ppp6c		
RBM39	Prkce		
RBMX	Prkch		
RNMT	Rac1		
SF3A2	Rasa1		
SF3A3	Rasa4		
SF3B5	Sos1		
SNRPB	Sos2		
SNRPE	Spry4		
SRRM1	Ywhab		
SRSF3	Ywhag		
SRSF4	Ywhah		
SRSF6	Ywhaq		
SRSF7			
SUPT5H			
TXNL4A			

Table 3 Genes from Select Pathways Enriched During Activation. Lists of genes from the KEGG mRNA Processing, FGF, ERK and p38 MAPK Signaling Pathways that are enriched during satellite cell activation (Table1, Table2).

FGF-2 signals through p38 and ERK MAPK pathways to promote satellite cell activaiton

The FGF, p38 and ERK1/2 MAPK pathways cooperate to promote satellite cell activation and self-renewal. Satellite cells express two FGF receptors: FGFR1 and FGFR4 (Flanagan-Steet et al. 2000; Zhao et al. 2006). Genetic ablation or inactivation of either receptor results in impaired muscle regeneration due to defective satellite cell activation (Flanagan-Steet et al. 2000; Zhao et al. 2006). FGF1, 2, 4 and 6 are expressed in skeletal muscle (Chakkalakal et al. 2012; Düsterhöft, Putman, and Pette 1999; Eash et al. 2007; Kästner et al. 2000). FGF1, 2, 6, and 7 are expressed by proliferating myoblasts and FGFs 5, 6 and 7 are expressed by in myotubes *in vitro* (Hannon et al. 1996). Mice null for either FGF6 or both FGF2 and FGF6 exhibit impaired satellite cell activation and muscle regeneration (Floss, Arnold, and Braun 1997; Neuhaus et al. 2003), while increased FGF2 expression results in loss of quiescence and depleation of the satellite cell pool (Chakkalakal et al. 2012). FGFs interact with their receptors through heperan sulfate proteoglycans (HSPG) to initiate signaling. The HSPGs Syndecan-3 and Syndecan-4 are expressed on satellite cells; loss of Syndecan-3 accelerates cell cycle entry whereas loss of Syndecan-4 delays activaiton of explanted satellite cells (D. D. W. Cornelison et al. 2004; D. D. Cornelison et al. 2001; Pisconti et al. 2010). *Syndecan-4-/-* satellite cells cultured with FGF2 fail to phosphorylate ERK1/2, indicating impaired FGF signaling (D. D. W. Cornelison et al. 2004).

p38α/β MAPK signaling is essential for satellite cell activation and self-renewal

FGF-2 stimulates p38 and ERK1/2 MAPK signaling to promote satellite cell activation. Proliferating MM14 myoblasts cultured with FGF-2 phosphorylate p38α/β (Jones et al. 2005).

p38α/β phosphorylation is the first detectable event of satellite cell activaiton (Jones et al. 2005, Hausburg et. al., in revision). Activated satellite cells asymmetrically phosphorylate p38α/β to coordinate cell fate among prospective daughter cells; the pp38α/β-expressing daughter cell proceedes through myogensis while the daughter cell lacking pp38α/β will self-renew and replenish the satellite cell population (Troy et al. 2012). Pharmacological inhibition of p38α/β signaling prevents cell cycle entry and MyoD expression, indicating a failure to activate (Jones et al. 2005).

Regulation of ERK1/2 signaling in quiescent and activated satellite cells

FGF-2 activation of ERK1/2 signaling is required for the G1 to S-phase transition of cultured myoblasts (Jones et al. 2005). In mice with satellite cell specific genetic deletion of *Sprouty1,* an ERK signaling inhibitor, the quiescent satellite cell pool was reduced 50% after regeneration, which was rescued through pharacological inhibiton of the ERK pathway (Shea et al. 2010). Furthermore, evelated ERK signaling was observed in *Sprouty1-/-* satellite cells that fail to return to quiescence (Shea et al. 2010). These observations suggest that downregulation of ERK signaling is required for satellite cell self-renewal. Paradoxically, uninjured *Erk1-/-* mice have a 40% reduction in satellite cells (Le Grand, Grifone, et al. 2012). Furthermore, Mice null for the ERK signaling inhibitor *Dusp6* exhibit a two-fold increase in quiescent satellite cells after regeneration (Le Grand, Grifone, et al. 2012). These disperate results are likely due to distinct effects of ERK signaling on satellite cell sub-populations. Serial injuries in satellite cell speicfic *Sprouty1-/-* mice fail to reduce the quiescent satellite cell population further than a single injury, indicating a sub-population that does not require *Sprouty1* for self-renewal (Shea et al. 2010).

Both the Sprouty and Dusp families are regulated during satellite cell activaiton. *Sprouty1, Sprouty4* and *Dusp8* decrease expression during satellite cell activation while *Dusp9* increases expression during satellite cell activation. As discussed above, *Sprouty1* is required for self-renewal in a sub-population of satellite cells and *Sprouty1* downregulation correlates with increased ERK signaling and proliferation (Shea et al. 2010; Sasaki et al. 2001). The Sprouty proteins form homo- and heterodimers to inhibit FGF-2, but not EGF, stimulated ERK signaling (Ozaki et al. 2005). The Sprouty1/4 heterodimer exhibits the strongest inhibiton of ERK signaling (Ozaki et al. 2005). Thus, *Sprouty1* and *Sprouty4* are likely quiescence genes that must be downregulated for satellite cell activation. The necessity of Sprouty4 for satellite cell quiescence, activation, and self-renewal has yet to be investigated.

Both Dusp8 and Dusp9 are expressed in adult skeletal muscle (Welle et al. 2004). Dusp8 inhibits ERK1 and p38a MAPK kinase activity in mammalan cells and dephosphorlylates ERK2 *in vitro* (Dickinson et al. 2002; Martell et al. 1995). Dusp9 attenuates phosphorylated p38 MAPK but not phosphorylated ERK in 3T3-L1 adipocytes (Bazuine et al. 2004); however, in mouse embryonic stem cells (ESC), BMP4 signaling upregulates Dusp9, which reduces ERK1/2 phosphorylation. This demonstrates that Dusp9 phosphatase activity is context dependent. To date, no studies have addressed roles of Dusp8 or Dusp9 in skeletal muscle or satellite cells.

Post-transcriptional regulation of adult satellite cell activation: microRNAs

Although many studied have identified microRNAs as regulators of skeletal muscle and satellite cell proliferation through differentiation (Kovanda, Režen, and Rogelj 2014), there is a sparsity of research examining microRNA regulation of activation. We recently demonstrated that miR-124, -133, and -206 are expressed in quiescent satellite cells and are rapidly

downregulated upon activation (Farina et al. 2012, Farina Thesis 2011). This expression pattern marks miR-124, -133 and -206 as putative quiescent genes. Indeed, *in vivo* inhibition of miR-133 or -206 prevents satellite cell self-renewal and muscle regeneration while injections of miR-1, -133, and -206 concurrent with injury increased the number of satellite cells and improved regeneration (Nakasa et al. 2010, Farina Thesis 2011). This is likely mediated through miR-133 and -206 regulation of Pax7 (Dey, Gagan, and Dutta 2011; Rosales et al. 2013; Nakasa et al. 2010). Additionally, miR-16, -93, and -107 are rapidly induced upon satellite cell activation. *In vitro* inhibition of miR-16 in explanted satellite cells on myofibers prior to their first division increases the Pax7+ proliferating population while inhibition of miR-106b and miR-124 increases the Pax7+/MyoD- quiescent population (Farina et al. 2012). These data demonstrate the role of microRNAs in regulating the cell fate decision between activation, proliferation, and self-renewal.

Post-transcriptional regulation of adult satellite cell activation: RNA Binding Proteins

We previously reported that Molecular Function Go term: RNA Binding and the biological network RNA-post transcriptional modification are enriched during satellite cell activation (Farina et al. 2012) (Chapter 3, Figure 3). We further examined the 154 RNA binding proteins (RNA-BPs) that change expression during activation and found that 69% are downregulated (Farina et al. 2012) (Chapter 3, Figure 4). Notably, this list contains known regulators of *Myod1* transcript stability: HuR, CELF1 and TTP (Farina et al. 2012) (Chapter 3, Figure Table 2). HuR and TTP bind to the AU-rich element (ARE) in the *Myod1* 3'UTR to either promote (HuR) or inhibit (TTP) *Myod1* transcript stability (van der Giessen et al. 2003; Figueroa et al. 2003, Hausburg et. al., in revision). A direct interaction between CELF1 and *Myod1* has not

been established; however, CELF1 binds to ARE and GU rich elements (GRE) to promote transcript decay and CELF1 knockdown in C2C12 myoblasts stabilizes ARE & GRE containing transcripts, including *Myod1* (Lee et al.; Luc Paillard et al.; Vlasova et al.; Vlasova and Bohjanen), suggesting that CELF1 may regulate *Myod1* transcript stability in myogenic cells.

HuR, TTP, CELF1, and CELF2 likely regulate expression of each other in quiescent and activated satellite cells. These four RNA-BPs have auto-regulatory functions by binding to AREs present in their own transcripts (Al-Ahmadi et al. 2009; Brooks, Connolly, and Rigby 2004; Dembowski and Grabowski 2009; Lee et al. 2010; Tchen et al. 2004). Furthermore, they compete amongst themselves to bind to and regulate common targets. For example, an alternatively poly-adenylated HuR transcript variant contains a class II ARE that TTP and HuR compete to bind to in HeLa cells (Al-Ahmadi et al. 2009). HuR expression also upregulates TTP translation (Tiedje et al. 2012). Additionally, HuR and TTP are downstream p38 MAPK targets; HuR is activated by phosphorylation, whereas, phosphorylation of TTP inactivates its transcript destabilizing function (Stoecklin et al. 2004; Tiedje et al. 2012). CELF1 binds to HuR, CELF1, and CELF2 in proliferating C2C12s, likely promoting decay (Lee et al. 2010) (Chapter 3, Table 4). Similarly to CELF1, CELF2 is expressed in quiescent satellite cells and decreases expression upon activation (Farina et al. 2012) (Chapter 3, Table 4). CELF2 out-competes HuR for binding to a shared target, the COX-2 ARE, resulting in inhibited COX-2 translation (Sureban et al. 2007). CELF2 is highly similar to CELF1 and may also target HuR, regulating expression (Barreau et al. 2006; Ladd et al. 2004; Pradhan, Samson, and Sun 2013).

Together, these data suggest a possible regulatory feedback mechanism in quiescent satellite cells. During quiescence, TTP, CELF1, and CELF2 may bind to the HuR transcript to promote decay and inhibit translation. Simultaneously, TTP protein inhibits TTP transcript

stability, reducing the levels of both (Hausburg et. al., in revision). Upon activation, p38 MAPK phosphorylates and inactivates TTP via MK2, transiently increasing TTP transcript levels (Sachidanandan, Sambasivan, and Dhawan 2002; Stoecklin et al. 2004). TTP, CELF1, and CELF2 are then downregulated, releasing HuR repression. HuR protein is phosphorylated and activated by p38 MAPK. Phosphorylated HuR may then bind to its own transcript, promoting stability in a positive feedback loop. Alternatively, HuR expression during activation may shift towards the short transcript variant that does not include a 3'UTR ARE, preventing TTP, CELF1, and CELF2-induced repression.

Putative TTP targets are downregulated during satellite cell activation

To determine whether TTP regulates satellite cell activation outside of HuR and *Myod1* interactions, I compared the satellite cell activation dataset to putative TTP targets. TTP is phosphorylated and inactivated by MK2 during satellite cell activation; thus, TTP target transcripts are expected to increase expression during satellite cell activation (Stoecklin et al. 2004). Lai *et. al.,* identified 250 stabilized transcripts in TTP deficient fibroblasts (W. S. Lai et al. 2006). Of the 250 putative TTP targets, 202 are expressed in satellite cells. The satellite cell activation dataset included 47 putative TTP targets (Table 4, *P*-value = 0.06); 5 increased expression (Table 4, *P*-value = 0.999) and 42 decreased expression during satellite cell activation (Table 4, *P*-value = 3.30×10^{-6}). Paradoxically, transcripts downregulated during satellite cell activation are statistically significantly enriched for putative TTP targets. Transcripts are frequently regulated by multiple post-transcriptional mechanisms that compete to determine gene expression (For example, see the discussion of COX-2 above (Bakheet, Williams, and Khabar 2003; Jiang, Singh, and Coller 2013; Murphy et al. 2011; Sureban et al. 2007). Such a scenario is

likely occurring during satellite cell activation when multiple RNA-BPs and microRNAs with shared targets (see below) are differentially expressed.

Change with Satellite Cell Activation			
Decreasing			Increasing
Adamts4	Ier5	Rreb1	Arfgef1
Atxn7	Jarid2	Smarca1	Arid4a
B4galt3	Kif16b	Socs5	Phactr1
Bmp4	LOC100044968	Sos2	Traf5
C230081A13Rik	Lmcd1	Spred2	Tsc22d2
Camsap1l1	Lmo7	Stard4	
Cited2	Ndel1	Synj1	
Cldn12	Pim3	Tmem57	
Ddx5	Pnrc2	Tob1	
Elf1	Ppp1r12a	Uvrag	
Ep300	Ppp1r12b	Vegfc	
Gadd45g	Ptpn12	Wasl	
Hivep1	Rabgef1	Zfp644	
Hspa1b	Rgs16	Zzz3	

Table 4 TTP targets that change expression during satellite cell activation. Putative TTP targets detected on the satellite cell activation dataset with expression changes during satellite cell activation indicated in the column headers. Genes annotated under the Molecular Function GO Term: SMAD binding are in bold.

GO analysis detected three putative TTP targets decreasing during satellite cell activation as SMAD binders (Cited2, Ep300, and Tob1, Table 4). Cited2 and Ep300 interact with SMAD3 as transcriptional co-activators to promote TGF-β-mediated upregulation of MMP-9 (Chou et al. 2006). MMP-9 is upregulated in skeletal muscle one day post-injury and is associated with myoblast migration *in vitro* (Kherif et al. 1999; Lewis et al. 2000). Tob1 associates with SMAD2 and SMAD4 in quiescent T-cells and must be downregulated for T-cell activation (Tzachanis et al. 2001). Additionally, Tob1 prevents proliferation of hepatocytes in uninjured liver and must be downregulated for normal liver regeneration (Tzachanis et al. 2001). This is consistent with Tob1 expression changes during satellite cell activation and suggests a role in maintaining quiescence.

CELF1/2/4 targets are downregulated during satellite cell activation

To determine whether CELF proteins are regulators of activation, I compared the activation dataset with CELF1/2/4 targets. Seven hundred ninety three CELF1/2/4 target transcripts were identified in proliferating C2C12 myoblasts through RIP-Chip using an anti-CELF antibody (Good et al. 2000; Lee et al. 2010; Roberts et al. 1997; Timchenko, Miller, et al. 1996). Of those, 717 (90.4%) were detectable by both microarrays used for the satellite cell activation and CELF1/2/4 RIP-Chip experiments (Chapter 3, Figure 5). Of the 4093 genes on the satellite cell activation dataset, 2199 (53.7%) are detectable by both arrays, including 846 (44.2%) genes increasing and 1353 (62.1%) genes decreasing during activation (Chapter 3, Figure 5). Approximately 23% CELF1/2/4 targets decrease expression during activation (165 genes, Chapter 3, Tables 3 and 4; Chapter 3, Figure 5, *P*-value = 1.4×10^{-18}). Additionally, one third of CELF 1/2/4 transcripts downregulated during activation contain at least one of two

known CELF1 binding motifs: GRE (UGUUUGUUUGU) and GU-repeat (UGUGUGUGUGU). These motifs are enriched in the 3'UTRs of short-lived transcripts in HeLa cells and C2C12 myoblasts and are stabilized upon CELF1 knockdown (Rattenbacher et al. 2010; Vlasova et al. 2008; Lee et al. 2010). This suggests that promoting mRNA decay may be a major mechanism of action for CELF1 during satellite cell activation.

CELF1/2/4 targets downregulated during satellite cell activation are enriched for microRNA regulators of satellite cell fate

To further investigate the role of CELF1/2/4 targets in satellite cell activation, I identified enriched biological networks. CELF1/2/4 targets downregulated during activation were analyzed by three independent enrichment algorithms: GSEA, ToppFun, and Ingenuity Pathway Analysis (IPA). These genes are enriched for putative miR-1/206, miR-124, miR-16 and miR-200B targets (Chapter 3, Table 5). miR-1/206, miR-124, miR-16 and miR-200B change expression during satellite cell activation (Farina et al. 2012; Farina Thesis 2011). We have recently demonstrated that miR-124, miR-16, and miR-206 regulate the satellite cell fate decision between proliferation and self-renewal (see next section) (Farina et al. 2012; Farina Thesis 2011). RNA-BPs and microRNAs can cooperate to increase the degradation rate of shared target transcripts (Jiang, Singh, and Coller 2013). Celf1 can promote transcript degradation and may cooperate with a cohort of microRNAs to maintain satellite cell quiescence (Vlasova et al. 2008). This suggests that satellite cell fate is post-transcriptionally regulated and involves the coordination of both microRNAs and RNA binding proteins.

CELF1/2/4 targets downregulated during satellite cell activation are enriched for the p38 MAPK signaling pathway

CELF1/2/4 targets decreasing expression during activation are enriched for the p38 α/β MAPK pathway (Table 5; Chapter 3, Figure 6). As discussed above, this pathway is required for satellite cell activation and self-renewal. CELF1/2/4 targets multiple members of the p38 α/β MAPK pathway during activation, including *Rac1* and *Cdc42* (Chapter 3, Table 7). These genes have roles in regulating myoblast proliferation, motility and differentiation (Bryan et al. 2005; Fiaschi et al. 2012; Kaminsky et al. 2011; Liu et al. 2011; Loh et al. 2012; Meriane et al. 2000). Additionally, CELF1/2/4 targets *Atf*2, a member of both the TGF-β and p38 MAPK pathways (Chapter 3, Table 7). ATF-2 forms a complex with SMAD3 and SMAD4 in C2C12 myoblasts upon p38 phosphorylation, correlating with reduced proliferation, increased p21 expression and terminal differentiation (Blank et al. 2009; Dziembowska et al. 2007; Hanafusa et al. 1999; Philip, Lu, and Gao 2005; Sano et al. 1999).

Pathway	P-value	Database
CDC42 signaling events	2.31E-06	BioSystems: Pathway Interaction Database
RAC1 signaling pathway	6.99E-06	BioSystems: Pathway Interaction Database
mTOR signaling pathway	1.29E-05	BioSystems: Pathway Interaction Database
ErbB1 downstream signaling	2.76E-05	BioSystems: Pathway Interaction Database
p38 MAPK Signaling Pathway	1.93E-04	BioSystems: WikiPathways
Signaling mediated by p38-alpha and p38-beta	2.17E-04	BioSystems: Pathway Interaction Database
Regulation of Actin Cytoskeleton	2.61E-04	BioSystems: WikiPathways
Ras Pathway	2.84E-04	PantherDB
Cytoskeletal regulation by Rho GTPase	3.04E-04	PantherDB
Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met)	3.71E-04	BioSystems: Pathway Interaction Database
RhoA signaling pathway	4.03E-04	BioSystems: Pathway Interaction Database
T cell activation	4.76E-04	PantherDB
Hedgehog signaling events mediated by Gli proteins	4.85E-04	BioSystems: Pathway Interaction Database
Hedgehog signaling pathway	5.36E-04	PantherDB
Huntington disease	6.43E-04	PantherDB
Signaling events mediated by the Hedgehog family	8.36E-04	BioSystems: Pathway Interaction Database
Parkinson disease	8.85E-04	PantherDB
TGF-beta signaling pathway	1.08E-03	PantherDB
S1P2 pathway	1.09E-03	BioSystems: Pathway Interaction Database
Physiological and Pathological Hypertrophy of the Heart	1.09E-03	BioSystems: WikiPathways

Table 5 Enriched Pathways from CELF1/2/4 targets decreasing expression during satellite cell activation. CELF1/2/4 targets downregulated during satellite cell activation were analyzed for pathway enrichment through ToppFun. The top twenty enriched pathways are listed along with p-values and the curation databases.

Alteration of satellite cell activation pathways with age

To identify genes differentially expressed with age, we performed unbiased genome-wide expression analysis between adult and aged satellite cells. RNA from FACS-isolated adult (3-6 months) and aged (20-25 months) satellite cells was collected and processed for microarray hybridization similarly to the activation experiment previously described (Farina et al. 2012; Bernet et al. 2014). To make my analysis more robust, I performed an identical analysis on another experimental dataset comparing genome-wide expression of satellite cells from adult (5-6 months), aged (20-24 months), and geriatric (28-32 months) mice (Sousa-Victor et al. 2014). Our aged satellite cell dataset clustered more closely with the geriatric dataset than did the other aged dataset (data not shown); I therefore used the geriatric dataset in our analysis.

To determine whether genes involved with satellite cell activation are dysregulated with age, I compared the satellite cell activation dataset to the old and geriatric datasets. Approximately 10% of genes downregulated with age decrease expression during satellite cell activation and 11% increase expression during satellite cell activation; however, these changes are not statistically significant (Table 6). Of 671 genes upregulated with age on both the old and geriatric datasets, 20% decrease expression during satellite cell activation (Table 6, P-value = 4.43×10^{-9}). This suggests satellite cell quiescence gene expression is elevated in aged satellite cells, in agreement with observations of delayed activation in aged satellite cells (Bernet et al. 2014; Yablonka-Reuveni et al. 1999; Zwetsloot and Childs 2013).

		Change with Age					
		Increasing	% of Total	P-value	Decreasing	% of Total	P-value
Change During Activation	Increasing	36	5.4%	1.00E+00	41	9.7%	8.63E-01
	Decreasing	138	20.6%	4.43E-09	47	11.1%	8.67E-01
Total Changing with Age		671			424		

Table 6 Genes upregulated with age are enriched for genes downregulated during satellite cell activation. Genes that are upregulated (671) or downregulated (424) with age on both the old and geriatric datasets were compared to the satellite cell activation dataset. A statistically significant number (138, 20% of 671) of genes upregulated with age are downregulated during satellite cell activation.

FGF signaling is impaired in aged satellite cells

FGF and the MAPK signaling pathways p38 and ERK1/2 are dysregulated in aged muscle and satellite cells, contributing to impaired satellite cell activation and regeneration (Bernet et al. 2014; Chakkalakal and Brack 2012). FGFR1 and FGFR4 are expressed in satellite cells although qRT-PCR of young and aged satellite cells shows no difference in FGFR1 or FGFR4 transcripts levels with age (Jump et al. 2009). Of the FGF ligands, only FGF-2 has been investigated in aged muscle regeneration. FGF-2 expression in skeletal muscle increases with age; however, aged satellite cells also express higher levels of Sprouty1, a negative regulator of FGF & ERK signaling (Shea et al. 2010; Chakkalakal et al. 2012). This likely contributes to the reduced FGF signaling, impaired activation and self-renewal observed in aged satellite cells (Chakkalakal et al. 2012; Shefer et al. 2006; Bernet et al. 2014).

The FGF pathway is dysregulated at the transcript level in aged satellite cells. FGF pathway members increase and decrease expression in both the old and geriatric datasets (Table 5). Seven FGF pathway members that change expression with age also change expression during satellite cell activation (Table 7, red and green shading). Of note, Sos1 and Sprouty4, which are differentially regulated during satellite cell activation, increase expression with age in both the

old and geriatric datasets (Table 7). Sprouty4 suppresses FGF-2 induced ERK signaling through association with Sos1, preventing Sos1 from binding with Grb2 and recruitment of the Grb2-Sos1 complex to either the FGF docking adaptor protein FRS2 or the phosphatase Shp2 (Ozaki et al. 2005; Hiroshi Hanafusa et al. 2002). Additionally, Sprouty2 is upregulated with age. Over-expression of Sprouty-2 in C2C12 myoblasts inhibited ERK1/2 activity and induced expression of the cell cycle inhibitor p21 (de Alvaro et al. 2005). Furthermore, ERK2 (MAPK1) itself is downregulated with age (Table 7). Together, these data suggest that upregulation of FGF signaling inhibitors and downregulation of ERK2 likely contribute to the abrogated FGF signaling and delayed activation of aged satellite cells.

Change in Old		Change in Geriatric	
Increasing	Decreasing	Increasing	Decreasing
araf	araf	bmp4	cdh1
braf	braf	eef2k	eif4e1b
il17rd	dusp6	elk4	etv2
sos1	eef2k	etv4	fgf14
spry2	map2k6	fgfr1op2	fgf21
spry4	map3k7	fos	fgf23
	mapk1	map3k7	fgf3
	mapkapk5	mapk1	fgfr2
		mapkapk5	gata5
		mycn	gbx1
		sos1	gbx2
		spry4	sh3gl2
			sox17

Table 7 The FGF pathway is dysregulated with age. Gene symbols of FGF pathway members that change expression with age in the old (first and second columns) and geriatric (third and fourth columns) datasets are listed. Genes that increase expression with age are listed in the first and third columns. Genes that decrease expression with age are listed in the second and fourth columns. Genes that increase (red) or decrease (green) expression during satellite cell activation are shaded. Bold genes (sos1 and spry4) appear on both the old and geriatric dataset.

Dysregulated expression of p38 MAPK pathway members in aged satellite cells

Recently, we showed that the loss of asymmetric division and self-renewal in aged satellite cells is due to elevated p38 MAPK signaling (Bernet et al. 2014). I next asked if the p38 MAPK pathway is dysregulated at the transcript level in aged satellite cells. The p38 MAPK pathway is significantly enriched among genes changing expression with age (Chapter 3, Table 9). In the old dataset, seven pathway members increase expression (*P* value = 6.5 $x10^{-3}$; Chapter 3,Table 10), and ten decrease expression with age (*P* value = 3.7 $x10^{-3}$; Chapter 3, Table 10), while in the geriatric dataset, twelve p38 MAPK genes increase expression (*P* value = 3.1 x 10^{-4}; Chapter 3, Table 10) and six decrease expression with age (*P* value = 4.3 x 10^{-2}; Chapter 3, Table 10). Furthermore, four of the twelve transcripts with elevated expression in aged satellite cells from the geriatric dataset decrease expression during adult satellite cell activation (*P* value = 7.0 $x10^{-3}$; Chapter 3, Table 12). This suggests that missexpression of multiple p38 MAPK pathway members is responsible for dysregulated p38 MAPK signaling in aged satellite cells.

RNA splicing and processing are dysregulated with age

To identify disrupted processes in aged satellite cells, the 671 genes upregulated with age in both the old and geriatric datasets were analyzed for enriched GO terms. Of the top ten enriched GO Term: Biological Process categories, eight involve either RNA splicing or processing (Table 8). Fifty percent of RNA-BPs regulated during satellite cell activation are involved with RNA splicing or processing (Farina et al. 2012) (Chapter 3, Figure 4). Additionally, 15/ 49 genes with mRNA processing functions that are regulated with age change expression during satellite cell activation (Table 9). Such mRNA processing factors include

HuR, Celf2, and Celf4 (Table 9). This suggests that mRNA processing involved with satellite cell activation is likely altered in aged satellite cells.

GO Term	P-value
RNA processing	3.88E-13
RNA splicing	1.20E-11
mRNA metabolic process	2.30E-11
mRNA processing	7.51E-11
RNA splicing, via transesterification reactions	2.77E-09
mRNA splicing, via spliceosome	6.31E-09
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	6.31E-09
mRNA splice site selection	1.05E-06
ER-nucleus signaling pathway	4.58E-05
cellular response to unfolded protein	6.13E-05

Table 8 RNA splicing and processing are dysregulated in aged satellite cells. Enriched GO Term: Biological Processing categories among 671 genes increasing expression with age in both the old and geriatric datasets.

Change with Age		
Increasing		Decreasing
brwd1	sf3b4	ankrd33b
cpsf4	sfpq	celf2
cstf3	sltm	celf4
ewsr1	snrpb	nufip1
fmr1	srpk2	rbm41
hnrnpc	srsf1	scaf8
hnrnpd	srsf10	srp54b
hur	srsf11	ybx1
lsm2	srsf6	
mak16	srsf7	
matr3	srsf9	
pabpn1	stau1	
prpf18	syncrip	
pskh1	tmed10	
ptbp2	trim21	
puf60	tsn	
qk	xrn2	
rbm39	zcrb1	
sf3a1	zfml	
sf3b2	zfp830	
	zrsr2	

Table 9 mRNA processing genes downregulated during satellite cell activaiton are elevated with age. List of genes in the mRNA Processing Wiki Pathway that are up- or downregulated with age in both the old and geriatric datasets. Genes that increase (red) or decrease (green) expression during satellite cell activation are shaded.

CELF1/2/4 targets p38 MAPK pathway members that are dysregulated with age

The *Celf1, 2 and 4* transcripts are dysregulated with age, as well as their p38 MAPK pathway targets. *Celf1* decreases expression with age in our dataset but increases expression in the geriatric dataset; whereas, *Celf2* and *Celf4* decrease expression with age in both datasets. Additionally, comparison of the CELF1/2/4 targets identified by Lee, *et. al.*, 2010 with both old and geriatric databases revealed a significant overlap between CELF1/2/4 targets and transcripts significantly increasing expression with age (52 CELF targets out of 505 upregulated transcripts;

Chapter 3, Table 12; *P* value = 2.28×10^{-4}). While CELF1/2/4 only targets two members of the p38 MAPK pathway that decrease expression with age in our dataset, half of the p38 MAPK pathways members upregulated in the geriatric dataset are CELF1/2/4 targets, and four of these decrease expression during satellite cell activation (Chapter 3, Table 13). Together, this suggests a role for the CELF family in delayed aged satellite cell activation through p38 MAPK signaling.

Discussion

Post-transcriptional regulation of gene expression is a critical component of myogenesis; however, few studies have addressed the role of post-transcriptional regulation in mediateing satellite cell activation. Here, I have reviewed the roles of microRNAs and RNA binding proteins in regulating the FGF, ERK1/2 and p38α/β MAPK signaling pathways during satellite cell activation and in aged satellite cells. Through investigating gene expression profiles of quiescent and activated adult satellite cells as well as aged satellite cells, I have identified several RNA-BPs that are likely regulators of satellite cell activation and self renewal. Furthermore, altered expression of these RNA-BPs may contribute to the delayed activation and imparied self-renewal observed in aged satellite cells. Future studies investigating the *in vivo* targets of RNA-BPs and microRNAs during satellite cell activation and in aged satellite cells will provide insight towards understanding muscle regeneration in healthy tissue as well as the progressive wasting and deficient regeneration of sarcopenic muscle.

Review: Molecular regulation of skeletal muscle by the CELF family of RNA binding proteins

Introduction

Skeletal muscle is essential for respiration, mobility, reproduction and metabolism. Deficits in muscle function due to disease, injury or age, reduce both quality of life and lifespan. Muscle function and regeneration is regulated by the highly coordinated expression of many genes. Investigations into the roles of RNA binding proteins in myogenesis have highlighted the importance of post-transcriptional regulation of gene expression in developing and adult muscle as well as muscle regeneration and disease (Apponi, Corbett, and Pavlath 2011; Ma, Hall, and Gallouzi 2012).

The CELF family of RNA binding proteins post-transcriptionally regulates developing and adult muscle. The CELF family has multiple roles in regulating mRNA processing and stability with functional consequences for muscle. In this review, I discuss our current understanding of the CELF family in regulating adult muscle function and how dysregulation of CELF proteins contributes to a specific myopathy, myotonic dystrophy type I.

Identification of the CELF family

The CELF family is conserved among metazoans, with essential roles in development and skeletal muscle. Mammalian CELF1 and 2 proteins were identified through their ability to $(CUG)_n$–RNA probes *in vitro* (Timchenko, Miller, et al. 1996; Timchenko, Timchenko, et al. 1996) as isoforms of the heterogeneous nuclear ribonuclear protein (hnRNP) hNab50. ETR-1, the *C. elegans* homologue of CELF1 & 2, is essential for animal viability and muscle

development (Milne and Hodgkin 1999). The CELF family is also homologous to two RNA binding protein families in *Drosophila*: Bruno (Bru) and embryonic lethal, abnormal vision (ELAV) proteins. Bru is essential for embryogenesis through translational repression of *oskar*, likely through interactions with the RNA helicase Vas (Webster et al. 1997; Good et al. 2000). ELAV proteins are essential for retina and neuronal development (Campos, Grossman, and White 1985; Good 1995; Homyk, Isono, and Pak 1985). CELF1 overexpression in *Drosophila* causes skeletal muscle disorganization and vacuolization (de Haro et al. 2006). The CELF family derives its name from homology to the ELAV family and the ability to bind CUG repeats: ***C***UG binding, ***e***lav type RNA-binding protein-3 ***l***ike ***f***actors (Ladd, Charlet, and Cooper 2001; Barreau et al. 2006). CELF1 regulates the conserved alternative splicing cardiac troponin T (cTNT) during muscle development (Philips, Timchenko, and Cooper 1998; Suzuki, Jin, et al. 2002). Together, these data highlight the importance of CELF proteins for appropriate muscle development and for muscle function.

Protein Structure and Conservation

The CELF family of RNA binding proteins consists of six members (CELF1-6). CELF1 and 2 are ubiquitously expressed, CELF 4 and 6 are expressed in many tissues while CELF3 and 5 are restricted to the brain (Choi et al. 1998, 1999; Good et al. 2000; Hwang, Hwang, and Liew 1994; Ladd et al. 2004, 2001; Li, Bachinski, and Roberts 2001; Lu, Timchenko, and Timchenko 1999; Meins et al. 2002; Roberts et al. 1997; Szafranski et al. 2007; Timchenko, Miller, et al. 1996; Zhang et al. 2002). The CELF family is highly conserved among metazoans with homologues present in humans, mice, chickens, flies, frogs, nematodes and fish, among others (Barreau et al. 2006; Brimacombe and Ladd 2007; Milne and Hodgkin 1999; Norris et al. 2014;

Tahara, Bessho, and Matsui 2013; Tan et al. 2012; Tang et al. 2012; Wu et al. 2010). CELF proteins contain three RNA recognition motifs (RRM), with the second and third separated by a highly variable linker region. When comparing amino acid sequences, the CELF proteins fall into two categories. CELF1 and 2 are highly similar; the three RRMs are 90-95% identical, with the linker region providing the most variability (29% identical) for an overall 76% sequence identity. CELFs 3-6 are more similar to each other than to CELFs 1 and 2 (42-46% identical); however, they are also not as well conserved amongst themselves (61-65% overall identical). As with CELF1 and 2, the RRMs are the most conserved between CELFs 3-6 (68-89% identical) with the linker region providing the most variability (28-49% identical) (Barreau et al. 2006; Kashyap, Sharma, and Bhavesh 2013; Ladd et al. 2004).

Functions of the CELF Family

The CELF family regulates gene expression via diverse mechanisms that include alternative splicing, RNA editing, deadenylation, mRNA transcript stability and translation. Despite overlapping expression patterns and extensive similarity in protein sequences, CELF proteins do not fully share mRNA targets and can interact with common targets in through multiple binding sites using different RRMs eliciting distinct outcomes. The best understood family members are CELF1 and 2 and the extent to which CELFs 3-6 are functionally distinct from CELF1 and 2 is unknown. A comprehensive list of identified CELF targets has been recently published by (Dasgupta and Ladd), and thus, I will focus on specific targets with implications for skeletal muscle function.

Alternative Splicing

CELF proteins have unique and apparently redundant functions in muscle. Even in the cases of functional redundancy, CELF proteins binding to target transcripts occurs via multiple RRMs and binding sites. As an example, all CELF proteins can bind to a muscle-specific enhancer 2 (MSE2) sequence in a cardiac troponin T (*cTNT*) minigene and promote inclusion of exon 5 *in vivo,* which increases calcium sensitivity and muscle contraction (Philips, Timchenko, and Cooper 1998; Faustino, Cooper, and Andre 2005; Cooper; Ryan and Cooper 1998; McAuliffe, Gao, and Solaro 1990; Godt et al. 1993; Faustino, Cooper, and Andre 2005). Exon 5 is included in embryonic muscle but excluded in the adult tissue (Ladd et al. 2004; Ladd et al. 2001). Interestingly, CELF2 and 4 use different domains to bind to MSE2 and activate splicing. CELF2 requires either the first two RRMs and 71 amino acids at the N-terminus of the linker or RRM3 and 119 amino acids at the C-terminus of the linker, while CELF4 binding requires RRMs 1, 2 and an adjacent 66 amino acids in the linker region. In each case, the RRMs were sufficient for RNA binding but the linker regions were required for splicing activity (Han and Cooper 2005; Ladd and Cooper 2004; Singh et al. 2004).

Exon 5 of *cTNT* is progressively excluded during skeletal muscle development and correlates with increasing CELF2 protein, which reaches maximal levels in adult skeletal muscle (Ladd, Charlet, and Cooper 2001). During this period, CELF2 undergoes a molecular weight shift, possibly due to post-translational modifications or alternative isoform expression, suggesting that modified or alternative forms of CELF2 may have alternative targets or functions. Indeed, an alternative isoform of CELF2 with a truncated RRM3 promotes inclusion of insulin receptor exon 11, which is excluded by full length CELF2; however, both isoforms have identical splicing activity on *α-actinin 1* (Suzuki, Takeuchi, et al. 2012).

The CELF family regulates cellular response to insulin through alternative splicing of the insulin receptor (*IR*). Skeletal muscle plays an important role in regulating glucose homeostasis and predominately expresses IR isoform B (includes exon 11). Exclusion of exon 11 results in decreased insulin sensitivity and signal capacity of the IR protein. CELF1 and 6 stimulate skipping of exon 11 in an *IR* minigene *in vivo* (Savkur, Philips, and Cooper 2001; Ladd, Nguyen, et al. 2004). *In vitro* UV crosslinking assays determined CELF1 binds to the IR transcript through an 100 nucleotide region between *IR* exons 10 and 11; however, this region is not required for CELF6 to induce exon 11 skipping, suggesting variability among the CELF family in RNA sequence recognition (Savkur, Philips, and Cooper 2001; Ladd, Nguyen, et al. 2004).

Additionally, CELF proteins regulate muscle excitability through alternative splicing of the skeletal muscle-specific chloride channel (*ClC-1*) (Steinmeyer, Ortland, and Jentsch 1991). CELF1 binds to the U/G rich motif within the 3' splice site for intron 2 *in vivo*, promoting intron 2 retention (A. Mankodi et al. 2002). Intron 2 contains a premature stop codon, resulting in *ClC-1* transcript degradation (A. Mankodi et al. 2002; Charlet-B et al. 2002). CELFs 3-6, but not CELF1 or 2, promote exon 7A inclusion of a human *ClC-1* minigene *in vivo*. Exon 7a also encodes a premature stop codon, which results in production of a truncated ClC-1 protein with reduced function (Kino et al. 2009). Diseases with impaired ClC-1 function through alternative splicing of either intron 2 or exon 7A, including DM1 and Huntington disease, exhibit muscle hyperexcitability and delayed relaxation (Waters et al. 2013; A. Mankodi et al. 2002). Although CELF1, 2 and 4 have been implicated in the *ClC-1* missplicing observed in DM1, their potential involvement in Huntington disease has yet to be examined (Kino et al. 2009; Ho et al. 2005).

CELF proteins are also involved in cytoskeletal and sarcomere structuring via alternative splicing of α-actinin. α-actinin is a component of the Z-band connecting adjacent sarcomeres and

functions to bind actin and distribute force along a myofibril (Luther 2000). α-actinin contains two exons that are alternatively spliced: a smooth-muscle exon (SM) and a non-muscle exon (NM). SM inclusion or exclusion of both SM and NM eliminates Ca^{2+}-dependent actin binding (Parr et al. 1992; Tang, Taylor, and Taylor 2001). CELF1 and 2 both promote inclusion of SM and skipping of NM while CELF4 promotes exclusion of both exons (Gromak et al. 2003). Alternative splicing of α-actinin in muscle may influence binding to other sarcomeric proteins, such as titin, and alter myofibril structural integrity (Tang, Taylor, and Taylor 2001; Atkinson et al. 2001). These binding and splicing experiments demonstrate that CELF regulation of alternative splicing has wide ranging developmental and functional consequences for skeletal muscle.

RNA Editing

To date, only one CELF protein has been shown to regulate RNA editing. CELF2 has been implicated in RNA editing of the apolipoprotein B (apoB) transcript. apoB functions in dietary lipid uptake and C to U RNA editing creates an in-frame stop codon, resulting in a truncated protein that is functionally distinct from the full length protein. The apoB transcript is edited by a holoenzyme containing apobec-1 and ACF. CELF2 interacts with both apobec-1 and ACF to form a functional holoenxyme capable of editing apoB mRNA; however, CELF2 likely limits the extent of editing as CELF2 knockdown in hepatoma and intestinal cell lines promotes editing while CELF2 inhibits editing in a cell-free system (Anant et al. 2001; Chen, Eggerman, and Patterson 2007).

Deadenylation and RNA stability

The CELF family also has roles in regulating transcript deadenylation and stability. In *Xenopus*, EDEN-BP, the homologue of CELF1/2, binds to EDEN (embryonic deadenylation element) and promotes the deadenylation and translational silencing of transcripts containing an EDEN binding site (U(A/G)) and AREs (AU rich element) (L Paillard et al. 1998; Paillard, Legagneux, and Beverley Osborne 2014). RIP-Chip of immunoprecipitated CELF1 RNAs from *X.* tropicalis egg extracts identified 158 target transcripts that were enriched for the EDEN binding motif in the 3'UTR, including regulators of oocyte maturation and cell cycle progression (Graindorge et al. 2008). Human CELF1 has also been shown to bind to the EDEN-BP deadenylation target c-Jun transcript *in vitro*, and is required for deadenylation of c-Fos and TNFα transcripts in HeLa cells (Luc Paillard et al. 2002; Paillard, Legagneux, and Beverley Osborne 2014; Moraes, Wilusz, and Wilusz 2006). This activity may be mediated through the PARN deadenylase, which co-immunoprecipitates with CELF1 (Moraes, Wilusz, and Wilusz 2006). c-Fos and c-Jun are both expressed in satellite cells early after injury and exercise but have different expression patterns during regeneration; both c-Fos and c-Jun repress MyoD and Myogenin function, and have roles in proliferation and differentiation of myogenic cells (Bengal et al. 1992; Daury et al. 2001; Kami, Noguchi, and Senba 1995; Li et al. 1992; Puntschart et al. 1998; Trouche et al. 1993). Regulation of c-Fos and c-Jun expression during myogenesis may be post-transcriptionally regulated through deadenylation and transcript decay.

CELF1 and 2 also regulate transcript stability. Presumably, some of these transcripts are destabilized via deadenylation. c-Jun, for example, is both deadenylated and destabilized when bound by CELF1 (Paillard et al. 2002; Vlasova and Bohjanen 2008; Vlasova et al. 2008). CELF1 binds to GRE (GU-rich elements)-containing reporter constructs in HeLa cells, enhancing mRNA decay; CELF1 knockdown stabilized transcripts steady-state levels (Vlasova and

Bohjanen 2008; Vlasova et al. 2008). In a global analysis of C2C12 myoblast RNA decay, 3'UTRs of short-lived transcripts were enriched for GREs and AREs. RIP-Chip for CELF1-associated transcripts in C2C12 and HeLa cells also showed enrichment for GREs; several of these transcripts, including the myogenic regulatory factor *Myod1*, are stabilized in CELF1 knockdown C2C12 cells or upon mutating GREs and loss of CELF1 binding (Lee et al. 2010; Rattenbacher et al. 2010). This unbiased, global identification of myogenic targets implicates CELF1 in regulation of a wide range of cellular processes, including: cell growth, motility, cell cycle, and apoptosis (Lee et al. 2010).

Additional transcripts regulated by the CELF family that are involved in skeletal muscle regeneration include the proinflammatory cytokine tumor necrosis factor (TNFα) and a prostaglandin synthesis enzyme, cyclooxygenase-2 (COX-2). CELF1 binds to TNFα mRNA via an ARE in the 3'UTR. At high levels in C2C12 myoblasts, TNFα activates p38 MAPK while inhibiting differentiation but at low levels TNFα promotes differentiation (Chen, Jin, and Li 2007; L. Zhang et al. 2008). The TNFα transcript is stabilized in CELF1 knockdown C2C12 myoblasts; however, it is also stabilized in myoblasts expressing a 960 CUG from the *DMPK* gene, where CELF1 protein levels are elevated. TNF stability in the latter case is dependent on PKC phosphorylation of CELF1; this suggests that post-translational modifications of CELF1 alter either transcript binding or the ability to stimulate mRNA decay and suggests a role for CELF1 in regulating myogenesis.

CELF2 stabilizes the COX-2 transcript while inhibiting its translation. COX-2 protein levels dramatically increase within one day of muscle injury; loss of COX-2 via chemical inhibition or genetic knockout reduces myoblasts number and myofiber size in regenerating muscle (Bondesen et al. 2004). CELF2 binds to AREs within the 3'UTR of COX-2 *in vitro* and

in vivo (Mukhopadhyay et al. 2003; Moraes, Monteiro, and Pacheco-Soares 2013; Sureban et al. 2007). Interaction with CELF2 increases stability of luciferase reporter plasmids expressing either the entire COX-2 3'UTR or only the ARE-containing region (first 60 nucleotides of 3'UTR containing six AUUUA repeats). Additionally, polysome fractionation shows that CELF2, but not CELF1, prevents loading of the reporter mRNA into polysomes, thereby inhibiting translation (Mukhopadhyay et al.2003). Furthermore, CELF2 binds to and competes with the mRNA-stabilizing RNA binding protein HuR in regulating COX-2 translation (Sureban et al. 2007). CELF2 and HuR bind with equal and high affinity to the COX-2 AREs and have opposite effects on translation; however, luciferase assays demonstrate that CELF2 is able to inhibit translation of the COX-2 full length 3'UTR luciferase reporter, *in vitro* and *in vivo*, even when present at half the levels of HuR (Sureban et al. 2007). HuR also stabilizes c-Fos, a CELF1 target for deadenylation; it remains to be seen if the antagonistic relationship between CELF1, 2 and HuR is paralleled for other CELF family members and other transcripts, such as c-Fos (Feng et al. 2013; Winzen et al. 2004).

An unbiased, global analysis of CELF4 targets in adult mouse cerebral cortex and hippocampus via individual nucleotide resolution UV-crosslinking and immunoprecipitation (iCLIP) identified ~2,000 genes, roughly 15% of the transcriptome, as CELF4 targets; the majority of binding occurred at (A/U)UGU sequences in 3'UTRs (Wagnon et al. 2012). Microarray analysis of 12,016 genes in *Celf4* null brain identified 144 CELF4 iCLIP target genes with significantly altered transcript steady-state levels, more than half of which decreased (Wagnon et al. 2012). RNASeq of CELF4 targets after polysome fractionation revealed enrichment in polysomes (Wagnon et al. 2012). These data suggest that, similar to CELF1 and 2, CELF4 regulates transcripts stability and translation.

Phosphorylation of CELF proteins and translational regulation of target transcripts

Phosphorylation alters CELF protein function and localization by altering protein interactions and transcript binding (Salisbury et al. 2008; Welm et al. 2000; Philips, Timchenko, and Cooper 1998; Timchenko, Wang, and Timchenko 2005; Timchenko, Salisbury, et al. 2006). Several kinases have been identified that phosphorylate CELF proteins. Akt phosphorylates CELF1 at serine 28 *in vitro* and in proliferating C2C12 myoblasts, increasing CELF1 binding with cyclin D1 mRNA in a phosphorylation-dependent manner (Salisbury et al. 2008). The Cyclin D3-cdk4/6 complex both *in vitro* and in differentiated C2C12 myotubes phosphorylates CELF1 at serine 302, increasing binding with eIF2 protein and binding of the CELF1-eIF2 complex to C/EBP and p21 mRNAs (Salisbury et al. 2008). CELF1 is hyperphosphorylated in differentiated C2C12 myotubes, as demonstrated by 2D-gel electrophoresis western blots, and likely contains additional phosphorylated residues that have yet to be identified. Distrophia myotonica-protein kinase (DMPK) also phosphorylates CELF1 and 2 *in vitro* and may contribute to their mislocalization the nucleus in DM1; however, phosphorylation via DMPK has not been directly linked to altered CELF function or localization (Roberts et al. 1997). PKCα and PKCβII directly phosphorylate CELF1 *in vitro* and in DM1 tissues (Kuyumcu-Martinez, Wang, and Cooper 2008; Verma et al. 2013). Co-localization of PKCα and CELF4 during mouse retinal development suggests that PKCα may also phosphorylate CELF4 (Karunakaran et al. 2013). CELF1 phosphorylation correlates with >4-fold increase in protein half-life and is necessary for some splicing events (Kuyumcu-Martinez, Wang, and Cooper 2008; Verma et al. 2013). In old livers and after partial hepatectomy, CELF1 is hyper-phosphorylated by cyclin D3/cdk4, promoting a phosphorylation-dependent interaction with eIF2α. This enhances C/EBP-β

translation and correlates with a switch of preferred translation start sites on the C/EBP-β transcript, shifting protein production from the major isoform produced in adults (LAP, liver-enriched activator protein) to a dominate-negative isoform (LIP, liver enriched inhibitory) (Descombes and Schibler 1991; Timchenko et al. 2006, 2005; Welm, Timchenko, and Darlington 1999). As C/EBP-β functions in proliferation (Greenbaum et al. 1998; Jin et al. 2013) and differentiation (Cao, Umek, and McKnight 1991; Darlington, Ross, and MacDougald 1998; Yeh et al. 1995) and is expressed in differentiated myotubes but not proliferating myoblasts (Salisbury et al. 2008), this suggests a potential mechanism by which CELF1 may regulate myoblast cell fate. These data demonstrate that CELF1 can promote translation in a phosphorylation-dependent manner and suggest a potential role in alternative translation start site selection. One can imagine that phosphorylation of multiple CELF1 residues alters localization, targeted transcripts, the function of CELF1 between splicing, transcript stability and translation, and is likely coordinated during myoblast proliferation and differentiation to direct cell fate.

CELF proteins and Myotonic Dystrophy

Myotonic dystrophy type I is an RNA microsatellite expansion disease

Myotonic dystrophy type I (DM1), the most prevalent form of adult-onset muscular dystrophy, is a multisystemic, dominantly inherited RNA microsatellite expansion disease, characterized by myotonia, progressive skeletal muscle degeneration, insulin resistance, cataracts, dilated cardiomyopathy, and cardiac conduction defects (Day and Ranum 2005; Khoshbakht et al. 2014; Ranum and Day 2004; Udd and Krahe 2012). RNA microsatellite expansion diseases, including amyotrophic lateral sclerosis (ALS) and fragile X syndrome among others, are characterized by expansion of a short, typically two to ten, nucleotide repeat

region in the genome (Mohan, Goodwin, and Swanson 2014; Vatovec, Kovanda, and Rogelj 2014; Verkerk et al. 1991; Wheeler and Thornton 2007). This expansion results in abnormally long transcripts that disrupt cellular functions. In DM1, the DMPK gene contains a CTG region in the 3'UTR that, when expanded past normally occurring 5-36 repeats, is pathogenic (Mahadevan et al. 1992; Tishkoff et al. 1998; Zerylnick et al. 1995). The extent of expansion negatively correlates with age of onset and positively correlates with symptom severity, with over 1000 repeats manifesting as both congenital and extremely severe (Takahashi et al. n.d.; Akiyama et al. 2008; Khoshbakht et al. 2014). The DMPK transcripts form hairpins and accumulate in nuclear foci (Michalowski et al. 1999; Taneja et al. 1995; Tian et al. 2000; Wang et al. 1995).

DM1 phenotypes are caused by toxic RNA

DMPK protein is reduced in DM1 skeletal muscle but this reduction cannot account for the disease phenotypes (Furling, Lemieux, et al. 2001; Furling et al. 2003; Pelletier et al. 2009; Sabouri et al. 1993; Wang and Griffith 1995; Wang et al. 1994). *DMPK*$^{-/-}$ mice display only mild myopathy and thus, cannot explain the myotonia and severe myopathy observed in DM1 (Reddy et al. 1996; Jansen et al. 1996). Transgenic mice carrying the human *DMPK* gene with an expanded CTG repeat region of over 300 CTG (DM300), an inducible skeletal muscle specific 960 CTG (EpA960/HSA-Cre-ER^{T2}), or 250 CTG repeats in the unrelated human skeletal actin gene (HSA^{LR}), exhibit myotonia and progressive myopathy (Mankodi et al. 2000; Orengo et al. 2008; Seznec 2001). Reduction of DMPK mRNA expression in human DMPK myoblasts, a transgenic mouse DM1 model carrying a 500 CTG repeat (DM500), and HSA^{LR} reduced the number of nuclear RNA foci and restored several missplicing events characteristic in DM1

(Mulders et al. 2009). These experiments demonstrate that the expanded transcript, not DMPK dysregulation, is the primary causal factor for the cellular and organismal symptoms of DM1.

CELF proteins likely mediate pathogenesis of toxic RNA

Studies of DM1 patient tissues and several transgenic mouse models of DM1 have provided clues for the mechanism involved in DM1's RNA-induced toxicity; these observations and experiments implicate CELF proteins in multiple missplicing and altered translation events. In DM1 skeletal and cardiac muscle, CELF1 is hyperphosphorylated and CELF 1 and 2 protein, but not mRNA, nuclear levels are increased (Nezu et al. 2007; Pelletier et al. 2009; Roberts et al. 1997). CELF1 protein levels correlate with the extent of CUG repeats and with disease severity, ranging from 3- to 11-fold higher than healthy individuals (Kim et al. 2014; Savkur et al. 2001; Timchenko, Cai, et al. 2001). In a cardiac-specific mouse model of DM1, expressing an inducible 960 CTG repeat from the *DMPK* gene (DMPK-CUG^{960}), CELF1 is hyper-phosphorylated and increased nuclear steady-state protein levels of CELF1 and CELF2 are observed in as little as 6-hours post induction; this is accompanied by missplicing of multiple CELF targets observed in DM1 patients (G.-S. S. Wang et al. 2007; Ho et al. 2005; Kuyumcu-Martinez, Wang, and Cooper 2008). These data demonstrate that upregulation of CELF1 and 2 are early events in response to CUG repeat RNA expression and are likely mediators of RNA toxicity in DM1 pathogenesis.

Developmental delay and fiber-type specification in DM1 skeletal muscle

Skeletal muscle is severely underdeveloped in congenital DM1; this phenotype is also seen in transgenic mice overexpressing CELF1. In two mouse lines, CELF1 is overexpressed in

cardiac and skeletal muscle under a modified β-actin promoter (β-CELF1), or under an MCK promoter (MCK-CELF1) (Furling et al. 2003; Ho et al. 2005; Timchenko et al. 2004). In both, the extent of CELF1 protein overexpression correlated with phenotype severity. Mice expressing 8-fold or greater levels of CELF1 protein (MCK-CELF1), or greater than 4 fold (β-CELF1) did not survive past postnatal day 7, while a 4-6 fold increase mimicked the delayed growth observed in a DM1 mouse model, DM300 (Ho et al. 2005; Seznec 2001; Timchenko et al. 2004). The size of newborn hindlimb muscles from CELF1 transgenic mice are also significantly reduced, suggest a role for CELF1 in primary myogenesis. Adult (6-8 months) and old (1.5 years) mice display wide variably in myofiber diameter, reduced number of myofibers (up to 6%) and a drastic increase in myofibers with internally located nuclei, up to 33%, with severity correlating with CELF1 overexpression and also observed in DM300 and EpA960/HSA-Cre-ER^{T2}mice, and human DM1 skeletal muscle (Ho et al. 2005; Mankodi 2000; Orengo et al. 2008; Orengo, Ward, and Cooper 2010; Sahgal et al. 1983; Seznec 2001; Thornell et al. 2009; Timchenko et al. 2004). These observations suggest that the muscle is immature and implicate CELF1 in the underdevelopment of DM1 skeletal muscle.

Interestingly, congenital human fetal DM1 skeletal muscle has delayed fiber-type specification and completely lacks slow myofibers; however, DM1 patients who live to adulthood exhibit elevated numbers of slow myofibers when compared to normal adults (Denis Furling et al. 2003; Sahgal et al. 1983; Thornell et al. 2009). This increase in slow myofibers is also observed in MCK-CELF1 and transgenic mice expressing a nuclear dominate-negative CELF protein, implicating CELF1, and possibly other CELF family members, in fiber type determination (Berger et al. 2011; Dilworth and Blais 2011; Ladd et al. 2005; Timchenko et al. 2004).

Alternative splicing defects in DM1 skeletal muscle

Increased expression and localization of CELF1 and 2 to the nucleus leads to missplicing of many transcripts including insulin receptor (IR), chloride channel 1 (ClC-1) and cardiac troponin T (cTNT) in both human DM1 tissues and mouse models of DM1 (Cardani et al. 2013; Llorian and Smith 2011; Lueck et al. 2007; Nezu et al. 2007; Orengo et al. 2008, 2010; Pelletier et al. 2009; Savkur et al. 2001; Timchenko, Miller, et al. 1996). Missplicing of these transcripts is sufficient to cause the insulin resistance and myotonia and contribute to the cardiac defects observed in DM1 patients (Dansithong et al. 2005; Mankodi et al. 2002; Philips et al. 1998; Savkur et al. 2001; Wheeler et al. 2007). Additionally, overexpression of CELF1 in mice is sufficient to reproduce the DM1 missplicing events of cTNT, myotubularin-related 1 phosphatase (Mtmr1), and ClC-1 (Buj-Bello et al. 2002; Charlet-B et al. 2002; Ho et al. 2005).

Cellular metabolism in DM1 is disrupted through splicing of the muscle pyruvate kinase transcript (Pkm). Pkm promotes glucose uptake and oxidative metabolism (Yang et al. 2012). During skeletal and cardiac muscle development, alternative splicing of Pkm shifts from the less active, embryonic isoform (Pkm2) to the constitutively active, adult isoform (Pkm1). In DM1 skeletal and cardiac muscle, Pkm2 is reexpressed. Induced Pkm2 reexpression in C2C12 myotubes and primary mouse hindlimb decreased the oxygen consumption rate of myotubes *in vitro* and increased the respiratory exchange ratio of mice during periods of activity, indicating a greater energy expenditure and a preference of glucose over fat for fuel (Gao and Cooper 2013). Furthermore, cardiac expression of an inducible 960-CTG transgene or cardiac-specific three-fold overexpression of CELF1 is sufficient to significantly increase the embryonic isoform of pyruvate kinase M2 (Pkm2) mRNA and protein expression in the adult heart (Gao and Cooper

2013). This suggests that increased expression of CELF1 in DM1 promotes reexpression of Pkm1, contributing to the altered metabolism of DM1 muscle. Experiments in diabetic hearts suggest that CELF1 may regulate metabolism through more than Pkm1 and supply additional splicing targets to investigate CELF-mediated metabolic regulation (Verma et al. 2013).

Dysregulation of differentiation factors in DM1 skeletal muscle

Skeletal muscle from DM1 patients and DM1 mouse models show elevated expression of MEF2A, C/EBP-β and p21 protein that occurs by CELF1 binding to these transcripts and promoting their translation (Iakova et al. 2004; Pelletier et al. 2009; Philips et al. 1998; Timchenko et al. 2004, 2006, 2005; Timchenko, Iakova, et al. 2001; Welm et al. 2000). Each of these proteins is involved with differentiation and their dsyregulation may explain differentiation defects in DM1 muscle (N. Liu et al. 2014; Yeh et al. 1995; P. Zhang et al. 1999). CELF1, via the third RNA recognition motif, directly binds to CAG repeats in the MEF2A transcript. In both cell-free and cultured cell assays, CELF1 induces translation of MEF2A (Timchenko, Patel, et al. 2004). MEF2A expression must be tightly regulated during myogenesis to ensure appropriate differentiation, as over- or under-expression of MEF2A impairs differentiation (Black, Lu, and Olson 1997; Gunthorpe, Beatty, and Taylor 1999; Molkentin et al. 1995).

Human DM1 myoblasts fail to exit the cell cycle when induced to differentiate in culture (Timchenko, Iakova, et al. 2001). Interestingly, DM1 and normal myoblasts express similar levels of p21 mRNA; however, DM1 myoblasts fail to express p21 protein, suggesting a translation defect (Timchenko, Iakova, et al. 2001). p21 translation is induced *in vitro* and *in vivo* by CELF1 binding to a GCN repeat in the 5' region of p21 mRNA (Timchenko, Iakova, et al. 2001; Timchenko, Patel, et al. 2004). Thus, the absence of p21 protein in DM1 myoblasts may

be due to the accumulation and mislocalization of CELF1 to the nucleus in DM1 myoblasts containing excessive CUG repeats.

In a separate DM1 myoblast study, CELF1 was found to have increased interactions with active Akt, which phosphorylates CELF1 and increase binding to cyclin D1. CELF1 phosphorylation by Akt inhibits interactions with eIF2 and eliminates binding to C/EBP-β mRNA and, therefore C/EBP-β translation. CELF1 phosphorylation by cyclin D3 overexpression in DM1 myoblasts rescued the CELF1-eIF2 complex, C/EBP-β transcript binding and delayed differentiation (Salisbury et al. 2008). Furthermore, a DM1 mouse model carrying an inducible toxic RNA transgene and null for CELF1 expressed normal MEF2A and C/EBP-β protein levels and showed improved muscle histopathology and improved muscle function (Kim et al. 2014). Additionally, β-CELF1 mice reproduce the elevated levels of myogenin, MEF2A, C/EBP-β, and p21 proteins (MCK-CELF1) in hindlimb muscle, similar to DM1 patients (Timchenko, Patel, et al. 2004). Inappropriate expression of MEF2A, C/EBP-β and p21 proteins disrupts differentiation and likely results in the immature myofibers observed in DM1 (Black et al. 1997; Gunthorpe et al. 1999; Molkentin et al. 1995). The disruption of skeletal muscle development and differentiation factors in mice overexpressing CELF1 suggests a role for CELF1 in regulating primary and secondary myogenesis.

CELF proteins and satellite cells

There is a conspicuous absence of research examining CELF proteins in satellite cells. Progressive muscle wasting is a hallmark of DM1 and likely mediated through impaired satellite cell function. Satellite cell numbers are increased in the more affected, distal muscle of DM1 patients; however, when cultured, they have significantly reduced proliferation and

differentiation capacity (Beaulieu et al. 2012; Beffy et al. 2010; Bigot et al. 2009; Furling, Coiffier, et al. 2001; Pelletier et al. 2009; Thornell et al. 2009). In one experiment, the differentiation defect in human DM1 satellite cells was due, in part, to increased COX-2 expression leading to increased prostaglandin E2 (PGE2) secretion (Beaulieu et al. 2012). As discussed above, CELF2 stabilizes *COX-2* mRNA and prevents translation of a reporter plasmid containing the *COX-2* 3'UTR (Mukhopadhyay et al. 2003). Additionally, CELF2 protein steady-state levels are increased in DMPK-CUG^{960} cardiac tissue (G.-S. S. Wang et al. 2007). This correlation suggests that CELF2 dysregulation in DM1, possibly by nuclear mislocalization or alternative phosphorylation, may derepress *COX-2* translation. As CELF1, 2, and 4 are expressed in satellite cells, exploring their roles in regulating regeneration may contribute to a mechanistic understanding of progressive muscle wasting diseases.

Conclusions

The CELF family of RNA binding proteins regulates skeletal muscle development and regeneration via multiple mechanisms that remain poorly understood. The majority of research on the CELF family has focused on CELF1 and CELF2 with much less emphasis on the CELF 3-6 subfamily. Several experiments summarized here demonstrate that 1) the CELF family is not completely functionally redundant, 2) individual CELF proteins target RNAs not regulated by other CELF family members, and 3) even shared targets can be differentially regulated.

Chapter 2: Adult Homeostatic Turn Over of Satellite Cells is Muscle Group Dependent

Background

Adult tissues are maintained and repaired by resident stem cells. Adult stem cells are primarily quiescent but have low levels of mitotic activity to maintain the long-term homeostasis of their associated tissue (Fuchs 2009). In skeletal muscle, this role is filled by satellite cells (Lepper and Fan 2010; Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011; Shea et al. 2010). Satellite cells reside above the sarcolemma of an adjacent myofiber but beneath the surrounding basal lamina (Mauro 1961). They are a rare population and comprise only 2-11% of muscle nuclei, depending on age and muscle group (Snow 1977; Schmalbruch & Hellhammer 1976; Allbrook et al. 1971; Cardasis & Cooper 1975; Megeney et al. 1996; Mauro 1961). Adult skeletal muscle where satellite cells have been genetically ablated fail to regeneration muscle *in vivo* (Lepper and Fan 2010; Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011; Shea et al. 2010). Furthermore, dystrophic and aged animals where the satellite cell pool is diminished also exhibit muscle wasting (Cerletti et al. 2008; Sacco et al. 2010). These studies demonstrate the necessity of satellite cells in muscle maintenance and suggest a required basal level of satellite cell contribution to healthy muscle.

Satellite cell contribution to muscle varies with age. During postnatal growth, satellite cells extensively contribute to the developing skeletal muscle (Cardasis and Cooper 1975; Kelly and Zacks 1969; Kelly 1978; Ross et al. 1987; Schultz 1976; Shea et al. 2010). In adult muscle, however, the majority of satellite cells have entered quiescence and only a small subset is cycling (2-4%) (Chakkalakal et al. 2012; Grounds and McGeachie 1987; McGeachie and Grounds 1987; Schultz 1976; Snow 1977). In aged muscle, satellite cell contribution is reduced below the level required to maintain muscle homeostasis, resulting in the muscle wasting disease sarcopenia

(Brack, Bildsoe, and Hughes 2005; Ryall, Schertzer, and Lynch 2008; Snow 1977; Verdijk et al. 2007).

Satellite cell contribution also likely differs among muscle groups. Muscle groups vary in the number of resident satellite cells and cycling satellite cells (Snow 1977; Regina M Crameri et al. 2004; Gibson and Schultz 1983; Umnova and Seene 1991; Renault et al. 2002; Kadi, Charifi, et al. 2004; Kadi, Schjerling, et al. 2004; Shefer et al. 2006; Mikkelsen et al. 2009; R M Crameri et al. 2007; Murphy et al. 2011). Presumably, differences in the satellite cell populations are due to distinct requirements for maintenance and repair. For example, ~11% of satellite cells are cycling in the plantaris muscle, as measured by BrdU incorporation (Dangott, Schultz, and Mozdziak 2000). In contrast, only ~1% of satellite cells are cycling in the soleus muscle (Mozdziak et al. 2000; Smith et al. 2001). These measurements, however, may not represent adult satellite cell homeostasis due to the age ranges used, which includes young animals where muscle is still growing. Additionally, these experiments do not define the point when satellite cells acquire quiescence or whether satellite cells in different muscle groups acquire quiescence at distinct times.

Although the reasons for satellite cell number variability among muscle groups remain unknown, it has been postulated that there is a connection to muscle fiber type (Smith et al. 2001). Muscle fibers fall into two major categories based on metabolism and contractile properties. Type I fibers are primarily oxidative and have short refractory periods between contractions. Type II fibers rely more on glycolytic metabolism and fatigue quickly. Some studies report that satellite cells preferentially associate with type I fibers (Mustafa 2014), although this pattern is not always observed (Verdijk et al. 2007). Additionally, there is a correlation between satellite cell number and type I fiber contribution to a muscle group (Gibson

and Schultz 1983). Furthermore, type II fibers atrophy and lose satellite cells to a greater extent than type I fibers in sarcopenic and dystrophic muscle (D'Antona et al. 2007; Lassche et al. 2013; Pedemonte et al. 1999; Verdijk et al. 2007). This suggests that the age and extent to which satellite cells become quiescent, and are no longer contributing to the muscle, may be fiber-type and muscle-group dependent.

To date, no comprehensive studies have addressed when satellite cells acquire quiescence or whether satellite cells in different muscles acquire quiescence at distinct times. In this study, I used an inducible *Pax7*^{IresCreERT2} driven fluorescent reporter to determine when satellite cells establish quiescence in multiple muscle groups. Satellite cell contribution to the tibialis anterior (TA), extensor digitorum longus (EDL), soleus, and gastrocnemius hindlimb muscles drops precipitously from 8 to 12 weeks of age and remains low through 27 weeks. Although satellite cell contribution to the extraocular muscles (EOM) significantly decreases from 8 weeks to 27 weeks, contribution remains extensive during all time points examined and does not plateau. This suggests that hindlimb satellite cells establish quiescence by 12 weeks; however, EOM satellite cells may not attain quiescence until much later, if at all.

Results

Satellite cells are efficiently labeled in Pax7^{ICreERT2}*; R26R*^{tdTomato} *mice*

To fluorescently label Pax7-expressing satellite cells, we crossed tamoxifen-inducible *Pax7*^{ICreERT2} mice with ROSA-lsl-tdTomato, which expresses tdTomato in response to Cre-mediated recombination. Recombination was induced through tamoxifen IP injections for five consecutive days. Tissues and cells were harvested two weeks after the final tamoxifen administration (Fig. 1A). In the absence of tamoxifen, no tdTomato was detected either in

satellite cells or in TA myofibers. To determine the efficiency of satellite cell labeling by tdTomato, I harvested satellite cells *en mass*, myofibers with their accompanying satellite cells, and whole muscles for sectioning. Similar to Murphy *et. al.,* 2011, recombination efficiency is high in satellite cells (Fig. 1, Fig. 2, Table 1). tdTomato labeled nearly all Syndecan4+ and Pax7+ cells in culture and on myofibers (Fig. 1). In TA muscle sections, ninety-eight percent of all sublaminar Pax7+ satellite cells expressed tdTomato (Fig. 3, Table 1). Recombination efficiency did not vary with age (4w, 8w, 12w, 27w) or muscle group (TA, EDL, Soleus, gastrocnemius, EOM) (Fig. 1, Fig. 2, Fig. 3, Table 1). Additionally, tdTomato did not mark interstitial cells or myonuclei (Fig. 2, Fig. 3).

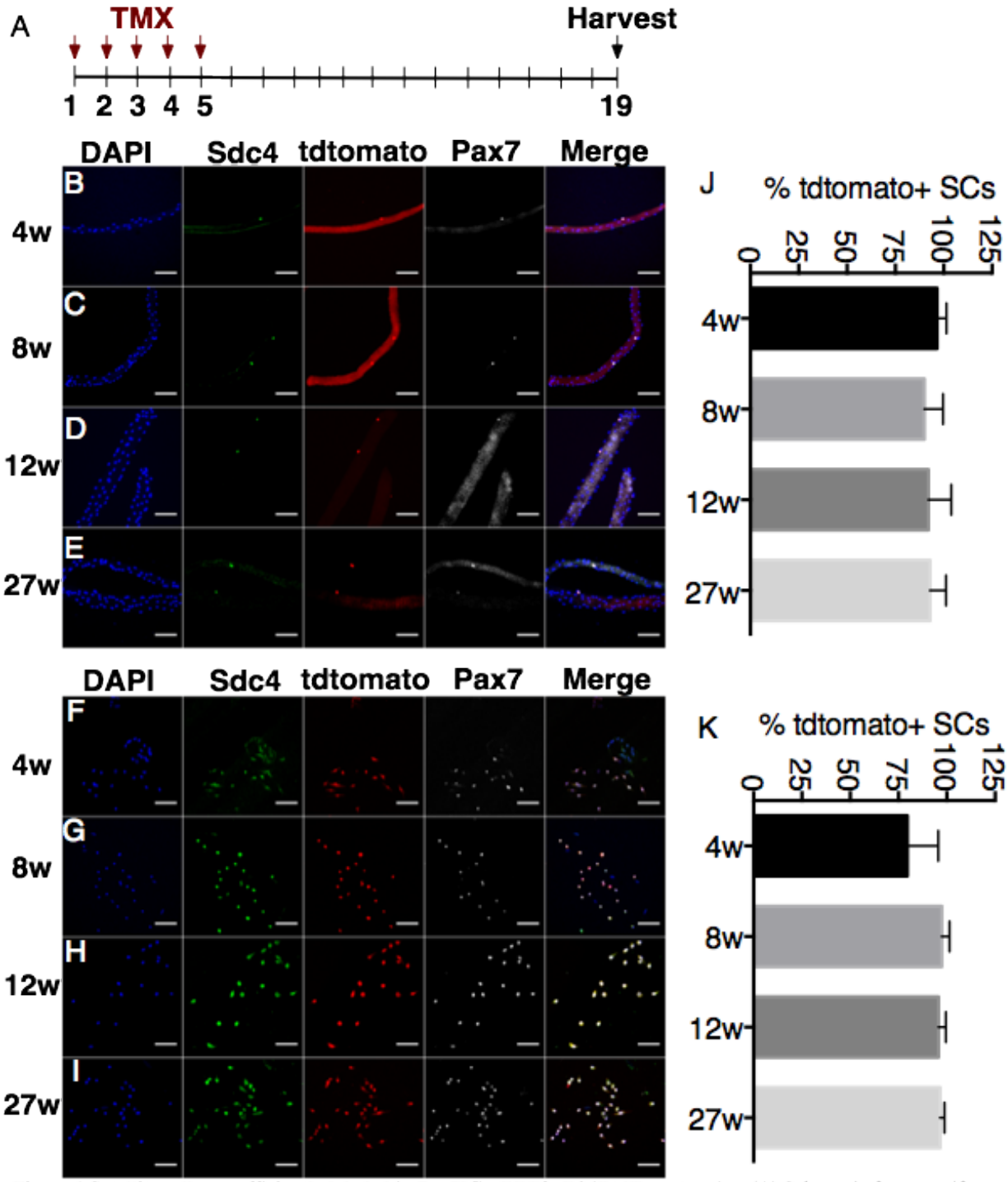
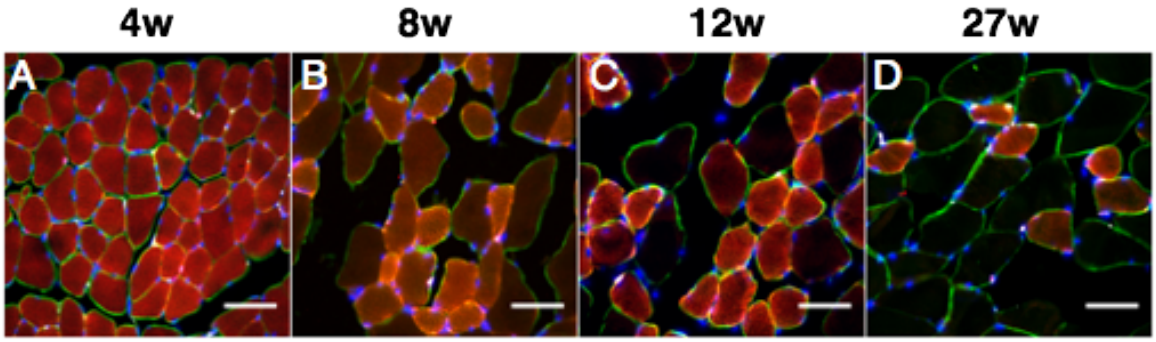


Figure 1 Satellite cells are efficiently labeled in Pax7ICreERT2; R26RtdTomato mice. (A) Schematic for tamoxifen treatment. EDL myofibers (B-E) and hindlimb satellite cells (F-I) harvested from 4w (B, F), 8w (C, G), 12w (D, H), and 27w (E, I) old mice after tamoxifen treatment as diagramed in (A). Quantification of recombined (tdTomato+) satellite cells on myofibers 24h post-isolation (J) or culture for three days (K). Pseudocolored images display DAPI in blue, laminin in green, tdTomato in red, and, Pax7 in white. Scale bar is 50μm. Error bars represent standard deviation. A minimum of three and maximum of six replicates were analyzed per time point.



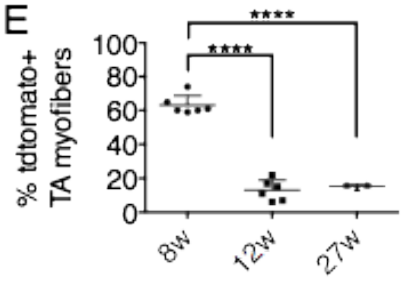


Figure 2 Tibialis anterior satellite cells extensively contribute to 4w and 8w old muscle. Sections of tibialis anterior after recombintion is induced at 4w (A), 8w (B), 12w (C), and 27w (D). Quantification of tdTomato+ myofibers for 8w, 12w, and 27w is shown in (E). Pseudocolored images display DAPI in blue, laminin in green, tdTomato in red, and, Pax7 in white. Scale bar is 50μm. **** = P-value <0.0001. Significance was determined by one-way ANOVA with Turkey post-hoc test.

% tdtomato+ SCs	8w	12w	27w	All Timepoints
EDL	100.0	95.2	100.0	98.7
Sol	94.7	100.0	94.4	97.1
EOM	95.0	100.0	96.6	97.1
Gastroc	100.0	97.1	100.0	99.4
TA	96.5	97.4	100.0	97.6
All Muscles	97.6	98.2	98.3	98.0

Table 1 Recombination efficiency does not vary with age or muscle group. Cross-sections of indicated muscle at indicated time points were stained for Pax7, laminin and counterstained with DAPI to mark nuclei. The number of tdTomato+ sublaminar, Pax7+ satellite cells was counted and divided by the total number of sublaminar, Pax7+ satellite cells. Between 20-80 satellite cells were counted at each time point per muscle. A total of 652 satellite cells were counted.

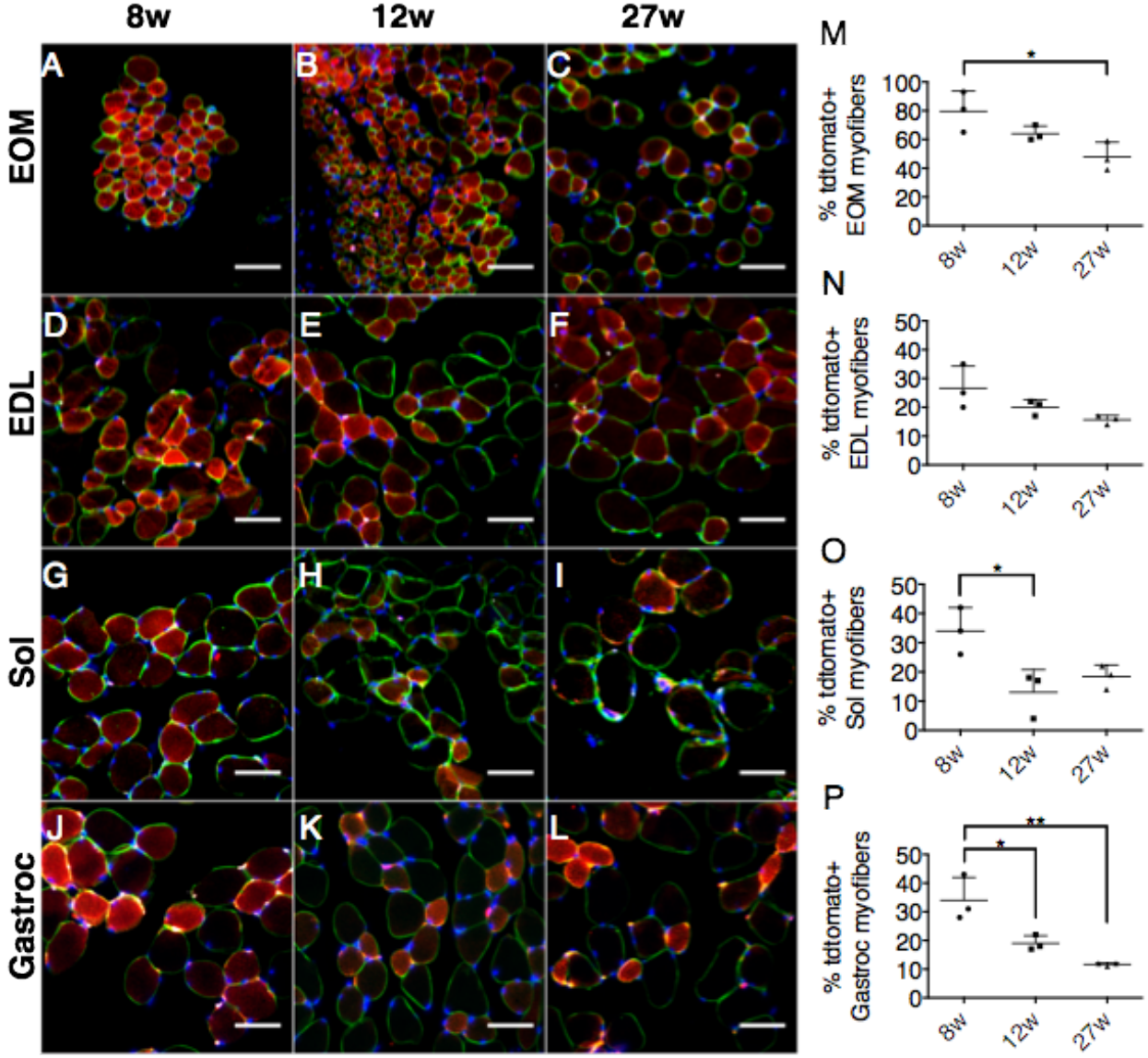


Figure 3 Satellite cell turnover is age and muscle group dependent. Recombination was induced at 8w, 12w or 27w as in Fig. 1A. Sections of extraocular (EOM, A-C), extensor digitorum longus (EDL, D-F), soleus (Sol, G-I), and gastrocnemius (Gastroc, J-L) muscle from mice with recombination induced at 8w, 12w or 27w old. Percent of tdTomato+ myofibers at 8w, 12w, and 27w from EOM (M), EDL (N), Sol (O), and Gastroc (P). Pseudocolored images display DAPI in blue, laminin in green, tdTomato in red, and, Pax7 in white. Scale bar is 50μm. * = P-value <0.05, ** = P-value <0.01. Significance was determined by one-way ANOVA with Turkey post-hoc test.

Satellite cell quiescence is age dependent

Recombination induces expression of tdTomato in Pax7-expressing satellite cells and is maintained in satellite cell progeny. If quiescent, satellite cells will express tdTomato exclusively. If, however, satellite cells are activated and contributing to the muscle, both satellite cells and myofibers will express tdTomato. By measuring the extent of tdTomato expression in myofibers over multiple time points, I can determine when satellite cells attain adult homeostatic levels of quiescence. By examining multiple muscles, I can determine whether satellite cell quiescence is muscle group dependent or correlates with muscle fiber type composition.

Mice from 6-12 weeks old are frequently used together for adult regeneration and satellite cell assays. However, mice continue to grow until 12 weeks of age, when growth plateaus (Eisen 1976; Somerville et al. 2004; White et al. 2010). Satellite cells are likely contributing to muscle growth during this period and, hence, are not quiescent. To determine when satellite cells attain quiescence, I measured satellite cell contribution to muscle at four time points: 4, 8, 12 and 27 weeks. At 4 weeks, satellite cells are extensively cycling and contribute to muscle growth (Cardasis and Cooper 1975; Kelly and Zacks 1969; Kelly 1978; Ross et al. 1987; Schultz 1976; Shea et al. 2010). By 27 weeks, adult muscles are fully formed and are no longer growing (Somerville et al.; Eisen). By comparing 8 and 12-week-old muscles, I can determine whether satellite cell quiescence is equivalent in these frequently combined ages. Furthermore, comparisons to 4 and 27-week time points will reveal whether that level of quiescence represents growing or adult muscle.

Hindlimb muscle satellite cells establish quiescence by 12 weeks

From the hindlimb, I examined the tibialis anterior (TA), extensor digitorum longus (EDL), soleus, and gastrocnemius. These muscles were selected for their diverse fiber type composition and frequent use as experimental regeneration models. The TA is exclusively comprised of type II fibers (Augusto, Padovani, and Campos 2004). Cycling satellite cells are present in the TA, although the reported amounts vary (Bjornson et al. 2012; Tatsumi, Anderson, et al. 1998). The EDL is located beneath the TA and includes up to 6% of type I fibers (Augusto, Padovani, and Campos 2004). The soleus is a mixed muscle and contains the highest percentage of type I fibers among hindlimb muscles (~37%, Augusto, Padovani, and Campos 2004). The soleus is also reported to have low levels of cycling satellite cells (<2%, (Augusto, Padovani, and Campos 2004; Mozdziak et al. 2000; Smith et al. 2001). The gastrocnemius is the largest muscle in the lower hindlimb, contains type I and all known type II sub-types.

The hindlimb muscles exhibit a similar pattern of establishing satellite cell quiescence. Both 4 and 8-week old TA myofibers displayed extensive contribution from satellite cells (99% and 63% tdTomato+ myofibers, respectively, Fig. 2). There was a significant reduction in tdTomato+ myofibers at 12 weeks (13% tdTomato+ myofibers, Fig. 2). Satellite cell contribution remained constant from 12 to 27 weeks (Fig. 2). Since satellite cells substantially contribute to 8-week old TA, I did not analyze 4-week time points for the remaining muscles. The EDL, soleus, and gastrocnemius displayed a similar drop in tdTomato+ myofibers from 8 to 12weeks, although the magnitudes vary (Fig. 3). Both the soleus and gastrocnemius exhibited 34% tdTomato+ myofibers at 8 weeks (Fig. 3). In the soleus, this drops two fold by 12 weeks (Fig. 3). Satellite cell contribution in the soleus is maintained from 12 to 27 weeks; whereas, contribution trends downward in the gastrocnemius over this period (1.8 fold decrease from 8 to 12w, 3 fold decrease from 8 to 27w, Fig. 3). However, the difference of satellite cell contribution to the

gastrocnemius between 12 and 27-week time points is not statistically significant (P-value = 0.23). In contract to the TA, soleus and gastrocnemius, there is no statistically significant difference in satellite cell contribution to the EDL over the time points examined (P-value > 0.05); however, there is a downward trend with age (Fig. 3). No correlation exists between fiber type composition of these muscles and extent of satellite cell contribution. Overall, satellite cell contribution to hindlimb muscles drops precipitously from 8 to 12 weeks and remains constant or slightly decreases from 12 to 27 weeks.

Satellite cells asymmetrical contribute to the gastrocnemius. In all time points examined, more myofibers located near the fibula and presumptive blood vessels expressed tdTomato than dorsally located myofibers (Fig. 4). Additionally, these myofibers were brighter, indicating a higher level of tdTomato expression and satellite cell contribution (Fig. 4).

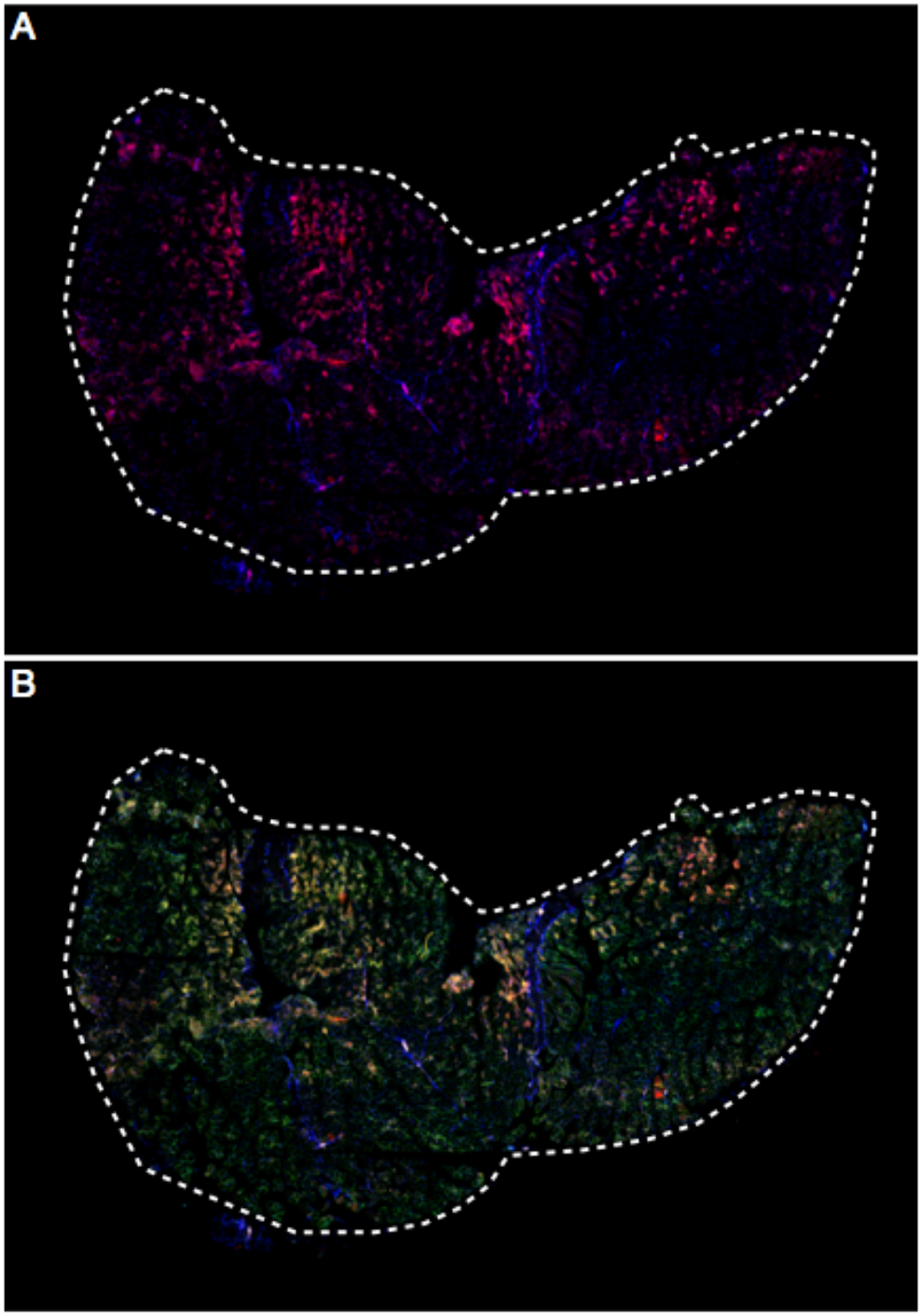


Figure 4 Uneven satellite cell contribution to the gastrocnemius. 8w gastrocnemius two weeks post-tamoxifen treatment shown with (B) and without (A) laminin for clarity. Dashed line indicates muscle boundary. Pseudocolored images display DAPI in blue, laminin in green, Pax7 in white, and tdTomato in red.

Extraocular muscles fail to establish quiescence by 27 weeks

The EOM is distinct from limb muscle in multiple aspects and is likely to have different satellite cell dynamics than those of the hindlimb muscles. The EOM and hindlimb muscles have different developmental origins. Craniofacial muscles, including the EOM, originate from the prechordal mesoderm; whereas, limb muscles develop from somites of the trunk mesoderm (Sambasivan, Kuratani, and Tajbakhsh 2011). EOMs are the fastest muscles in the body, with contraction times up to 20x quicker than limb muscle (Li, et al. 2011). EOM contains six fiber types, including embryonic and EOM specific fibers (Hwang, Huan, and Kim 2011; McLoon, Rios, and Wirtschafter 1999; Rashed, El-Alfy, and Mohamed 2010; Rubinstein and Hoh 2000; Wicke et al. 2007). The cross-sectional area of EOM myofibers is up to an order of magnitude smaller than those of the gastrocnemius (Li, et al. 2011). Unlike limb muscles, the EOM is constantly engaged, even during sleep (Li, et al. 2011). Additionally, several short-term BrdU labeling studies have reported more cycling satellite cells in EOM compared to hindlimb muscle (McLoon and Wirtschafter 2002, 2002, 2003; Rodgers et al. 2014).

The EOM displays high satellite cell contribution throughout the time points examined. At 8 weeks, 80% of EOM myofibers are tdTomato+ (Fig. 3). This falls to 64% by 12 weeks and 48% by 27 weeks (Fig. 3). The magnitude of change is equivalent from 8 to 12 weeks and 12 to 27 weeks (1.3 fold decrease, Fig. 3). Although there is a statistically significant difference between satellite cell contribution at 8 and 27 weeks, there is no indication that contribution has plateaued.

Discussion

Skeletal muscle requires satellite cell contribution for maintenance throughout the life of the organism. During postnatal growth, satellite cells are mitotically active and extensively contribute to muscle (Cardasis and Cooper 1975; Kelly and Zacks 1969; Kelly 1978; Ross et al. 1987; Schultz 1976; Shea et al. 2010). In adult muscle, however, satellite cells are predominately quiescent and only a small subset activate for muscle maintenance in uninjured tissue (Bjornson et al. 2012; Crameri et al. 2004; Dangott et al. 2000; Lepper et al. 2009; Mackey et al. 2009; Mikkelsen et al. 2009; Mozdziak et al. 2000; Schmalbruch and Lewis 2000; Smith et al. 2001; Tatsumi et al. 1998). To date, no comprehensive studies have determined when satellite cells acquire quiescence or whether satellite cells in different muscles acquire quiescence at distinct times.

Here, I used $Pax7^{ICreERT2}$; $R26R^{tdTomato}$ to genetically label satellite cells and indirectly determine when satellite cells attain quiescence in the TA, EDL, soleus, gastrocnemius and EOM. All hindlimb muscles examined display substantial satellite cell contribution at 8 weeks of age, which drops by 12 weeks and remains constant from 12 to 27 weeks (Fig. 2, Fig. 3). This indicates that hindlimb satellite cells acquire adult homeostatic levels of quiescence between 8 and 12 weeks. Frequently, 6 -12 week old mice are used for adult regeneration and satellite cell studies. These data suggest that using mice younger than 12 weeks old is inappropriate for studying adult regeneration as satellite cell contribution to muscle is above adult homeostatic levels.

Despite the overall trend in establishing satellite cell quiescence, there are notable differences among the hindlimb muscles. Of the four hindlimb muscles examined, approximately 30% of EDL, soleus and gastrocnemius myofibers receive satellite cell contribution at 8 weeks, in contrast to ~63% of TA myofibers (Fig. 2, Fig. 3). It is unlikely that muscle fiber type

composition causes this discrepancy as the TA, EDL and gastrocnemius all have relatively equal proportions of type I and type II fibers (>90% type II) (Augusto, Padovani, and Campos 2004). Furthermore, as these muscles functionally cooperate in locomotion, they are likely exposed to similar stresses and resultant degeneration (Hardt 1978; Lieber, Shah, and Fridén 2002; Maas, Baan, and Huijing 2001). However, in dystrophic muscle, the TA is damaged to a greater degree than other hindlimb muscle, including the soleus and gastrocnemius (Gabellini et al. 2006). This suggests that the TA may be more susceptible to damage and may require additional repair to maintain function in healthy muscle.

Satellite cells asymmetrical contribute to the gastrocnemius. I find a higher percentage of myofibers located near the fibula and presumptive blood vessels have incorporated satellite cells than dorsally located myofibers. Additionally, the ventrally located myofibers have incorporated more satellite cells, as evident by higher tdTomato expression. This may be caused by influx of systemic signals that form a concentration gradient while diffusing through the muscle.

Satellite cells contribute extensively to the EOM at all time points examined. At 8 weeks, satellite cells have contributed to 80% of EOM myofibers (Fig. 3). Contribution decreases constantly to 27 weeks with no indication that plateauing. This suggests that either EOM satellites do not become quiescent or that quiescence is attained after 27 weeks. These observations are consistent with several studies reporting more cycling satellite cells in EOM compared to hindlimb muscle (McLoon and J. D. Wirtschafter 2002; McLoon and J. Wirtschafter 2002; McLoon and Wirtschafter 2003; Rodgers et al. 2014). EOM likely has a higher capacity for regeneration than limb muscle. Satellite cells in the EOM have enhanced proliferation *in vitro* (Pacheco-Pinedo et al. 2009) and the EOM remains functionally intact in multiple muscular dystrophies (Kaminski et al. 1992; Khurana et al. 1995).

These observations illustrate the heterogeneity among satellite cells from different muscle groups. Gene expression profiles examining satellite cells from EOM and pharyngeal muscle, EDL and masseter muscle, and multiple head muscles revealed distinct genetic programs for satellite cells derived from different muscles (Harel et al. 2009; Ono et al. 2010; Sambasivan et al. 2009). Additionally, satellite cells from different muscles are functionally heterogeneous. Satellite cells derived from the EDL, soleus, masseter, and TA have different transplantation efficiencies as well as proliferation and differentiation capabilities *in vitro* (Collins et al 2005.; Yusuke Ono et al. 2010).

This is the first comprehensive study of satellite cell homeostasis in multiple uninjured muscles. These data support the hypothesis that satellite cells are a functionally heterogeneous population among muscles of both similar and distinct developmental origins. Additionally, this work cautions researchers against using mouse hindlimb muscle younger than 12 weeks for adult regeneration and satellite cell studies as the satellite cell population has yet to establish quiescence.

Methods

Mice

Mice were housed in a pathogen-free environment at the University of Colorado at Boulder. The University of Colorado Institutional Animal Care and Use Committee (IACUC) approved all animal protocols. $Pax7^{CreERT2}$ mice (Murphy et al. 2011) were crossed with ROSA-lsl-tdTomato mice (Madisen et al. 2010) to generate $Pax7^{ICreERT2}; R26R^{tdTomato}$. Recombination was induced by five daily intraperitoneal injections of tamoxifen (Sigma Aldrich) in corn oil dosed at 2mg tamoxifen/ 20g mouse weight. Tissues were collected from mice two weeks after

the final dose of tamoxifen. Wild type mice were C57BL/6J x DBA/2J (B6D2F1/J, Jackson Labs). Mice were sacrificed by cervical dislocation prior to tissue harvest. Both male and female mice were used in this study.

Satellite cell isolation and culture

For satellite cell isolations, hindlimb muscles were dissected, minced, and digested in 400U/mL collagenase in Ham's F-12C at 37°C for 1 hour with periodic vortexing. Collagenase was inactivated with 15% horse serum and debris was removed by sequential filtering through 100μm and 70μm cell strainers (BD Falcon). Cell pellets were re-suspended in 2ml of red blood cell lysing buffer hybrid-max (Sigma) and incubated for 2 minutes at room temperature to remove red blood cells. Cells were washed with Ham's F-12C supplemented with 15% horse serum and filtered through a 40μm cell strainer (BD Falcon). Satellite cells were enriched by pre-plating on a gelatin-coated 15cm plate in Ham's F-12C for 2 hours at 37°C. Cultured cells were grown in Ham's F-12C supplemented with 15% horse serum and 0.5 nM FGF-2 at 6% O_2 on gelatin-coated surfaces for 72 hours prior to fixation and immunostaining.

Myofiber isolation and culture

Extensor digitorum longus and soleus muscles were dissected, connective tissue removed, and muscle groups separated followed by enzymatic digestion in 400U/mL collagenase at 37°C 1.5 hours Collagenase was inactivated by the addition of Ham's F-12C supplemented with 15% horse serum. Individual myofibers were gently isolated and maintained in Ham's F-12C supplemented with 15% horse serum and 0.5 nM FGF-2 at 6% O_2. Myofibers were fixed 24 hours post-isolation.

Muscle sections

Tibialis anterior, extensor digitorum longus, soleus, gastrocnemius, tongue, diaphragm and extraocular muscles were harvested, fixed in 4% paraformaldehyde for 2 hours on ice, and incubated in 30% sucrose overnight. Muscles were mounted for cryosectioning in sufficient O.C.T. (Tissue-Tek®) to cover the tissue. Cryosectioning was performed on a Leica Cryostat and sections were between 8-12nm. Tissues and sections were stored at -80°C until ready for use. Tissues were equilibrated to -20°C overnight prior to sectioning. Sections were post-fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed three times for five minutes each in PBS. Heat-induced epitope retrieval (HIER) was used to improve Pax7 staining. For HIER, post-fixed slides were placed in citrate buffer, pH 6.0 and subjected to six minutes of high pressure-cooking in a Cuisinart model CPC-600 pressure cooker. HIER-treated sections were incubated with 30% H_2O_2 for five minutes at room temperature reduced tissue autofluorescence.

Immunofluorescence

Primary satellite cells and explanted myofibers were processed for immunofluorescence in the same manner. Cells were fixed with 4% paraformaldehyde (PFA) on ice at the time of harvest and washed in phosphate buffered saline (PBS). Cells were permeabilized with 0.2% Triton-X100 (Sigma) in PBS followed by blocking with 5% bovine serum albumin (Sigma) in PBS. Incubation with primary antibody occurred at 4°C overnight followed by incubation with secondary antibody at room temperature for 1hr in 3% bovine serum albumin (BSA) in PBS. Primary antibodies were 1:200 mouse anti-Pax7, 1:200 chicken anti-syndecan-4, 1:150 rat or rabbit anti-laminin (Sigma), neat mouse IgG1 anti-MyHC IIA (clone SC-71, Developmental Studies Hybridoma Bank), 1:20 mouse IgM anti- MyHC IIX (clone 6H1, Developmental Studies Hybridoma Bank), neat mouse IgM anti-MyHC IIB (clone BF-F3, Developmental Studies Hybridoma Bank) and mouse IgG2b anti-MyHC I (clone BA-D5, Developmental Studies

Hybridoma Bank). All MyHC antibodies were generously provided by Leslie Leinwand (MCDB, University of Colorado Boulder). Secondary antibodies against IgG of the appropriate species were conjugated to Alexa-488, Alexa-555, or Alexa-647 (Molecular Probes) and used at 1:500. Cells were incubated with 1μg/mL DAPI for 10 minutes at room temperature then mounted in mowiol supplemented with DABCO (Sigma-Aldrich) as an anti-fade agent.

Microscopy and image processing

Images were captured on a Leica DMRXA upright spinning disc confocal microscope. Objectives were 10x/0.3NA HC Plan Fluotar, 20x/0.7NA HC Plan Apo or 40x/0.85NA HC X Plan Apo (correction collar). Leica was equipped with a Yokagawa CSU10B spinning disk and images were taken with a Hamamatsu ImagEM EM-CCD. Muscle sections were imaged on either the Leica or a Zeiss 510 LSM. Objectives used on the Zeiss were 10x/0.3NA EC Plan Neofluar, 20x/0.8NA Plan Apo Chromat or 63x/1.4NA oil differential interference contrast Plan Apo Chromat M27 lens (Carl Zeiss). Images were processed using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) or the FIJI ImageJ version 1.47 package (NIH) with the additional MacMaster BioPhotonics Facility plugin set. For morphological measurements, individual fibers were outlined and cross sectional area, maximum diameter, and minimum diameter measured in ImageJ. Confocal stacks were projected as maximum intensity images for each channel, background subtracted, and merged into a single image in ImageJ. Brightness and contrast was adjusted for the entire image as necessary. Images were either cropped or merged as necessary and individual color channels were extracted without color correction or ɣ-adjustment. Images were adjusted and counted manually or with ImageJ macro MyoCount (Appendix X: Annotated MyoCount Script), designed to perform the same functions.

Chapter 3: mRNA post-transcriptional regulation mediates satellite cell activation and cell fate decision between proliferation and self-renewal

This chapter includes data that were published on October 9th, 2012 in the scientific journal *Skeletal Muscle* as part of a manuscript entitled: *A role for RNA post-transcriptional regulation in satellite cell activation*. This work identifies genes involved in skeletal muscle satellite cell activation (referred to as the activation dataset) and determines that RNA binding proteins are significantly enriched among these genes. A subset of the activation dataset was applied to microRNA target prediction algorithms to identify potential microRNA regulators of activation. *In vitro* inhibition of miR-16 in satellite cells on myofibers prior to their first division increases the Pax7+ proliferating population, while inhibition of miR-106b and miR-124 increases the Pax7+/MyoD- quiescent population. From this work, we suggest that RNA post-transcriptional regulation plays a role in regulating the satellite cell fate decision between proliferation and self-renewal after injury. My primary contributions to this work include gene expression analysis of *Sdc4-/-* and wild type satellite cells at uninjured and 12-hours post-injury time points (Figures 1E, 2A, 2B), gene ontology characterization (Figures 3, 5), and qRT-PCR validation (Tables 1, 5) of the satellite cell activation dataset. Figures that include published data will be noted in the figure footnote.

Background

Satellite cells are a population of adult stem cells that repair and maintain skeletal muscle (Murphy et al. 2011). These cells reside adjacent to myofibers, above the sarcolemma but beneath the basal lamina and comprise 2-11% of all myonuclei (Snow 1977; Schmalbruch and Hellhammer 1976; Allbrook, Han, and Hellmuth 1971; Cardasis and Cooper; Megeney et al. 1975; Mauro 1961). In healthy, uninjured muscle, satellite cells are mitotically quiescent (Snow 1977; Schultz 1976). Upon myotrauma, extrinsic signals including HGF, nitric oxide, FGF2, and TNFα, trigger satellite re-entry into the cell cycle, a process termed activation (Do et al. 2012; Filippin et al. 2009; S.-E. Chen et al. 2007). Activation induces p38α/β MAPK signaling (Troy et al. 2012; Bernet et al. 2014), leading to expression of the myogenic regulatory factor MyoD (Jones et al. 2005) and proliferation. Upon activation, satellite cells proceed through an initial cell cycle within 48h (D. D. W. Cornelison et al. 2004; Troy et al. 2012). A subset will self-renew, replenishing the satellite cell pool for future regeneration (Troy et al. 2012). The majority, however, will form a rapidly proliferating population of myoblasts that will fuse with each other and with damaged myofibers, repairing the muscle. The molecular mechanisms regulating this process are not well understood.

To elucidate satellite cell regulatory genes, multiple satellite cell gene expression profiling studies have been performed. These gene expression analyses have compared freshly isolated satellite cells to cultured, diseased and neonatal satellite cells (Bentzinger et al. 2013; S. Fukada et al. 2007; LaFramboise et al. 2009; Pallafacchina et al. 2010; Seale et al. 2004). However, to identify activation-specific genes, a comparison of gene expression profiles between *quiescent* satellite cells and activated or proliferating satellite cells requires that that majority of isolated satellite cells are quiescent and not cycling. The majority of published expression

profiling analyses isolated satellite cells at ages when the cells are extensively contributing to the muscle and are not quiescent (<12 weeks, see Chapter 3 Fig. 3). Although these studies provide insight into the molecular mechanism regulating satellite cells during development, disease, proliferative and differentiated states, they do not adequately address the transcriptional changes occurring in the transition from a quiescent satellite cell to a proliferating myoblast.

The satellite cell transition from quiescence or G_0 into G_1 is assumed to involve a significant increase in transcription since the cells are mitotically quiescent and transitioning into DNA synthesis. Consistent with the hypothesis that satellite cells have low metabolic and transcriptional activity, electron microscopy (EM) morphological characterization of quiescent satellite cells revealed heterochromatin, a low cytoplasm volume to nuclear volume ratio, few organelles and ribosomes, an absence of polysomes, rough endoplasmic reticulum, and Golgi cisternae (Hanzlíková, Macková, and Hník 1975; Lu, Huang, and Carlson 1997; Ontell 1975; Sakai 1977; Schultz 1976). Satellite cells actively maintain quiescence (Pallafacchina et al. 2010) and can exist in distinct quiescent states, a G_0 and an 'alert' G_0 state (Rodgers et al. 2014). The 'alert' satellite cells display increased mitochondrial activity and distinct gene expression profiles from satellite cells isolated from uninjured animals and proliferating myoblasts (Rodgers et al. 2014).

To better understand the molecular mechanisms involved in satellite cell activation and entry into S-phase, we performed an unbiased, global gene expression analysis of satellite cells transitioning from quiescence to proliferating myoblasts *in vivo*. Specifically, we isolate satellite cells from uninjured mice as well as 12 hours post-injury. We identified genes in wild-type satellite cells that are up- or downregulated during this transition. To eliminate gene expression changes not directly related to satellite cell activation, we excluded genes that change expression

in activation-deficient *Sdc4-/-* satellite cells. These genes, termed the *activation dataset*, were enriched for RNA binding proteins.

I further compared the activation dataset to two independent gene expression profiles of aged satellite cells. I identified several pathways involved in activation that are dysregulated in aged satellite cells, including the p38 MAPK pathway. Additionally, I identified the CELF family of RNA binding proteins as potential regulators of satellite cell activation and cell fate decision between proliferation and self-renewal. CELF proteins likely regulate satellite cell function through interaction with the p38 MAPK pathway. Enhanced p38 MAPK signaling and impaired proliferation and self-renewal of aged satellite cells may result from dysregulation of CELF1, 2 and 4. The analysis presented in this chapter suggests that satellite cells 1) actively maintain the quiescent state, 2) are primed to quickly respond to injury, and 3) dysregulation of these processes contributes to impaired activation, proliferation, and self-renewal in aged satellite cells.

Results

Identification of gene expression changes during satellite cell activation in vivo

Unbiased, global gene expression analysis was performed to identify genes involved in the satellite cell transition from quiescence to proliferating myoblasts after muscle injury *in vivo.* Syndecan-3 positive satellite cells from wild-type mice, either uninjured or 12 hours post-$BaCl_2$ injury were isolated using fluorescence activated cell sorting (FASC) (Farina et al. 2012) (Figure 1C). The 12-hour time point was chosen to identify early changes in gene expression post-injury that prime satellite cells for re-entry into the cell cycle. We identified 5162 genes that significantly change expression >= 2-fold from uninjured to 12-hour post-injury (Figure 2A). To

eliminate gene expression changes not specific to activation, we compared the transcriptome changes of wild type to *syndecan-4*$^{-/-}$ (*Sdc4-/-*) satellite cells (Figure 1E). *Sdc4-/-* satellite cells fail to activate, proliferate and express MyoD within 48 hours in culture and fail to regenerate muscle (Farina et al. 2012; D. D. W. Cornelison et al. 2004) (Figure 1A, B). *Sdc4-/-* satellite cells were similarly isolated via FASC using anti-Syndecan-3 (Figure 1D). Hierarchical clustering of *Sdc4-/-* and wild type satellite cell gene expression profiles shows that at all time points post-injury *Sdc4-/-* satellite cells cluster with freshly isolated wild type satellite cells (Figure 1E). This supports our observation that *Sdc4-/-* satellite cells are activation deficient (Figure 1A, B). We subtracted from the wild type list genes that significantly change expression in activation deficient, *Sdc4-/-* satellite cells (1069 genes), resulting in 4093 gene expression changes specific to activation, hereafter referred to as the 'activation dataset' (Figure 2A). The majority of genes within the activation dataset decrease expression during activation (56%, Figure 2B). Genes downregulated during activation show a greater magnitude of change, ranging up to 64-fold, than upregulated genes, the majority of which only increase expression up to 4-fold (Figure 2C). Additionally, genes in the activation dataset involved in cell cycle progression are regulated as expected for cell cycle re-entry. Genes regulating the G1/S transition are upregulated (Figure 2D, green). In contrast, cell cycle inhibitors are downregulated (Figure 2D, red). These observations suggest that satellite cell quiescence is actively maintained by a set of 'quiescence genes' that must be downregulated for cell cycle re-entry. This hypothesis is consistent with recent studies comparing freshly isolated and 'alert' satellite cells to proliferating myoblasts (Rodgers et al. 2014).

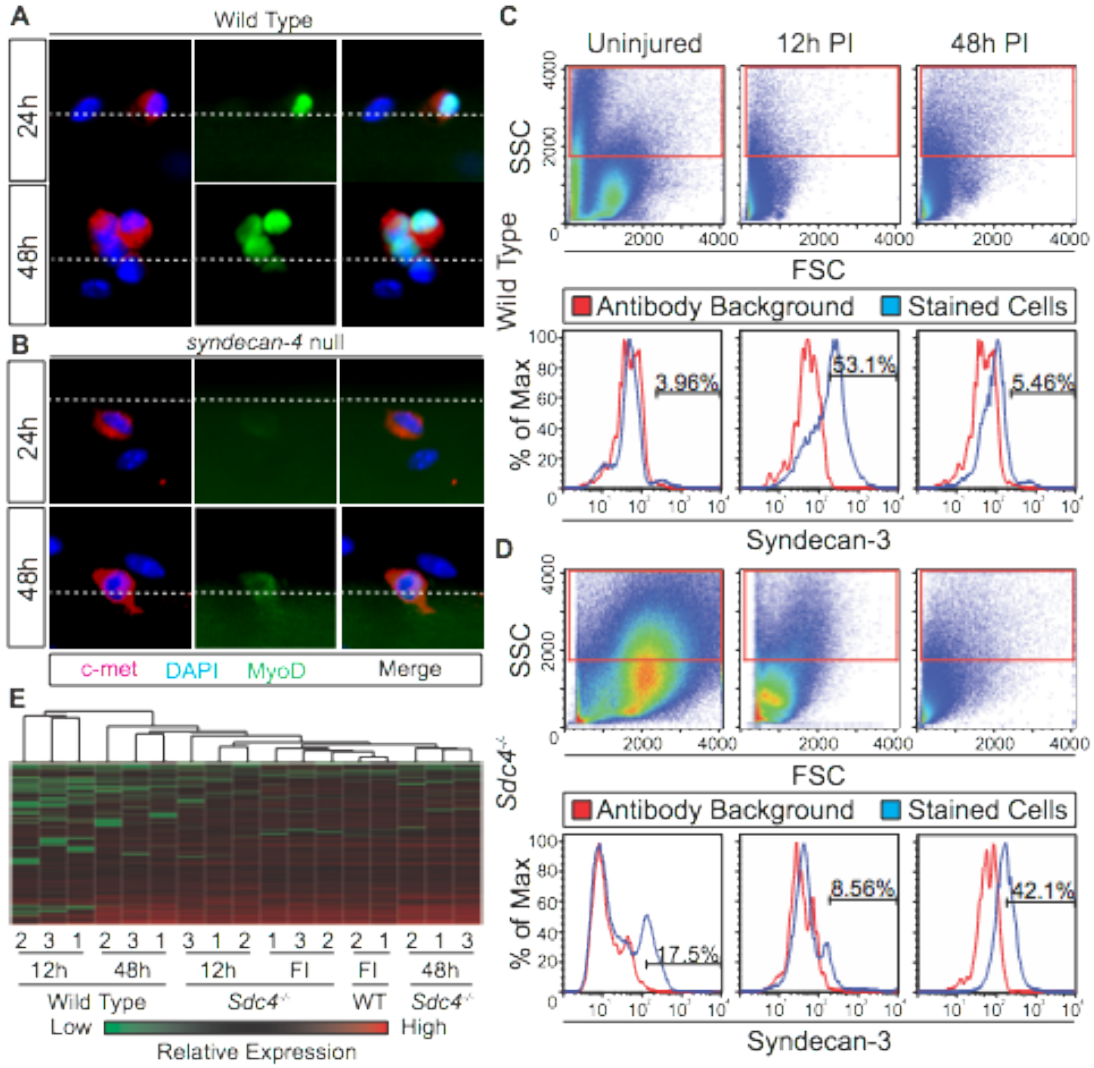


Figure 1 Sdc4–/– satellite cell gene expression post-muscle injury is similar to freshly isolated satellite cells. Myofiber-associated satellite cells are immunoreactive for MyoD 24 h and 48 h after isolation from wild type mice but not Sdc4–/– mice (A, B). Wild type but not Sdc4–/– cells divide by 48 h in culture (B) where c-met (red), MyoD (green), and DAPI (blue) identify satellite cells and a dashed line indicates the position of the myofiber membrane (A, B). Flow cytometry histograms of wild type (C) and syndecan-4 null (D) mononuclear cells from uninjured and injured skeletal muscle 12 h and 48 h post-injury plotted for cell size (FSC) vs. internal complexity (SSC), where the red box indicates gating for further analysis to remove debris (upper panels). Syndecan-3 immunoreactive cells present in the gate were isolated from wild type mice (C, lower panel) and Sdc4–/– mice (D, lower panel) where the percentages indicate satellite cells (blue lines) relative to other events with false-positives set to an antibody background<0.1% (red lines). A hierarchical dendrogram constructed with Spotfire DecisionSite using Affymetrix GeneChip data reveals that Sdc4–/– satellite cells cluster most closely to freshly isolated wild type satellite cells while injured wild type satellite cells either 12 h post-injury or 48 h post-injury cluster independently (E). Red depicts high relative gene expression and green depicts low relative expression in the hierarchical cluster dendrograms (UPGMA, Euclidean distance). FI, freshly isolated; PI, post-injury. This figure was published as Figure 1 of Farina, *et. al.*, 2012.

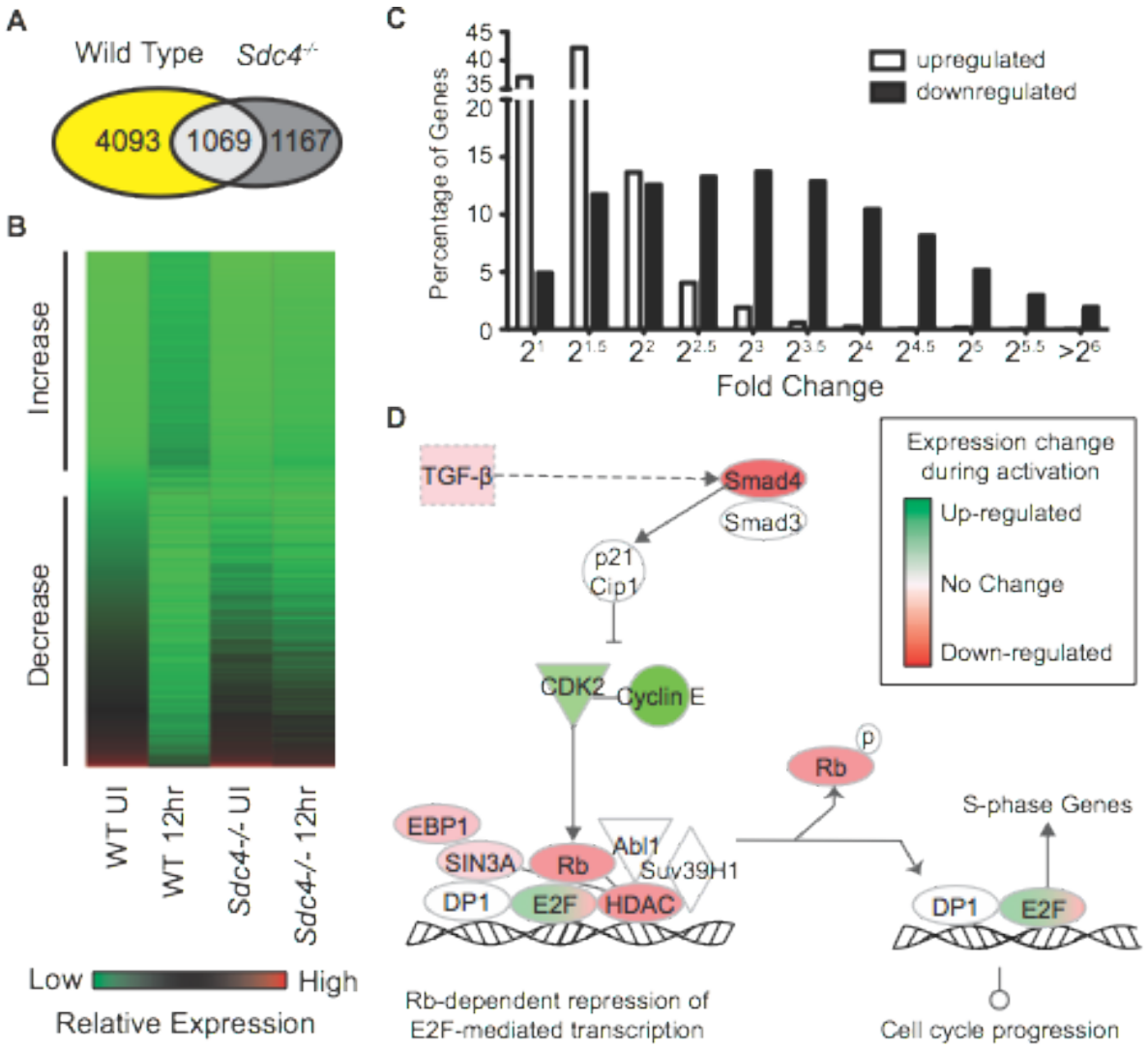


Figure 2 Gene expression changes occurring during satellite cell activation. The genes significantly regulated between freshly isolated satellite cells and satellite cells isolated 12 h post-injury from wild type and Sdc4–/– mice were plotted as a Venn diagram to identify genes unique to wild type satellite cells (A, yellow, ANOVA P≤0.01, ≥ two-fold change). A heat map depicting changes in relative expression of genes unique to wild type satellite cells with more than half (56%) of the transcripts decreasing during the first 12 h following satellite cell activation (B, red is high relative expression and green low relative expression). The frequency of genes that decrease (▪) more than four-fold (22)is significantly higher than the frequency of genes that increase (▫) more than four-fold (22) during the first 12 h post-muscle injury (C). Further analysis of gene expression data using IPA 9.0 (IngenuityW Systems, www.ingenuity.com) demonstrate that genes promoting cell cycle progression increase (green) while genes that inhibit the G1/S phase transition decrease (red) in wild type satellite cells 12 h post-muscle injury (D, relative intensity depicts the fold change with higher color intensity denoting a greater fold change). This figure was published as Figure 2 of Farina, *et. al.*, 2012.

Genes involved with mRNA post-transcriptional regulation are significantly enriched during satellite cell activation

Gene Ontology (GO) analysis was used to identify potential regulatory mechanisms of satellite cell activation. Unique gene identifiers from the activation dataset were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) to identify enriched GO terms. The general Molecular Function GO categories: Binding (*P* value = 7.03×10^{-43}), Structural molecule activity (*P* value = 4.97×10^{-4}), Catalytic activity (*P* value = 1.46×10^{-3}), and Enzyme regulator activity (*P* value = 0.0521) were significantly enriched in the activation dataset compared to the mouse genome (Figure 3A). The specific Molecular Function GO terms unfolded protein binding, actin binding, and RNA binding were enriched at least 1.5 fold in the activation dataset, as determined by three independent enrichment algorithms: DAVID, ProfCom and FunNet (Figure 3B, Table 1). The enrichment of unfolded protein binding is likely in preparation for the increased translation that occurs during proliferation, while the enrichment of actin binding is likely related to increased satellite cell motility after skeletal muscle injury (Ishido and Kasuga 2011; Klein-Ogus and Harris 1983; Maltin, Harris, and Cullen 1983; Schultz, Jaryszak, and Valliere n.d.). The enrichment of RNA binding genes suggests a role for post-transcriptional regulation of satellite cell quiescence and activation.

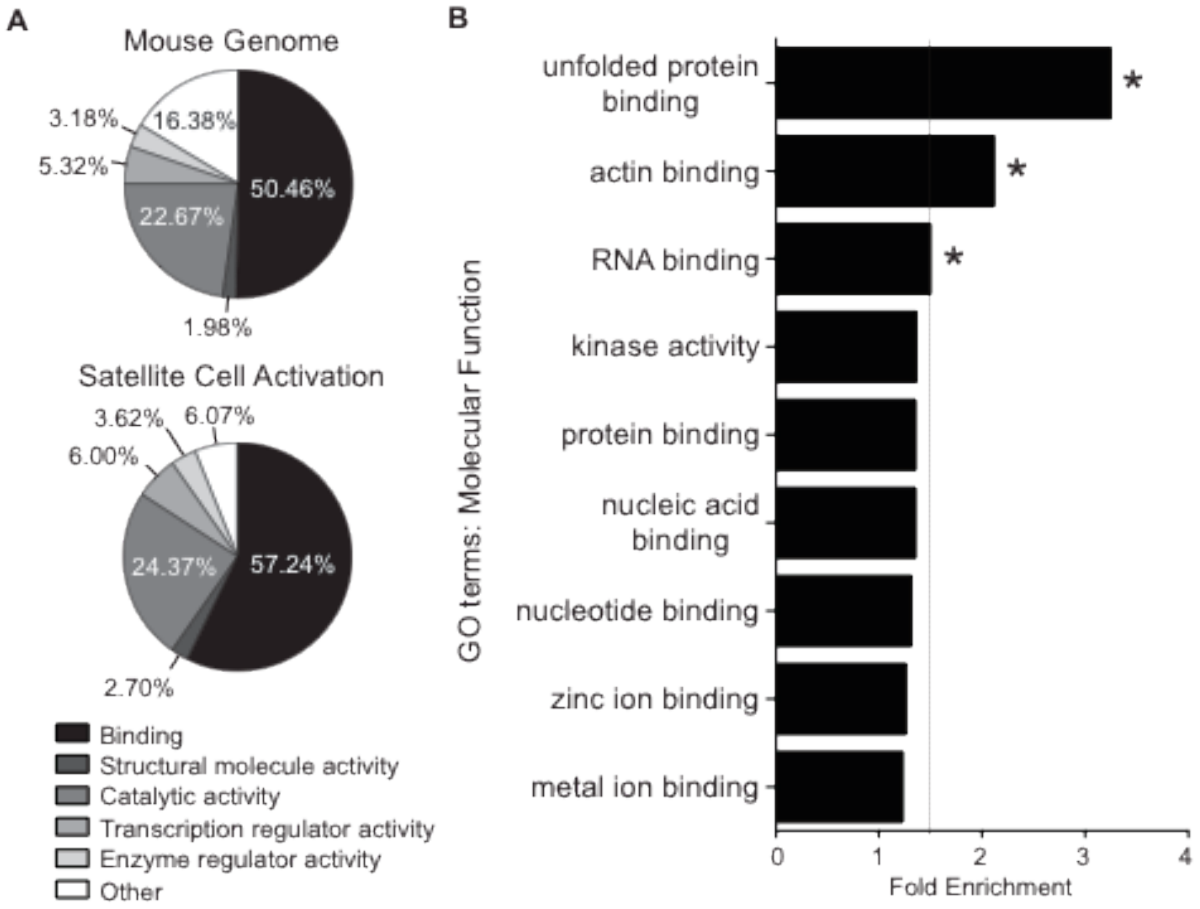


Figure 3 Binding genes are enriched during satellite cell activation. Gene expression changes unique to wild type satellite cells occurring within 12 h post-muscle injury were further analyzed by gene ontology. The general Molecular Function GO categories of Binding (P=7.03×10-43), Structural molecule activity (P=4.97×10-4), Catalytic activity (P=1.46×10-3), Transcription regulator activity (P=0.0229), and Enzyme regulator activity (P=0.0521) were identified by DAVID as enriched when comparing satellite cells isolated 12 h post-muscle injury to freshly isolated satellite cells (A). Specific Molecular Function GO terms including RNA binding, unfolded protein binding, and actin binding were enriched an average of 1.5-fold when comparing satellite cells from injured and uninjured muscle as identified by three independent algorithms including ProfCom, FunNet, and DAVID (B). The dotted line marks a 1.5-fold enrichment threshold. Asterisks mark GO terms with an average enrichment≥1.5-fold. This figure was published as Figure 3 of Farina, *et. al.*, 2012.

GO Term	DAVID	*P* value	ProfCom	*P value2*	FunNet	*P value3*
Unfolded protein binding	2.27	2.74E-05	4.65	2.90E-08	2.83	2.64E-08
Actin binding	1.83	2.00E-09	2.6	2.10E-12	1.92	5.03E-10
RNA binding	1.3	3.25E-04	1.82	9.80E-10	1.39	6.98E-05
Kinase activity	1.25	7.15E-04	1.54	4.30E-08	1.31	1.48E-04
Protein binding	1.25	8.42E-29	1.52	5.90E-70	1.3	1.86E-21
Nucleic acid binding	1.13	2.38E-04	1.63	7.50E-10	1.31	7.34E-05
Nucleotide binding	1.21	6.45E-07	1.5	6.10E-18	1.23	2.76E-06
Zinc ion binding	1.19	1.83E-05	1.38	1.80E-15	1.23	5.01E-05
Metal ion binding	1.17	7.66E-09	1.37	5.20E-23	1.16	4.83E-05

Table 1 Identified Molecular Function GO Terms. Fold enrichment and associated p-values for the Molecular Function GO terms shared between three independent gene ontological analyses using DAVID (http://david.abcc.ncifcrf.gov), profcom (http://webclu.bio.wzw.tum.de/profcom/), and FunNet (http://www.funnet.info). This table was published in Farina, et. al., 2012.

Of the 154 RNA binding proteins identified through GO analysis of the satellite cell activation dataset, the majority regulate mRNA splicing or processing (Figure 4) and many have known roles in stem cell, satellite cell or skeletal muscle function or disease (Farina et al. 2012). This list contains three known regulators of *Myod1* transcript stability: HuR, CELF1, and TTP. TTP, an mRNA destabilizing protein, and CELF1, a negative regulator of *Myod1*, are downregulated during activation, while HuR, an mRNA stabilizing protein, is upregulated (van der Giessen et al. 2003; Lee et al. 2010; Manuscript et al. 2003; Di Marco et al. 2005). The change in expression from *Myod1* transcript-destabilizing RNA-BPs to *Myod1* transcript-stabilizing RNA-BPs during satellite cell activation may explain the rapid up-regulation of

MyoD protein in satellite cells after injury (Srikuea et al. 2010; Sachidanandan, Sambasivan, and Dhawan 2002, Hausburg, *et. al.* in revision). I confirmed the expression changes of a subset of RNA binding protein via qRT-PCR and found a correlation of ~80% with the microarray data (Table 2). The presence of known regulators of stem cell, satellite cell, and muscle biology on the satellite cell activation dataset validates our approach to identify novel regulators of satellite cell activation.

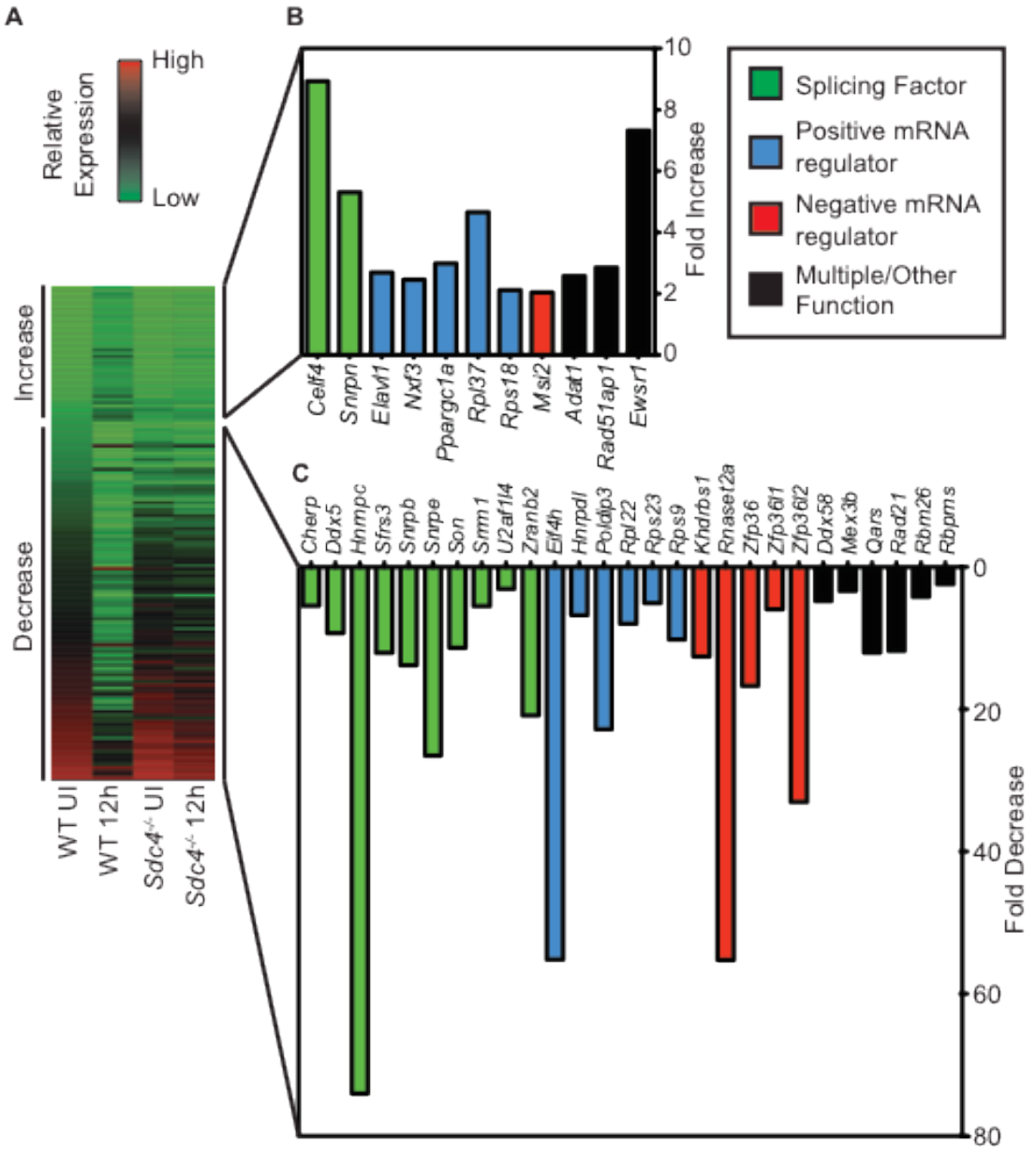


Figure 4 Classification of top quartile RNA binding proteins significantly regulated 12 h post-injury in satellite cells. Genes categorized with the GO term Molecular Function: RNA Binding that change expression≥two-fold (ANOVA P≤0.01) comprise 22% (154 of 716) of the total GO category in wild type satellite cells but do not change significantly in Sdc4–/– satellite cell during the first 12 h post-muscle injury (A). A minority of the identified genes increase in relative expression (B), while the majority of these genes decrease in their relative expression 12 h post-muscle injury (C). Upregulated and downregulated genes were further classified and plotted as splicing factors (green bars), positive mRNA regulators (blue bars), negative mRNA regulators (red bars), or multiple/ other functions (black bars). The values plotted are for fold increase (B)or fold decrease (C)unique to wild type satellite cells occurring in the first 12 h post-muscle injury. This figure was published as Figure 5 of Farina, et. al., 2012.

Gene	Microarray	qPCR	Fold change	Correlation	Biological function
Celf4	↑	↑	2.51	Yes	Splicing factor
Pabpn1	↓	↓	-1.8	Yes	Positive mRNA regulator
Ppargc1a	↑	↑	1.34	Yes	Positive mRNA regulator
Mbnl1	↓	↓	-1.14	Yes	Splicing factor
Matr3	↓	↑	11.79	No	Negative mRNA regulator
Sfrs3	↓	↑	19.11	No	Splicing factor
Zfp36	↓	↓	-2.87	Yes	Negative mRNA regulator
Zfp36l1	↓	↓	-4.48	Yes	Negative mRNA regulator
Zfp36l2	↓	↓	-1.63	Yes	Negative mRNA regulator
Elavl1	↑	↑	3.01	Yes	Positive mRNA regulator
Cdk2	↑	↑	10.28	Yes	Promotes cell cycle entry
E2F3	↑	↑	12.9	Yes	Promotes cell cycle entry

Table 2 Microarray and qPCR expression trends correlate for RNA binding proteins. Eight of ten RNA binding proteins and two cell cycle genes validate expression changes between wild type satellite cells isolated from uninjured TA muscle and from the TA 12 h post-muscle injury. Arrows show increase (↑) and decrease (↓) for both microarray and qPCR during satellite cell activation. Fold change is from qPCR data where positive values indicate increased expression and negative valued indicate decreased expression between satellite cells isolated from uninjured TA muscle and from the TA 12 h post-muscle injury. Quantitative PCR data is normalized to GAPDH or 18S. This table was published as Table 5 of Farina, et. al., 2012.

The CELF family of RNA binding proteins is enriched during satellite cell activation

The CELF family of proteins, which regulate mRNA splicing, stability, and translation, consists of six members (CELF1-6) (Ladd et al. 2001; Lu et al. 1999; Meins et al. 2002; Roberts et al. 1997; Szafranski et al. 2007; Wheeler et al. 2007). Of the six CELF family members, CELF1, 2 and 4 are present in the activation dataset. Additionally, CELF1, 2 and 4 are among genes whose transcripts undergo the largest magnitude expression change during satellite cell activation. Following the first 12h post muscle-injury, CELF1 decreases 30 fold (93rd percentile), CELF2 decreases 13 fold (69th percentile), and CELF4 increases 9 fold (98th percentile) in satellite cells. The expression patterns observed during satellite cell activation

suggest that the CELF family may be involved in both maintaining quiescence (CELF1 and 2) and promoting activation (CELF4).

CELF1/2/4 target transcripts are preferentially downregulated during satellite cell activation

To determine if CELF proteins are likely regulators of activation, I compared the satellite cell activation dataset with CELF1/2/4 targets. RIP-Chip using an anti-CELF antibody revealed 793 CELF1/2/4 target transcripts in proliferating C2C12 myoblasts (Good et al. 2000; Lee et al. 2010; Roberts et al. 1997; Timchenko, Miller, et al. 1996). Of those, 717 (90.4%) were detectable by both microarrays used for the satellite cell activation (Affymetrix 430v2 GeneChip) and CELF1/2/4 RIP-Chip (Affymetrix Mouse Gene 1.0 array) experiments (Figure 5A). Of the 4093 genes on the activation dataset, 2199 (53.7%) are detectable by both arrays, including 846 (44.2%) genes increasing and 1353 (62.1%) genes decreasing during activation (Figure 5A). Thirty-seven (5%) CELF1/2/4 targets increase expression during activation (Table 3, Figure 5A, not significant). Additionally, 165 (23%) CELF1/2/4 targets decrease expression during activation (Table 4, Figure 5A, P value = 1.4×10^{-18}). The number of CELF1/2/4 targets upregulated during activation is not statistically significant; however, CELF1/2/4 targets are upregulated to a higher magnitude than the majority of upregulated transcripts on the activation dataset and do not follow a random distribution (Figure 5B). Conversely, downregulated CELF1/2/4 targets tightly follow the fold change distribution of downregulated transcripts (Figure 5C). These data show that CELF1/2/4 targets are significantly enriched among genes regulated during satellite cell activation; therefore, the CELF family may play an important role in both maintaining quiescence and transitioning satellite cells to a proliferative state.

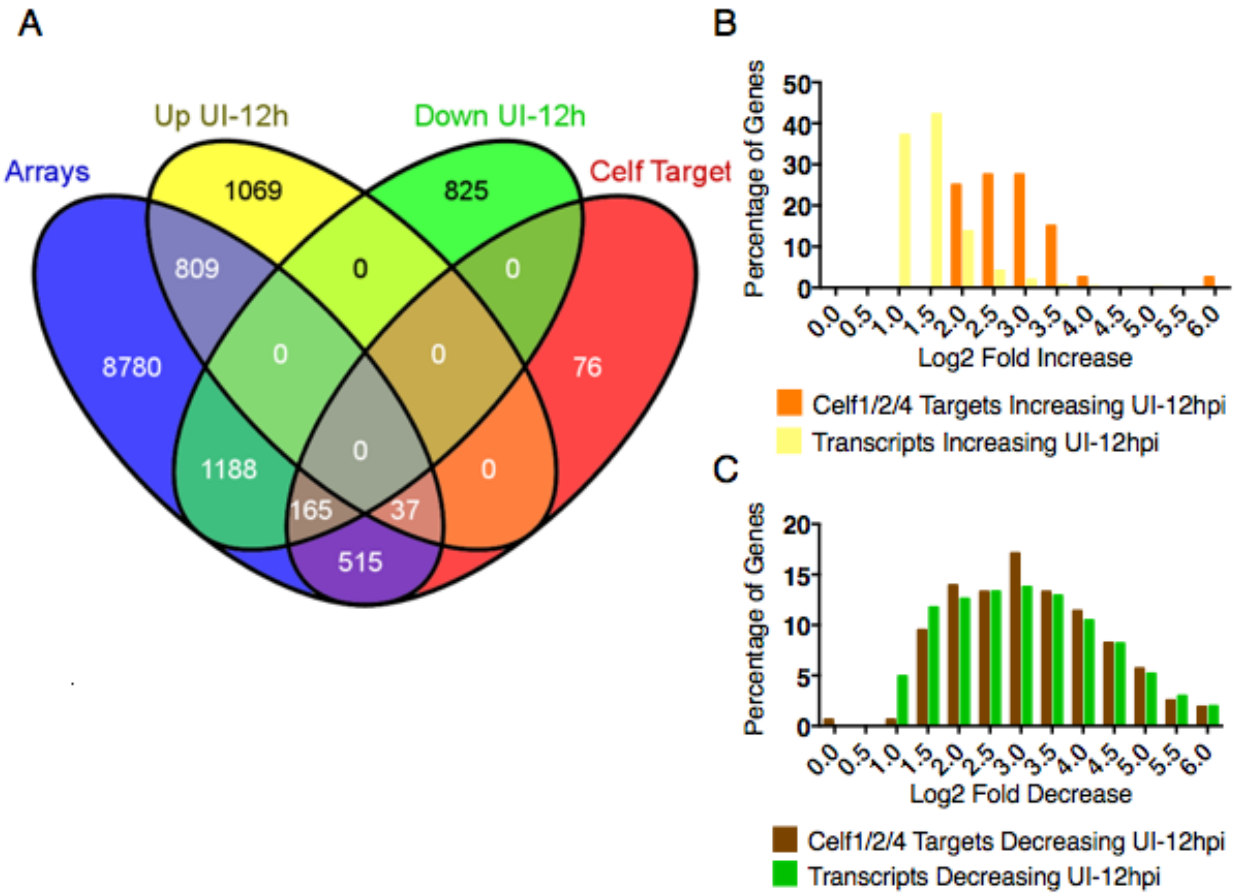


Figure 5 CELF1/2/4 targets are enriched during satellite cell activation. Gene expression changes unique to wild type satellite cells occurring within 12 h post-muscle injury were compared to putative CELF1/2/4 targets identified by CELF RIP-Chip of proliferating C2C12 myoblasts (Lee, 2012). (A) A total of 11494 unique genes were detectable by both microarrays used for the satellite cell activation (Affymetrix 430v2 GeneChip) and CELF1/2/4 RIP-Chip (Affymetrix Mouse Gene 1.0 array) experiments (Arrays, blue). Ninety percent (717/793) of CELF1/2/4 targets (Celf Target, red) were detectable by both arrays (red/blue overlap). Fifty four percent (2199/4093) of genes on the satellite cell activaiton dataset were detectable by both arrays (yellow/blue and green/blue overlap). Of these, 846/1915 increased (Up UI-12h, yellow/blue overlap) and 1353/2178 decreased (Down UI-12h, green/blue overlap) during activation. Five percent (37) of CELF1/2/4 targets increase with age (red/yellow/blue overlap, not significant) while twenty three percent (165) CELF1/2/4 targets decrease with age (red/green/blue overlap, P-value = 1.4E-18). (B) Activation fold change histograms for all transcripts (yellow) and CELF1/2/4 targets (orange) upregulated 12h post-injury. CELF1/2/4 targets are upregulated to a higher magnitude than the majority of transcripts upregulated during activation. (C) Activation fold change histograms for all transcripts (green) and CELF1/2/4 targets (brown) downregulated 12h post-injury. Significance was determined by hypergeometric distribution.

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
TSPAN31	1455853_x_at	3.934	6.561	6.176	67125
PHF21A	1418391_at	3.332	5.270	3.83	192285
LUC7L2	1445717_at	2.931	4.746	3.519	192196
CORO1C	1449660_s_at	3.756	5.562	3.496	23790
TUBB3	1415978_at	2.558	4.362	3.493	22152
CSNK2A1	1453427_at	2.871	4.665	3.469	12995
LPCAT1	1424459_at	4.004	5.743	3.338	210992
LFNG	1449943_at	2.415	4.145	3.316	16848
UHMK1	1446120_at	2.589	4.244	3.149	16589
DZIP3	1458439_a_at	2.764	4.383	3.073	224170
DDX39B	1437367_at	2.551	4.170	3.072	53817
ARHGAP29	1441618_at	2.590	4.208	3.07	214137
GATAD2B	1425075_at	3.451	4.986	2.898	229542
TMED2	1455968_x_at	10.869	12.379	2.847	56334
RBFOX2	1418245_a_at	2.744	4.241	2.822	93686
NR4A2	1433423_at	3.417	4.911	2.816	18227
SIX4	1439753_x_at	2.710	4.201	2.811	20474
ASB1	1444127_at	2.941	4.428	2.803	65247
RCC2	1426897_at	2.499	3.970	2.771	108911
ELAVL1	1428653_x_at	2.900	4.328	2.692	15568
SLC35A5	1419972_at	2.885	4.301	2.669	74102
E2F3	1427462_at	3.006	4.404	2.635	13557
CERS5	1447308_at	2.831	4.209	2.599	71949
TCF3	1427764_a_at	2.414	3.761	2.545	21423
KRAS	1426229_s_at	2.643	3.924	2.429	16653
UBE2C	1452954_at	2.819	4.079	2.395	68612
MBOAT1	1441824_at	2.945	4.185	2.361	218121
SF1	1422321_a_at	2.263	3.458	2.29	22668
TERF1	1431332_a_at	3.047	4.236	2.279	21749
PFDN5	1422297_at	3.063	4.246	2.271	56612
ZFAND3	1452275_at	2.572	3.737	2.242	21769
RNF219	1431624_a_at	3.494	4.658	2.24	72486
ZDHHC2	1452655_at	2.968	4.119	2.22	70546
ELP2	1415774_at	2.946	4.077	2.191	58523
MOB1B	1430564_at	2.835	3.925	2.128	68473
TSC22D2	1453012_at	3.246	4.304	2.081	72033
LDLR	1431947_at	2.818	3.867	2.07	16835
BCL2L1	1426191_a_at	2.642	3.681	2.054	12048
CCT8	1436973_at	2.471	3.502	2.043	12469
ASB8	1424225_at	2.366	3.370	2.005	78541

Table 3 CELF1/2/4 targets upregulated during activation. CELF1/2/4 targets identified by RIP-Chip in C2C12 myoblasts (Lee, et. al., 2012) were compared to the satellite cell activation dataset. 37 of 717 (5%) detectable CELF1/2/4 targets are upregulated upon activation and are listed here by gene symbol. Affymetrix refers to the associated Affymetrix probeset from the activation dataset. Relative expression levels for wild type (WT) satellite cells are listed for freshly isolated (uninjured, UI) and 12h post-injury (12hpi) time points in log2 along with expression fold change from UI to 12hpi. Entrez gene IDs are listed for their corresponding genes.

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
GRN	1438629_x_at	12.610	4.062	-374.342	14824
VAMP3	1437708_x_at	11.346	3.253	-273.148	22319
ELOVL5	1437211_x_at	11.368	4.241	-139.774	68801
EFHD2	1437478_s_at	9.508	2.863	-100.09	27984
CNOT6	1426682_at	9.870	3.526	-81.218	104625
MFF	1456736_x_at	11.416	5.105	-79.418	75734
VEZF1	1429085_at	9.054	2.935	-69.493	22344
FAM8A1	1433475_a_at	10.964	4.869	-68.329	97863
FNBP1L	1434339_at	10.007	4.031	-62.925	214459
STX7	1418436_at	8.969	3.125	-57.457	53331
YWHAB	1455815_a_at	12.637	6.887	-53.804	54401
BUB3	1416815_s_at	9.259	3.622	-49.791	12237
RAB1A	1416082_at	9.763	4.194	-47.48	19324
TPM2	1419738_a_at	11.816	6.276	-46.522	22004
CALR	1417606_a_at	8.857	3.374	-44.699	12317
RDX	1416179_a_at	11.052	5.662	-41.93	19684
MYADM	1439389_s_at	11.243	5.923	-39.97	50918
JUN	1448694_at	10.173	4.902	-38.592	16476
DNAJC5	1436180_at	8.740	3.514	-37.434	13002
POLDIP3	1437837_x_at	9.316	4.099	-37.199	73826
STT3A	1455824_x_at	9.655	4.532	-34.833	16430
HIST2H2BE	1418072_at	9.080	4.036	-32.986	68024|319181|319179
RTN3	1418101_a_at	8.214	3.188	-32.568	20168
H3F3A/H3F3B	1420376_a_at	10.656	5.656	-31.994	15078|15081
P4HB	1437465_a_at	8.708	3.759	-30.886	18453
SYPL1	1422881_s_at	10.284	5.350	-30.559	19027
CELF1	1426407_at	9.007	4.075	-30.529	13046
GOLGA7	1415672_at	7.995	3.106	-29.626	57437
TUBB2A	1427347_s_at	9.219	4.436	-27.531	22151
FAM174A	1419170_at	8.322	3.596	-26.472	67698
CDC42	1415724_a_at	8.400	3.676	-26.439	12540
RAB2A	1419945_s_at	8.354	3.689	-25.381	59021
VMA21	1434232_a_at	7.610	2.961	-25.09	67048
RAC1	1451086_s_at	8.062	3.423	-24.911	19353
DNAJB1	1416756_at	8.772	4.171	-24.265	81489
KIAA1191	1448100_at	9.010	4.417	-24.139	97820
PSMA5	1434356_a_at	9.600	5.014	-24.007	26442

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
TXNDC9	1436951_x_at	8.210	3.632	-23.878	98258
CMTM6	1423792_a_at	10.940	6.402	-23.229	67213
SNX3	1422480_at	8.818	4.283	-23.19	54198
PDLIM5	1427475_a_at	8.227	3.825	-21.145	56376
TMEM41B	1437402_x_at	7.602	3.230	-20.705	233724
GNA13	1433749_at	7.402	3.061	-20.267	14674
ARPC5	1448129_at	8.694	4.375	-19.964	67771
PPP3CB	1433835_at	6.973	2.693	-19.431	19056
TSN	1416908_s_at	8.367	4.110	-19.116	22099
PSAP	1415687_a_at	9.395	5.143	-19.057	19156
SPIN1	1436809_a_at	11.755	7.536	-18.619	20729
REEP3	1424781_at	7.842	3.658	-18.179	28193
ACVR2A	1437382_at	7.050	2.873	-18.085	11480
RNF2	1424873_at	7.900	3.746	-17.8	19821
IER5	1417612_at	8.630	4.490	-17.63	15939
TULP4	1448548_at	6.802	2.695	-17.232	68842
SLC44A1	1433645_at	8.637	4.579	-16.653	100434
PPP6C	1424347_at	6.420	2.368	-16.577	67857
CRYZ	1438610_a_at	7.101	3.126	-15.718	12972
TOR1AIP2	1435526_at	7.161	3.200	-15.574	240832
LAMP2	1428094_at	9.808	5.891	-15.1	16784
DRAM2	1424782_at	7.601	3.695	-14.989	67171
COA5	1426355_a_at	7.528	3.638	-14.825	76178
CITED2	1452207_at	7.380	3.524	-14.483	17684
RBBP4	1434892_x_at	10.131	6.279	-14.44	19646
HNRNPK	1460547_a_at	12.503	8.660	-14.352	15387
SPCS2	1450907_at	7.425	3.583	-14.333	66624
TM9SF3	1416509_at	10.161	6.325	-14.279	107358
ZC3H14	1426999_at	6.935	3.113	-14.139	75553
ACTR10	1417157_at	8.384	4.580	-13.969	56444
ZZZ3	1438487_s_at	7.081	3.279	-13.951	108946
MYEOV2	1434823_x_at	8.495	4.717	-13.712	66915
SNX6	1447234_s_at	8.770	5.046	-13.214	72183
ARID5B	1434283_at	8.526	4.815	-13.094	71371
SPRED2	1434403_at	7.391	3.687	-13.036	114716
COMMD3-BMI1	1448733_at	7.281	3.586	-12.947	12151
RBM39	1438420_at	7.861	4.175	-12.874	170791
URI1	1433913_at	7.174	3.507	-12.704	19777
SRSF3	1416150_a_at	9.768	6.170	-12.101	20383

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
SLC35A3	1429648_at	6.876	3.285	-12.056	229782
1810037I17Rik/Gm2036	1424365_at	7.871	4.279	-12.052	100039078|67704
Hmgn1	1422495_a_at	8.981	5.392	-12.029	15312
BGN	1416405_at	7.103	3.525	-11.943	12111
TMEM167A	1425780_a_at	6.675	3.157	-11.456	66074
TRAM1	1423732_at	8.627	5.124	-11.339	72265
TIMP3	1449335_at	6.542	3.052	-11.235	21859
NEDD9	1447885_x_at	5.923	2.478	-10.89	18003
EIF4EBP1	1434976_x_at	7.986	4.543	-10.87	13685
ATP1B3	1423126_at	7.742	4.315	-10.76	11933
MYLIP	1424988_at	7.649	4.260	-10.478	218203
RBM3	1422660_at	8.889	5.504	-10.441	19652
Ly6a	1417185_at	11.398	8.038	-10.268	57248|546644|110454|
TTC33	1423958_a_at	6.083	2.766	-9.966	67515
SOCS5	1423349_at	6.247	2.952	-9.814	56468
ITPRIPL2	1435777_at	8.893	5.622	-9.658	319622
PJA2	1434383_at	6.567	3.295	-9.656	224938
PAK2	1454887_at	6.903	3.644	-9.574	224105
DDX5	1423645_a_at	9.446	6.226	-9.317	13207
YWHAH	1416004_at	6.753	3.561	-9.136	22629
SERINC1	1448108_at	9.687	6.508	-9.056	56442
PANK3	1433613_at	5.960	2.782	-9.054	211347
SUMO3	1422457_s_at	7.502	4.329	-9.017	20610
CANX	1415692_s_at	9.345	6.197	-8.861	12330
PABPN1	1422848_a_at	8.119	4.980	-8.805	54196
AMOT	1454890_at	6.137	3.007	-8.751	27494
MORF4L2	1415778_at	7.382	4.273	-8.626	56397
HOXA9	1455626_at	7.781	4.673	-8.619	15405
AP3S1	1422593_at	6.462	3.369	-8.532	11777
SMIM7	1426646_at	5.733	2.642	-8.522	66818
RBMS1	1434005_at	6.872	3.792	-8.453	56878
RAB11B	1435253_at	6.223	3.147	-8.43	19326
ARL5B	1455166_at	6.757	3.698	-8.333	75869
PRKAR1A	1452032_at	7.643	4.594	-8.281	19084
INPP5A	1433605_at	5.794	2.781	-8.068	212111
SCAMP1	1426775_s_at	5.818	2.843	-7.86	107767
UBAP2L	1454643_at	6.240	3.360	-7.357	74383
PTCH1	1428853_at	7.248	4.410	-7.151	19206

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
SLC35B1	1448769_at	5.422	2.603	-7.054	110172
ATXN7	1436175_at	6.702	3.907	-6.938	246103
GAS1	1416855_at	6.123	3.345	-6.856	14451
HNRNPDL	1428224_at	6.143	3.370	-6.838	50926
PDZD8	1435440_at	5.193	2.424	-6.815	107368
SHISA5	1437503_a_at	10.735	7.989	-6.711	66940
GNB1	1454696_at	6.158	3.419	-6.676	14688
RAB5C	1424684_at	5.957	3.219	-6.669	19345
ATXN7L3B	1428125_at	7.114	4.388	-6.613	382423
TWF1	1420875_at	6.293	3.577	-6.569	19230
SLC10A7	1436302_at	5.366	2.712	-6.298	76775
ATF2	1426583_at	6.394	3.774	-6.146	11909
FAM91A1	1454705_at	5.800	3.233	-5.926	210998
C9orf85	1416841_at	6.494	4.003	-5.619	66206
Cbx3	1416884_at	7.313	4.846	-5.528	12417
TWSG1	1450388_s_at	5.939	3.482	-5.491	65960
B4GALT1	1418014_a_at	5.640	3.186	-5.477	14595
CRIP2	1417311_at	5.154	2.758	-5.261	68337
SLC30A4	1418843_at	6.075	3.710	-5.151	22785
COQ10B	1460510_a_at	5.287	2.942	-5.081	67876
FBXL14	1417407_at	5.490	3.188	-4.931	101358
ARHGDIA	1451168_a_at	5.306	3.009	-4.912	192662
MAT2B	1448196_at	5.625	3.352	-4.834	108645
LRRC8A	1434694_at	7.352	5.102	-4.758	241296
DPYSL3	1454613_at	5.269	3.032	-4.716	22240
TMEM55B	1454797_at	4.826	2.610	-4.644	219024
ATP8B2	1434026_at	4.865	2.689	-4.521	54667
ANGPTL2	1455090_at	6.002	3.827	-4.515	26360
OSBP	1460350_at	4.648	2.476	-4.507	76303
NUP93	1424291_at	5.213	3.057	-4.456	71805
F2R	1450852_s_at	6.357	4.253	-4.298	14062
SMARCAD1	1452276_at	5.411	3.319	-4.266	13990
LAMTOR1	1434124_x_at	6.520	4.450	-4.197	66508
FBXW11	1425462_at	7.296	5.227	-4.196	103583
CDON	1434957_at	4.568	2.511	-4.162	57810
EIF1B	1428272_at	7.426	5.372	-4.153	68969
LDLRAD3	1438666_at	5.824	3.784	-4.112	241576
PTPN9	1451037_at	4.919	2.894	-4.07	56294
SMAD7	1443771_x_at	6.057	4.033	-4.068	17131

Gene Symbol	Affymetrix	WT UI	WT 12hpi	Fold Change	Entrez Gene ID
CHTOP	1423408_a_at	7.786	5.866	-3.784	66511
SIDT2	1426940_at	5.376	3.461	-3.772	214597
ATXN10	1456466_x_at	4.783	2.891	-3.711	54138
SRP68	1433708_at	4.675	2.860	-3.519	217337
MIDN	1449188_at	6.786	5.133	-3.145	59090
GOSR2	1419371_s_at	7.730	6.115	-3.063	56494
CXXC5	1448960_at	4.195	2.647	-2.923	67393
UBE2V2	1429131_at	4.477	2.939	-2.903	70620
KCTD9	1418399_at	5.152	3.624	-2.884	105440
TMEM98	1424133_at	4.116	2.605	-2.85	103743
AMOTL2	1452387_a_at	5.500	4.044	-2.744	56332
COX8A	1448222_x_at	5.038	3.632	-2.65	12868
PARP11	1434139_at	5.313	3.939	-2.592	101187
CCSER2	1452223_s_at	5.133	3.862	-2.412	72972
GDI1	1451070_at	5.570	4.406	-2.241	14567

Table 4 CELF1/2/4 targets downregulated during activation. CELF1/2/4 targets identified by RIP-Chip in C2C12 myoblasts (Lee, et. al., 2012) were compared to the satellite cell activation dataset. 165 of 717 (23%) detectable CELF1/2/4 targets are downregulated upon activation and are listed here by gene symbol. Affymetrix refers to the associated Affymetrix probeset from the activation dataset. Relative expression levels for wild type (WT) satellite cells are listed for freshly isolated (uninjured, UI) and 12h post-injury (12hpi) time points in log2 along with expression fold change from UI to 12hpi. Entrez gene IDs are listed for their corresponding genes.

I next asked if CELF1/2/4 target transcripts that change expression during activation were enriched for CELF binding sites. CELF proteins bind to GU-rich regions (GREs) and GU-repeats (Faustino et al. 2005; Lee et al. 2010; Marquis et al. 2006; Rattenbacher et al. 2010; Vlasova et al. 2008). GREs and GU-repeats are enriched in the 3'UTRs of short-lived transcripts in HeLa cells and C2C12 myoblasts and are stabilized upon CELF1 knockdown (Rattenbacher et al. 2010; Vlasova et al. 2008; Lee et al. 2010). I searched the 3'UTRs of each transcript represented in the activation dataset for the GRE (UGUUUGUUUGU) and GU-repeat (UGUGUGUGUGU) 11mers. One third of genes decreasing expression during activation contain at least one of these

motifs. This is a significant enrichment over the mouse genome (mm9 build, 22.4%, *P* value = 9.99 x10^{-16}), suggesting that promoting mRNA decay may be a major mechanism of action for CELF1 during activation.

CELF1/2/4 targets downregulated during satellite cell activation are enriched for microRNA regulators of satellite cell fate

To further investigate the role of CELF1/2/4 targets in satellite cell activation, I identified enriched biological networks. CELF1/2/4 targets decreasing expression during satellite cell activation were uploaded to the Broad Institute's Gene Set Enrichment Analysis (GSEA), ToppFun, and Ingenuity Pathway Analysis (IPA). These genes are enriched for putative miR-1/206, miR-124, miR-16 and miR-200B targets (Table 5). All of these microRNAs change expression during satellite cell activation (Farina et al. 2012; Farina Thesis 2011). We have recently demonstrated that miR-124, miR-16 and miR-206 regulate the satellite cell fate decision between proliferation and self-renewal (Farina et al. 2012; Farina Thesis 2011). These observations suggests that satellite cell fate is post-transcriptionally regulated and involves the coordination of both microRNAs and RNA binding proteins.

microRNA & Seed Sequence	GSEA Rank	GSEA p-value	ToppFun Rank	ToppFun p-value
TGCCTTA,MIR-124A	1	1.59E-12	45	6.46E-07
CAGTATT,MIR-200B,MIR-200C,MIR-429	2	1.61E-12	37	3.04E-07
GCATTTG,MIR-105	3	8.52E-12	6	9.44E-09
ACATTCC,MIR-1,MIR-206	5	1.81E-10	1	3.09E-11
CTACTGT,MIR-199A	9	1.09E-07	30	2.31E-07
TTGGGAG,MIR-150	11	3.78E-07	43	4.41E-07
TGCTGCT,MIR-15A,MIR-16,MIR-15B,MIR-195,MIR-424,MIR-497	12	3.87E-07	7	2.02E-08
CAGTGTT,MIR-141,MIR-200A	15	5.53E-07	49	1.01E-06
ATGCAGT,MIR-217	18	1.60E-06	52	1.49E-06
ATGTAGC,MIR-221,MIR-222	23	0.0429	29	2.27E-07

Table 5 CELF1/2/4 targets downregulated during satellite cell activation are enriched for microRNA regulators of satellite cell fate. CELF1/2/4 targets downregulated during satellite cell activation were analyzed for microRNA target enrichment through GSEA and ToppFun. The top ten enriched targeting microRNAs that overlap between both algorithyms are listed along with their seed sequence, ranks, and p-values.

CELF1/2/4 targets downregulated during satellite cell activation are enriched for TGF-β and p38 MAPK signaling pathways

CELF1/2/4 targets decreasing expression during activation are enriched for two pathways known to regulate satellite cell function: TGF-β (P-value = 1.08×10^{-3}, ToppFun) and p38 MAPK (P-value = 3.66×10^{-4}, ToppFun) (Table 6). The TGF-β pathway inhibits both proliferation and differentiation in satellite cells through SMAD-mediated induction of cell cycle inhibitors and myogenic regulatory factors (Allen and Boxhorn 1987, 1989; Hsu et al. 2011; Li, McFarland, and Velleman 2008; Liu, Black, and Derynck 2001; Ono et al. 2011). Reduction of TGF-β signaling during activation is likely required for cell cycle entry (Carlson, Hsu, and Conboy 2008). Here, the CELF1/2/4 target *Smad7* decreases expression during activation. Although SMAD7 expression is elevated in satellite cells from chickens with muscle weakness, impaired satellite cell proliferation and differentiation, the role of SMAD7 in satellite cells remains unclear (Li, McFarland, and Velleman 2008).

Pathway	P-value	Database
CDC42 signaling events	2.31E-06	BioSystems: Pathway Interaction Database
RAC1 signaling pathway	6.99E-06	BioSystems: Pathway Interaction Database
mTOR signaling pathway	1.29E-05	BioSystems: Pathway Interaction Database
ErbB1 downstream signaling	2.76E-05	BioSystems: Pathway Interaction Database
p38 MAPK Signaling Pathway	1.93E-04	BioSystems: WikiPathways
Signaling mediated by p38-alpha and p38-beta	2.17E-04	BioSystems: Pathway Interaction Database
Regulation of Actin Cytoskeleton	2.61E-04	BioSystems: WikiPathways
Ras Pathway	2.84E-04	PantherDB
Cytoskeletal regulation by Rho GTPase	3.04E-04	PantherDB
Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met)	3.71E-04	BioSystems: Pathway Interaction Database
RhoA signaling pathway	4.03E-04	BioSystems: Pathway Interaction Database
T cell activation	4.76E-04	PantherDB
Hedgehog signaling events mediated by Gli proteins	4.85E-04	BioSystems: Pathway Interaction Database
Hedgehog signaling pathway	5.36E-04	PantherDB
Huntington disease	6.43E-04	PantherDB
Signaling events mediated by the Hedgehog family	8.36E-04	BioSystems: Pathway Interaction Database
Parkinson disease	8.85E-04	PantherDB
TGF-beta signaling pathway	1.08E-03	PantherDB
S1P2 pathway	1.09E-03	BioSystems: Pathway Interaction Database
Physiological and Pathological Hypertrophy of the Heart	1.09E-03	BioSystems: WikiPathways

Table 6 Enriched Pathways from CELF1/2/4 targets decreasing expression during satellite cell activation. CELF1/2/4 targets downregulated during satellite cell activation were analyzed for pathway enrichment through ToppFun. The top twenty enriched pathways are listed along with p-values and the curation databases.

p38 α/β MAPK signaling is required for proliferation, differentiation, asymmetric division, and self-renewal of satellite cells (Bernet et al. 2014; Jones et al. 2005; Lovett et al. 2010; Shi et al. 2010). Six members of the p38 MAPK pathway are downregulated during activation (Figure 6) and four are CELF1/2/4 targets, including *Rac1* and *Cdc42* (Figure 6, Table 7). These genes have roles in regulating myoblast proliferation, motility and differentiation (Bryan et al. 2005; Fiaschi et al. 2012; Han et al. 2011; H. Liu et al. 2011; Loh et al. 2012; Meriane et al. 2000). Additionally, CELF1/2/4 targets *Atf*2, a member of both the TGF-β and p38 MAPK pathways (Figure 6, Table 7). ATF-2 forms a complex with SMAD3 and SMAD4 in C2C12 myoblasts upon p38 phosphorylation, correlating with reduced proliferation and increased p21 expression (Blank et al. 2009; Dziembowska et al. 2007; Hanafusa et al. 1999;

Philip et al. 2005; Sano et al. 1999). Taken together, CELF1/2/4 targeting of both the TGF-β and p38 MAPK pathways places the CELF proteins in signaling networks that regulate satellite cell fate decisions.

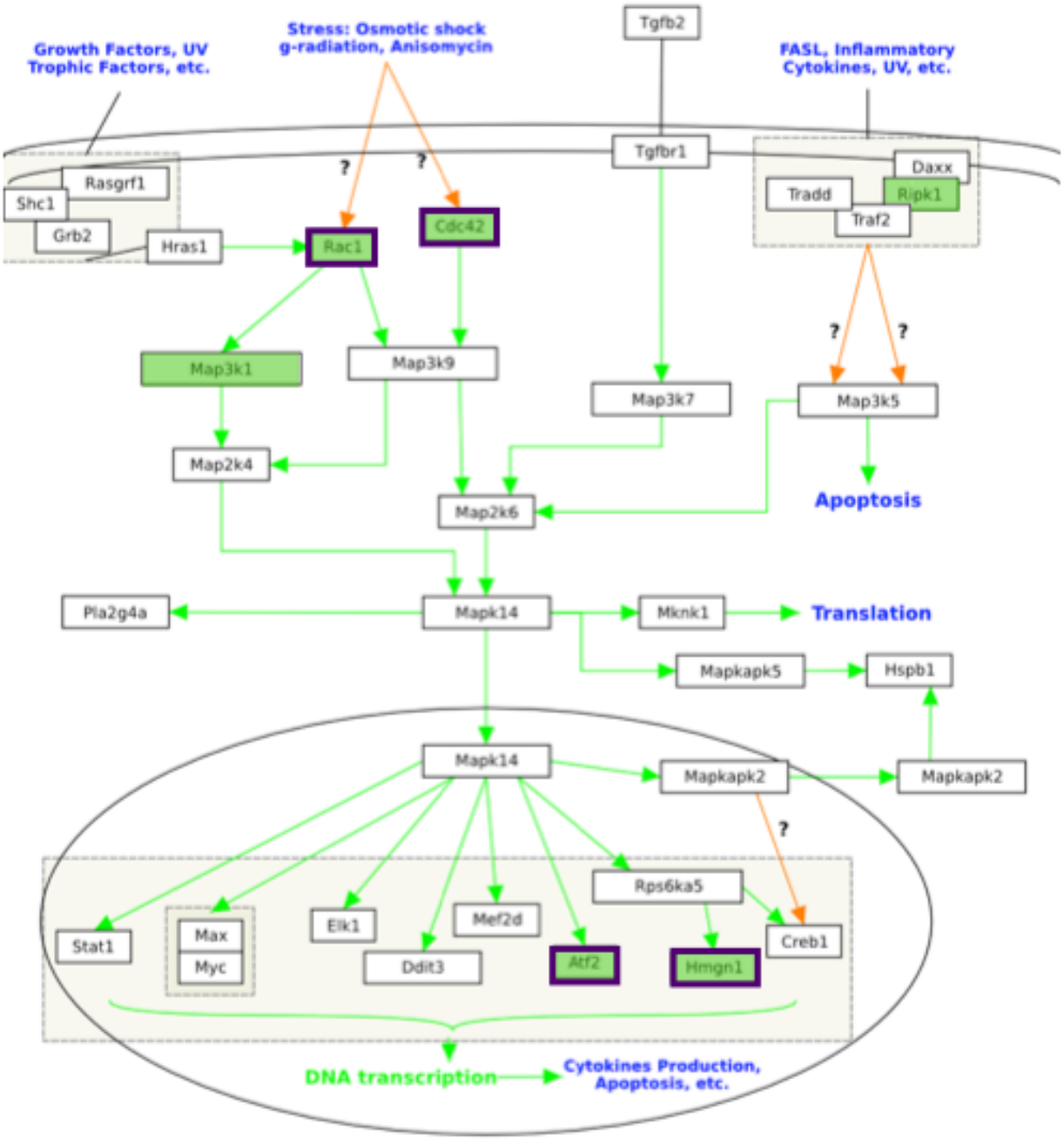


Figure 6 CELF1/2/4 targets the p38 MAPK pathway during satellite cell activation. CELF1/2/4 targets regulated during satellie cell activation are enriched for the p38 MAPK pathway. Six members of the p38 MAPK pathway are downregulated during satellite cell activation (green shading). CELF1/2/4 targets four p38 MAPK pathway members downregulated during satellite cell activation: Rac1, Cdc42, Aft2, and Hmgn1 (purple boxes).

p38 MAPK pathway	TGF-β pathway
Cdc42	Atf2
Hmgn1	JUN
Atf2	SMAD7
Rac1	CDC42
	ACVR2A

Table 7 CELF1/2/4 targets downregulated during satellite cell activation in the p38 MAPK and TGF-β signaling pathways.

Celf1, 2 and 4 expression correlates with self-renewal in multiple cell types

The CELF family's role in stem cell self-renewal has not been studied; however, the *Celf* homologues *bru* and *elav* in *Drosophilia* and *Smed-bruli* in the planarian *S. mediterranea*, regulate self-renewal and cell polarity in germline stem cells, neruoblasts, and neoblasts, respectively (Guo, Peters, and Newmark 2006; Lai et al. 2012; Xin et al. 2013). I next asked whether mammalian *Celf1*, *2* or *4* expression correlates with changes in self-renewal capacity. I performed a Gene Expression Omnibus (GEO) Profile search and examined *CELF1, 2* and *4* transcript expression changes in nine microarrays assaying self-renewal phenotypes in various cell types (Table 8, Table 9). I found that *Celf4* expression positively correlated, while *Celf2* expression inversely correlated with self-renewal capacity (Table 8, Table 9). Furthermore, *Celf1* expression frequently changed with self-renewal but the direction of change varied, indicating that cellular context influences the role of *Celf1* in self-renewal. Together, this suggests that CELF1, 2 and 4 may regulate satellite cell self-renewal.

Increasing Self Renewal	Change in CELF Expression	Cell Type	Alteration	Technology	GEO DataSet ID	Relevant PMID
CELF1	Mixed - Decrease	bone marrow progenitors	Tet2 KO	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS4287	21723200
CELF2	Mixed					
CELF4	Mixed - Decrease					
CELF1	Increase	memory B cells	Immunization with NP-CGG	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS1695	16492737
CELF2	Increase					
CELF4	Mixed - Increase					
CELF1	Decrease	granulocyte-macrophage progenitors	acute myelogenous leukemia stem cells induced by oncoprotein MLL-AF9 expression	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS3839	20339075
CELF2	Decrease					
CELF4	Increase					
CELF1	Mixed	neuronal stem cells	PlagL2-transduced, p53-null	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS3767	20478531
CELF2	Decrease					
CELF4	Increase					
CELF1	Increase	embryonic fibroblasts	FGF2 treatment	Sentrix Mouse-6 Expression BeadChip	GDS2421	17038665
CELF2	Decrease					
CELF4	Increase					
Decreasing Self Renewal	**Change in CELF Expression**	**Cell Type**	**Alteration**	**Technology**	**GEO DataSet ID**	**Relevant PMID**
CELF1	no change	embryonic stem cell	Zfx KO	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS2718	17448993
CELF2	not detected					
CELF4	Decrease					
CELF1	Mixed - Increase	embryonic stem cell	Oct4 KD	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS1824	16518401
CELF2	Increase					
CELF4	Decrease					
CELF1	Mixed	embryonic stem cell	Nanog KD	[Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array	GDS1824	16518401
CELF2	Increase					
CELF4	Decreased or no change					
CELF1	Decrease	myoblasts	Aged	[MG_U74Av2] Affymetrix Murine Genome U74A Version 2 Array	GDC1079	15569352
CELF2	Decrease					
CELF4	Increase					

Table 8 Expression changes in CELF1, 2, and 4 during modification of self-renewal in various cell types. Results of a Gene Expression Omnibus (GEO) Profile search for Celf1, 2, and 4 in experiments assaying self-renewal phenotypes in various cell types.

	Change with Increasing Self Renewal	Change with Decreasing Self Renewal
CELF1	Mixed	Mixed
CELF2	Decreasing (3/5)	Increasing (2/4)
CELF4	Increasing (4/5)	Decreasing (3/4)

Table 9 Celf1, 2 and 4 expression correlates with self-renewal in multiple cell types.
Summary of the data presented in Table 7.

Dysregulated expression of p38 MAPK pathway members in aged satellite cells

Recently, we showed that the loss of asymmetric division and self-renewal in aged satellite cells is due to elevated p38 MAPK signaling (Bernet et al. 2014). I next asked if the p38 MAPK pathway is dysregulated at the transcript level in aged satellite cells. To identify genes differentially expressed with age, we performed an unbiased genome-wide expression analysis between adult and aged satellite cells. RNA from FACS-isolated adult (3-6 months) and old (20-25 months) satellite cells was collected and processed for microarray hybridization similarly to the activation experiment previously described (Bernet et al. 2014; Farina et al. 2012). To make my analysis more robust, I performed an identical analysis on another experimental dataset comparing genome-wide expression of satellite cells from adult (5-6 months), aged (20-24 months), and geriatric (28-32 months) mice (Sousa-Victor et al. 2014). Our old satellite cell dataset clustered more closely with the geriatric dataset than did the other aged dataset (data not shown); therefore, I used the geriatric dataset in our analysis.

The p38 MAPK pathway is significantly enriched among transcripts changing expression with age (Table 10). In our dataset, seven pathway members increase expression (*P* value = 6.5 $x10^{-3}$, Table 10), and ten decrease expression (*P* value = 3.7 $x10^{-3}$, Table 11), while in the

geriatric dataset, twelve p38 MAPK pathway transcripts increase expression (*P* value = 3.1 x 10^{-4}, Table 11) and six decrease expression with age (*P* value = 4.3 x 10^{-2}, Table 11). Furthermore, four of the twelve transcripts with elevated expression in aged satellite cells from the geriatric dataset decrease expression during adult activation (*P* value = 7.0 x10^{-3}, Table 11). This suggests that missexpression of multiple p38 MAPK pathway members is responsible for dysregulated p38 MAPK signaling in aged satellite cells.

Pathway	Total # Genes in Pathway	# Genes Changing Expression in Old Dataset	P value for Old Dataset	# Genes Changing Expression in Geriatric Dataset	P value for Geriatric Dataset
TNF-alpha/NF-kB Signaling Pathway	184	45	3.06E-10	65	5.45E-10
mRNA processing	551	109	1.08E-09	176	5.58E-10
TGF-beta Receptor Signaling Pathway	150	36	8.64E-09	52	4.29E-10
Delta-Notch Signaling Pathway	84	17	5.27E-04	27	8.28E-06
MAPK signaling pathway	159	24	3.49E-03	37	6.69E-04
p38 MAPK Signaling Pathway	34	7	6.52E-03	12	3.07E-04
Toll Like Receptor signaling	33	7	1.60E-02	19	5.33E-02
Wnt Signaling Pathway	109	16	1.99E-02	25	5.60E-03
MAPK Cascade	29	6	3.23E-02	10	3.67E-03
Notch Signaling Pathway	47	8	3.39E-02	17	6.29E-05

Table 10 Pathways elevated in aged satellite cells. List of top ten Wiki pathways enriched in genes upregulated from adult to old and adult to geriatric satellite cells.

Increasing with Age - Old Dataset	Decreasing with Age - Old Dataset	Increasing with Age - Geriatric Dataset	Decreasing with Age - Geriatric Dataset
Creb1	Elk1	Atf2	Elk1
Map3k1	Map2k4	Cdc42	Map3k5
Map3k9	Map2k6	Hmgn1	Map3k9
Myc	Map3k7	Map3k7	Pla2g4a
rRpk1	Mapk14	Mapk14	Rasgrf1
Stat1	Mapkapk5	Mapkapk5	Stat1
Tradd	Mef2d	Mef2d	
	Rps6ka5	Myc	
	Shc1	Rac1	
	Tgfb2	Tgfb2	
		Tradd	
		Traf2	

Table 11 p38 MAPK Pathway Members Change Expression with Age. List of genes in the p38 MAPK pathway that are up- or downregulated with age.

CELF1/2/4 targets p38 MAPK pathway members that are dysregulated with age

The *Celf1, 2 and 4* transcripts are dysregulated with age, as well as their p38 MAPK pathway targets. *Celf1* decreases expression with age in our dataset but increases expression in the geriatric dataset; whereas, *Celf2* and *Celf4* decrease expression with age in both datasets. Additionally, comparison of the CELF1/2/4 targets identified by Lee, *et. al*, 2010 with both databases revealed a significant overlap between CELF1/2/4 targets and transcripts significantly increasing expression with age (52 CELF targets out of 505 transcripts increasing, Table 12 , *P* value = 2.28×10^{-4}). While CELF1/2/4 only targets two members of the p38 MAPK pathway that decrease with age in the old dataset, half of the p38 MAPK pathways members increasing expression in the geriatric dataset are CELF1/2/4 targets, four of which decrease expression during satellite cell activation (Table 13). Together, this suggests a role for the CELF family in regulating satellite cell activation through p38 MAPK signaling.

CELF1/2/4 Targets Increasing Expression with Age					
Adrb2	Gna13	Pik3r1	Senp1	Tmed10	Ywhaz
Arhgap29	Hif1a	Prpf18	Shisa5	Tmed2	Zwint
Atp6v0b	Laptm4b	Psma5	Slc25a44	Tsn	
Atp6v1g1	Lnpep	Qtrtd1	Slc44a1	Tubb5	
Calr	Lsm6	Rbbp4	Smad7	Twsg1	
Ciapin1	Mapre1	Rbm39	Snhg1	Ugcg	
Cxxc5	Mboat1	Rnf2	Spin1	Wdr1	
Dad1	Pabpn1	Rnf219	Stambp	Wdr70	
Elavl1	Pcgf5	Rrp1b	Sypl	Xiap	
F2r	Pgam1	Safb	Tcf7l2	Ywhab	

Table 12 CELF1/2/4 targets increase expression with age. List of 52 CELF1/2/4 targets that increase expression with age in both the old and geriatric satellite cell datasets. A total of 505 transcripts are upregulated with age in both datasets.

Increasing with Age - Geriatric Dataset	Increasing with Age & Decreasing During Activation - Geriatric Dataset	Decreasing with Age - Old Dataset
Atf2	Atf2	Mapk14
Cdc42	Cdc42	Mef2d
Hmgn1	Hmgn1	
Mapk14	Rac1	
Mef2d		
Rac1		

Table 13 CELF1/2/4 targets in the p38 MAPK pathway are regulated during age and activation. List of CELF1/2/4 targets that increase expression with age (geriatric dataset, left column), increase expression with age and decrease expression during activation (geriatric dataset, middle column), and decrease expression with age (old dataset, right column).

miR-133 may regulate Celf4 expression in quiescent satellite cells

Next, I searched for potential regulators of the CELF family. Recently, several miRNAs expressed in quiescent, but not activated or proliferating, satellite cells with roles in satellite cell fate decision were identified (Farina et al. 2012, Farina Thesis 2011, Zhang et al. 2012). I identified binding sites for miRNA-107, -200bc, and -93 in the *Celf2* 3'UTR and binding sites

for miRNA-133 in the *Celf4* 3'UTR. The predicted miRNA binding sites within the *Celf2* 3'UTR had low probability of conserved targeting (P_{CT}) and poor context+ scores (likelihood of miRNA binding to the predicted binding site as influenced by the surrounding nucleotides, Garcia et al. 2011). Additionally, *Celf2* and miRNA-107, -200bc, and -93 have similar expression patterns during activation, making these microRNAs unlikely regulators of *Celf2* in this context. In contrast, the two miR-133 binding sites within the *Celf4* 3'UTR are highly conserved among metazoans, have high probability of conserved targeting, and are in the top five predicted targets by two independent algorithms, making *Celf4* a likely target of miR-133 (Figure 7, Table 14). Furthermore, miR-133 and *Celf4* have opposite expression changes during satellite cell activation. miR-133 is only detectable during quiescence, when *Celf4* transcript levels are low; upon activation, miR-133 is rapidly downregulated while *Celf4* expression increases (Zhang et al. 2012, Farina et al. 2012, Farina Thesis 2011). This suggests that miR-133 inhibits the *Celf4* transcript in quiescent satellite cells and that, upon activation, miR-133 downregulation releases *Celf4* inhibition, allowing CELF4 to interact with members of the p38 MAPK pathway, promoting the transition to a cycling myoblast.

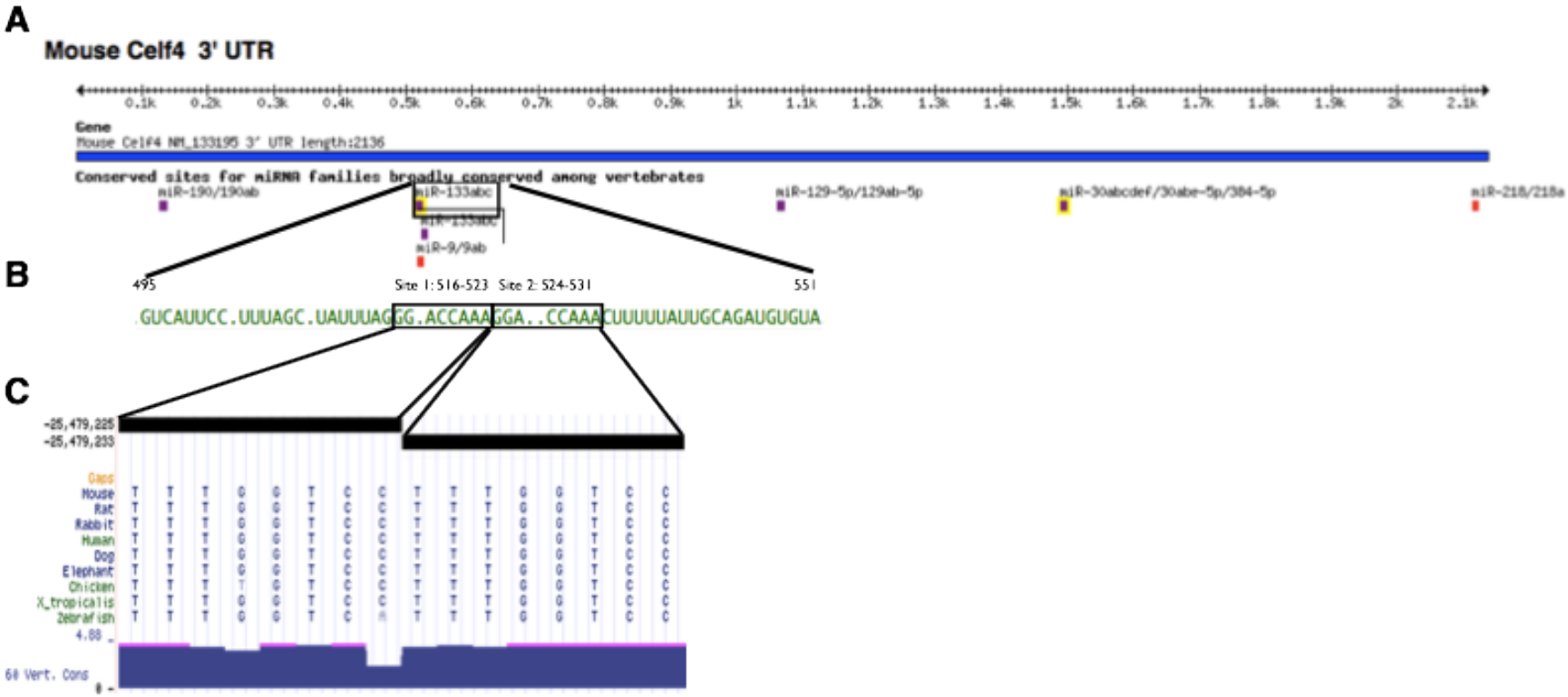


Figure 7 Celf4 3'UTR contains two highly conserved microRNA-133 binding sites. (A) Screenshot of the Celf4 3'UTR and predicted microRNA binding sites taken from TargetScan. (B) Sequence of the two predicted microRNA-133 binding sites and surrounding nucleotides. Nucleotide location within the 3'UTR is indicated for the start and end of the enlarged sequence as well as above the predicted microRNA-133 target sites (C) 60 vertebrate basewise conservation by phastCons and phylop (UCSC/Penn State Bioinformatics comparative genomics alignment pipeline) shows high conservation of both predicted microRNA-133 binding sites among vertebrates. Sequences are shown for select species. Overall conservation is indicated by height of the blue bar beneath each nucleotide (bottom).

miRanda Rank	Target Gene	# Binding Sites	Total Context Score	Aggregate PCT	mirSVR Score
1	4930519F24Rik	2	N/A	N/A	-4.45
2	**Celf4**	2	-0.63	0.95	-4.39
3	**Lhfp**	2	-0.62	0.97	-4.07
4	Ifi44	2	N/A	N/A	-3.46
5	Zfp874	3	N/A	N/A	-3.45
6	Acat3	3	-0.37	0.87	-3.42
7	Enc1	2	-0.5	0.43	-3.18
8	Rdh12	2	N/A	N/A	-3.11
9	**Slc6a1**	3	-0.67	0.9	-3.04
10	Sec14l2	2	N/A	N/A	-2.98
TargetScan Rank	**Target Gene**	**# Binding Sites**	**Total Context Score**	**Aggregate PCT**	**mirSVR Score**
1	Gabpb2	5	-0.82	0.88	N/A
2	**Slc6a1**	3	-0.67	0.9	-3.012
3	Edem1	3	-0.65	0.91	-2.3298
4	**Celf4**	2	-0.63	0.95	-4.39
5	**Lhfp**	2	-0.62	0.97	-4.069
6	Kndc1	3	-0.6	< 0.1	-1.5674
7	Fgf12	3	-0.58	0.59	-2.2232
8	Tspan15	2	-0.56	0.61	-1.0568
9	Smarcd1	1	-0.54	0.89	-2.4824
10	Maml1	3	-0.54	0.97	-1.7348

Table 14 Celf4 is a predicted microRNA-133 target. Celf4 is within the top 10 predicted microRNA-133 targets by two independent algorithms: miRanda and TargetScan. Targets are ordered by miRanda or TargetScan ranking. # Binding Sites indicates the quantity of predicted miR-133 target sites within the 3'UTR of the respective gene. Total Context Scores is the sum of context+ scores for each predicted miR-binding site and represent the likelihood of miRNA binding to the predicted binding site as influenced by the surrounding nucleotides (higher likelihood indicated by more negative number) (Grimson, 2007; García, 2011). Aggregate probability of conserved targeting (PCT) is the sum of PCT for each predicted miR-binding site and represents the likelihood that a miR-binding site is conserved due to selective maintenance of miRNA targeting rather than from random chance (higher likelihood at values nearing 1) (Friedman, 2009). The mirSVR Score is calculated from a machine-learning algorithm based decreased mRNA expression following microRNA transfections (more negative scores indicate a greater probability of miR targeting) (Betel, 2010). Targets that appear on both lists are in bold.

Discussion

Morphological characteristics of quiescent satellite cells including heterochromatin, sparse organelles and a low cytoplasm volume to nuclear volume ratio suggest limited metabolic and transcriptional activity (Hanzlíková et al. 1975; Lu et al. 1997; Ontell 1975; Sakai 1977; Schultz 1976). Indeed, many quiescent cells have reduced metabolic activity and transcription rates (Choder 1991; Arai, and Suda n.d.). However, recent studies have demonstrated that quiescent satellite cells are a heterogeneous population with varying levels of metabolic activity (Rocheteau et al. 2012; Rodgers et al. 2014). The increase in cell size, mobility and delay

through the initial cell cycle suggest that satellite cells would require transcriptional upregulation of an extensive group of genes. However, studies of serum-starved fibroblasts suggest that transcriptional upregulation alone fails to account for the increased expression of multiple cell cycle regulators, suggesting that post-transcriptional mechanisms are involved in the transition to a mitotically active fibroblast (Cortner and Farnham 1991; Bork et al. 1992). Similarly, others and we have hypothesized that satellite cells are not transcriptionally silent and that, instead, satellite cells actively maintain quiescence while awaiting signals to trigger cell cycle re-entry (Pallafacchina et al. 2010; Rodgers et al. 2014; Farina et al 2012).

In this study, my goal was to identify the broad molecular pathways regulating satellite cell activation. Through comparison of unbiased, genome-wide expression profiling of satellite cells from uninjured and injured muscle, I identified expression changes specific to activation. Supporting our hypothesis that satellite cells actively maintain quiescence, I found that the majority of transcripts expressed in satellite cells decrease steady-state level upon activation. Moreover, Gene Ontology analysis revealed enrichment for mRNA post-transcriptional regulation in the activation dataset. Many RNA binding proteins in the activation dataset have known roles in muscle function, disease and stem cells, supporting this approach.

To further investigate how mRNA post-transcriptional regulation is involved in satellite cell activation, I analyzed the RNA binding proteins in the activation dataset. Included among these RNA binding proteins are CELFs 1, 2 and 4. Dysregulation of CELF proteins contributes to DM1 pathogenesis and yet the roles of this family in regulating satellite cell function remain largely uninvestigated. Here, I show that transcripts decreasing expression during activation are enriched for CELF1/2/4 targets, including members of the p38 MAPK and TGF-β pathways. These pathways regulate multiple aspects of satellite cell function. Recently, we showed that

elevated p38 MAPK signaling is responsible for reduced proliferation and self-renewal in aged satellite cells (Bernet et al. 2014). Comparisons of the satellite cell activation dataset with gene-expression profiles of aged satellite cells revealed dysregulation of p38 MAPK transcripts targeted by CELF1/2/4. Additionally, the *Celf4* 3'UTR contains two highly conserved binding sties for miR-133, a miRNA that we recently discovered is essential for satellite cell self-renewal *in vivo.* Together, this places the CELF family in signaling pathways that regulate satellite cell function and suggests that CELF4 has a role in regulating the satellite cell fate decision between proliferation and self-renewal.

In conclusion, this research provides evidence for mRNA post-transcriptional regulation by RNA binding proteins as a general mechanism regulating satellite cell activation and the cell fate decision between proliferation and self-renewal.

Methods

Mice

Mice were housed in a pathogen-free facility at the University of Colorado at Boulder. The University of Colorado Institutional Animal Care and Use Committee (IACUC) approved all animal protocols. Male and female mice were sacrificed between 3-6 months of age. Wild type mice were C57BL/6J x DBA/2J (B6D2F1/J, Jackson Labs) and *syndecan-4-/-* and *syndecan-3-/-* mice carry homozygous deletion of *syndecan-4* or *syndecan-3,* respectively in the C57BL/6 background (Echtermeyer et al. 2001). Dr. Heikki Rauvala at the University of Helsinki donated the *syndecan-3-/-* mice. Mice were sacrificed by cervical dislocation prior to muscle harvest.

Microarray hybridization

Satellite cell RNA was isolated using the PicoPure RNA Isolation kit (Arcturus) followed

by two rounds of linear T7-based amplification (RiboAmp HA kit: Arcturus). The RNA equivalent of 5000 cells was hybridized to Affymetrix mouse 430v2 GeneChips (MOE430v2) according to manufacturers' instructions. GeneChips were scanned at the University of Colorado at Boulder on an Affymetrix GeneChip Scanner 3000 and spot intensities were recovered in the GeneChip Operating System (Affymetrix). The raw CEL data files for wild type and *Sdc4-/-* satellite cell injury time-course are in the GEO database as Series GSE38870.

Microarray Data Processing and Analysis

All analysis was performed using Spotfire™ DecisionSite 2 for Microarray Analysis or GeneSpring GX version 12.6 (Agilent Technologies). The MOE430v2 raw CEL data files were normalized using GC Robust Multi-array Analysis (GCRMA). One wild type freshly isolated satellite cell replicate consistently clustered with the wild type satellite cells 12hr post injury replicates (via hierarchical, Self-Organizing Map (SOM), k-means) indicating myogenic commitment and was removed from our analysis. The hierarchical cluster and associated dendrogram were generated using the $\log_2$-value for relative probe intensity using the Unweighted Pair Group Method with Arithmatic Mean (UPGMA) with Euclidean distance as the similarity measure. The significance between genotypes and time points was determined using the multifactor analysis of variance (ANOVA) with a false discovery rate (FDR) ≤ 0.05 and Bonferroni correction for multiple hypothesis testing. Fold change was calculated as absolute(difference)^2 where difference is the $\log_2$ difference between compared samples. Venn diagrams were generated in Spotfire™ using the list comparison function or by importing lists into Venny at (http://bioinfogp.cnb.csic.es/tools/venny, (Oliveros et al 2007). Graphs were created using GraphPad Prism version 6.0e.

Gene ontology and biological pathway analysis

Unique gene identifiers (gene symbol, entrez gene IDs, RefSeq IDs or Affymetrix probeset IDs) were uploaded to the Database for Annotation, Visualization and Integrated Discovery version 6.7 (DAVID, http://david.abcc.ncifcrf.gov, (Huang et al. 2009), the Broad Institute's Gene Set Enrichment Analysis Molecular Signatures Database version 4.0 (GSEA, http://www.broadinstitute.org/gsea/msigdb/index.jsp, (Subramanian et al. 2005), the Cincinnati's Children's Hospital Medical Center's functional enrichment tool ToppFun (https://toppgene.cchmc.org/enrichment.jsp, (Chen et al. 2007), Profiling of Complex Functionality (ProfCom) (http://webclu.bio.wzw.tum.de/profcom/index.php, (Antonov et al. 2008), Ingenuity Pathway Analysis (IPA, Agilent Technologies), and GeneSpring Pathway Analysis (Agilent Technologies). The mouse genome (mm9 build) reference database for each algorithm was used as background for satellite cell activation analysis. For identification of enriched Gene Ontology (GO) terms and biological pathways of comparisons between microarrays (activation vs. aged satellite cells, CELF targets vs. activation &/or aged satellite cells), overlapping gene identifiers detectable between both arrays were used as background. The default settings for each algorithm were used to identify enriched GO terms, gene sets, and biological pathways. Pathway maps were created through or modified from GeneSpring Pathway Analysis, KEGG Pathway Database (http://www.kegg.jp/kegg/pathway.html, (Kanehisa et al. 2000) or WikiPathways (http://wikipathways.org, (Kelder et al. 2011).

Identification of RNA-binding protein target enrichment during satellite cell activation

Published microarray data for CELF (Lee et al. 2010) and TTP (Wi et al. 2006) was obtained through NCBI's GEO database (http://www.ncbi.nlm.nih.gov/geo/). Hypergeometric

distribution was used to determine enrichment of RNA-binding protein targets in the satellite cell activation dataset. Overlap of total detectable unique gene IDs for Affymetrix mouse 430v2 GeneChips (MOE430v2) (satellite cell microarray) and Affymetrix mouse gene 1.0 ST arrays (CELF array, Lee et al. 2010) or Affymetrix mouse 430v2 GeneChips (MOE430v2)

GEO Datasets for adult and aged satellite cell expression analysis

Raw CEL data files for published geriatric, old and adult satellite cell microarrays Series GSE47104 (Bernet et al. 2014), Series GSE53728 (Sousa-Victor et al. 2014) were downloaded from NCBI's GEO database (http://www.ncbi.nlm.nih.gov/geo/). The raw CEL data files for wild type and *Sdc4-/-* satellite cell injury time-course are in the GEO database as Series GSE38870. Hypergeometric distribution was used to determine enrichment of satellite cell activation genes and RNA-binding protein targets in aged satellite cell datasets. Overlap of total detectable unique gene IDs for Affymetrix mouse 430v2 GeneChips (MOE430v2) (GSE47104 and GSE38870 microarrays) and Agilent-028005 SurePrint G3 Mouse GE 8x60K Microarray (GSE53728 microarrays) were used as the total populations for their respective calculations. RNA-binding protein (CELF or TTP) targets or satellite cell activation genes detectable by both arrays were used to define samples and successes.

RNA isolation for RT-PCR

mRNA was extracted from satellite cells using the RNeasy Kit according to the manufacturers protocol (Qiagen). miRNA was extracted using mirVana miRNA isolation kit according to the manufacturers protocols (Ambion) RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Quantitative RT-PCR

The Superscript III First Strand cDNA Synthesis kit was used to generate cDNA from mRNA according to manufacturer's instructions (Invitrogen). Briefly, RNA was DNase I-treated to eliminate contaminating genomic DNA. After heat-inactivation of DNase I, RNA was poly-adenylated with Poly-A polymerase followed by cDNA transcription with Superscript III reverse transcriptase using an equal amount of Oligo-dT and poly-A primers. Quantitative RT-PCR was performed using SYBR-Green (Applied Biosystems) or SYBR-GreenER (Invitrogen) on either an ABI 7500 Fast or ABI 7900 Real-Time PCR machine (Applied Biosystems). Primer efficiency was determined using a five-point standard curve of mouse universal cDNA (BioChain). 18S or GAPDH were used as normalization control and data was analyzed using the delta-delta Ct method (Livak et al. 2001), correcting for primer efficiency.

Satellite cell isolation and culture

For satellite cell isolations, hindlimb muscles were dissected, minced, and digested in 400U/mL collagenase in Ham's F-12C at 37°C for 1.5hr with periodic vortexing or gentle, continuous rocking. Collagenase was inactivated with 15% horse serum and debris was removed by sequential filtering through 70μm and 40μm cell strainers (BD Falcon) and washed with Ham's F-12C supplemented with 15% horse serum. Satellite cells were isolated at the interface of a 40%/70% percoll gradient or preplated on a gelatin-coated 15cm plate in Ham's F-12C for 1-24 hours at 37°C and harvested for RNA isolation. Cultured cells were grown in Ham's F-12C supplemented with 15% horse serum and 0.5 nM FGF-2 on gelatin-coated surfaces for various times prior to RNA isolation or fixation.

Flow cytometry and Fluorescent activated cell sorting

For primary satellite cells, hindlimb muscle was dissected, digested, and filtered as

described above. Cultured cells were trypsinized to remove from culture and washed in Hank's Balanced Salk Solution (HBSS) supplemented with 5% horse serum. Cells were incubated with fluorophor-conjugated primary antibody at 4°C in HBSS supplemented with 5% horse serum for 1h, rotating at 4°C. When secondary antibodies were required, cells were incubated for 45minutes-1h, rotating at 4°C. Antibodies included 1:200 PE-conjugated rat anti-CD45 (BD Bioscience), 1:200 PE-conjugated rat anti-CD31 (BD Bioscience), 1:200 647-conjugated rat anti-alpha-7-integrin (UBC ABlab), 1:100 chicken anti-syndecan-4 and anti-chicken Alexa-488, 1:500 mouse anti-Flag (clone M2, Sigma) and anti-mouse Alexa-555. All Alexafluors were used at 1:500. Cells were incubated in 1μg/mL DAPI with secondary antibodies. Cell were sorted directly into RNA lysis buffer (RNAqueous-micro kit, Ambion) on a MoFlo XDP cell sorter (Dako Cytomation) or analyzed on a CyAn ADP flow cytometer (Beckman Coulter). Raw flow data was analyzed using FlowJo software (TreeStar).

Cell culture

MM14 cells were maintained in growth media (Ham's F-12C supplemented with 15% horse serum and 0.5-4 nM FGF-2 every 12h) on a gelatin-coated surface at less then 40% confluency. Differentiation was induced by washing at least twice with phosphate buffer saline (PBS) and adding differentiation media (Ham's F-12C supplemented with 15% horse serum). C2C12 cells were maintained in growth media (Dulbecco's Modified Eagle's Medium [DMEM] + 10% horse serum) at less than 40% confluency. Differentiation was induced by washing at least twice with phosphate buffer saline and adding differentiation media (DMEM + 5% horse serum). For clonal analysis, cells were plated at fewer then 500 cells per $1mm^2$ on gelatin-coated coverslips in either growth or differentiation media and cultured for various times prior to

fixation. Primary satellite cells were isolated as above and cultured at 6% O_2 in Ham's F-12C supplemented with 15% horse serum with 0.5 nM FGF-2 added daily (growth media) or Ham's F-12C supplemented with 15% horse serum (differentiation media).

Myofiber explant culture

Hindlimb muscle was dissected, connective tissue removed, and muscle groups separated followed by enzymatic digestion in 400U/mL collagenase at 37°C 1.5h. Collagenase was inactivated by the addition of 15% horse serum. Individual myofibers were gently isolated and maintained in Ham's F-12C supplemented with 15% horse serum and 0.5 nM FGF-2 at 6% O_2. Myofibers were either transferred to new media daily or the media was gently changed until fixation 24-120 hours post-isolation.

Muscle sections

Whole tissues were harvested, fixed in 4% paraformaldehyde for 1.5-2h on ice, and incubated in 30-40% sucrose overnight. Muscles were mounted for cryosectioning in sufficient O.C.T. (Tissue-Tek®) to cover the tissue. Cryosectioning was performed on a Leica Cryostat and sections were between 8-12nm. Tissues and sections were stored at -80°C until ready for use. Tissues were equilibrated to -20°C overnight prior to sectioning. Sections were post-fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed three times for five minutes each in PBS. Heat-induced epitope retrieval (HIER) was used to improve Pax7 staining. For HIER, post-fixed slides were placed in citrate buffer, pH 6.0 and subjected to six minutes of high pressure-cooking in a Cuisinart model CPC-600 pressure cooker. Incubation with 30% H_2O_2 for five minutes at room temperature reduced tissue autofluorescence.

Transfection

MM14 cells, C2C12 cells, primary satellite cells, and myofiber explant culture were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturers' protocol with a 2.5:1 or 3:1 ratio of Lipofectamine (μl): nucleic acid (μg). miR-133 over-expression plasmid was co-transfected with $1/10^{th}$ molar ratio of pEGFP-N1 to mark transfected cells. Myc-DDK tagged *Celf4* transcript 3 (MR220695) and 5 (MR220696) over-expression plasmids were purchased from Origen (Rockville, MD).

Immunofluorescence

MM14 cells, C2C12 cells, primary satellite cells, and explanted myofibers were processed for immunofluorescence in the same manner. Cells were fixed with 4% paraformaldehyde (PFA) on ice at the time of harvest and washed in phosphate buffered saline (PBS). Cells were permeabilized with 0.2% Triton-X100 (Sigma) in PBS followed by blocking with 5% bovine serum albumin (Sigma) in PBS. Incubation with primary antibody occurred at 4°C overnight followed by incubation with secondary antibody at room temperature for 1hr in 3% bovine serum albumin (BSA) in PBS. Primary antibodies were 1:5 mouse anti-Pax7 (hybridoma) or concentrated 1:200 mouse anti-Pax7, neat mouse anti-Myogenin (F5D), 1:500 rabbit anti-Myogenin (M-225 Santa Cruz), neat mouse anti-MyHC (MF20), 1:500 rabbit anti-MyoD (C-20 Santa Cruz), 1:500 chicken anti-syndecan-4, 1:150 rat or rabbit anti- laminin (Sigma), 1:500 mouse anti-Flag (M2, Sigma). Secondary antibodies against IgG of the appropriate species were conjugated to Alexa-488, Alexa-555, or Alexa-647 (Molecular Probes) and used at 1:500. Coverslips were mounted with Vecta-Shield plus DAPI (Vector Labs) or incubated with 1μg/mL DAPI for 10 minutes at room temperature then mounted in mowiol

supplemented with DABCO (Sigma-Aldrich) as an anti-fade agent.

Microscopy and image processing

Images of MM14, C2C12, primary satellite cells, and myofibers for quantification were captured on an epifluorescent Nikon Eclipse E800 microscope or a Leica DMRXA upright spinning disc confocal. Objectives were either 20x/0.50 Ph1 DLL Plan Fluor or 40x/0.75 differential interference contrast M on the Nikon or 10x/0.3NA HC Plan Fluotar, 20x/0.7NA HC Plan Apo or 40x/0.85NA HC X Plan Apo (correction collar) on the Leica. Leica was equipped with a Yokagawa CSU10B spinning disk and images were taken with a Hamamatsu ImagEM EM-CCD. Confocal images of muscle sections were captured on either the Leica or a Zeiss 510 LSM. Objectives used on the Zeiss were either 10x/0.3NA EC Plan Neofluar or 20x/0.8NA Plan Apo Chromat (Carl Zeiss). Nomarsky images were captured on a Zeiss 510 LSM with a 63x/1.4NA oil differential interference contrast Plan Apo Chromat M27 lens (Carl Zeiss). Images were processed using Slidebook (Intelligent Imaging Innovations, Inc.), MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) or the FIJI ImageJ version 1.47 package (NIH) with the additional MacMaster BioPhotonics Facility plugin set. For morphological measurements, individual fibers were outlined and cross sectional area, maximum diameter, and minimum diameter measured in ImageJ. Confocal stacks were projected as maximum intensity images for each channel, background subtracted, noise removed, and merged into a single image in ImageJ. Brightness and contrast was adjusted for the entire image as necessary. Images were either cropped or merged as necessary and individual color channels were extracted without color correction or ɣ-adjustment. Images were adjusted and counted manually or with ImageJ macros designed to perform the same functions (see Chapter 4 for a detailed explanation of ImageJ macro MyoCount).

miRNA Target Prediction

To identify potential microRNA regulators of the CELF family, miRanda (http://www.microrna.org/microrna/home.do) (Betel et al. 2010) and TargetScan (http://www.targetscan.org/) (Lewis et al. 2005; Garcia et al. 2011; Friedman et al. 2009) databases were searched for CELF family members and microRNAs with known roles in satellite cell function.

Chapter 4: Discussion

Skeletal muscle is essential for respiration, mobility, reproduction and metabolism. Deficits in muscle function due to disease, injury or age reduce both quality of life and lifespan. Skeletal muscles are long-lived tissues, surviving for an average of 15 years (Spalding et al. 2005). Such enduring tissues require maintenance from resident adult stem cells to retain functional integrity (Rando and Wyss-Coray 2014; Rando 2006). Loss of regenerative capacity results in progressive muscle wasting, which is a hallmark of muscular dystrophies and sarcopenia (Pagotto and Silveira 2014; Malatesta 2012). Muscular dystrophies are a collection of genetic myopathies with a combined prevalence of ~1/2700 (CDC 2014); however, sarcopenia affects the majority of elderly persons (Batsis et al. 2014; Pagotto and Silveira 2014). With a rapidly aging population, sarcopenia is increasingly a concern as the number of individuals at risk nears one billion (United Nations 2013). An understanding of muscle regeneration will provide insight necessary to develop treatments for degenerative myopathies.

Satellite Cell Homeostasis

Adult tissues are maintained and repaired by resident stem cell populations. Stem cells are primarily quiescent and infrequently divide to preserve DNA integrity and avoid cellular senescence (Hayflick 2000; Finkel and Holbrook 2000). The frequency of stem cell turnover relates to the longevity of the tissue. Skeletal muscles are long-lived tissues, surviving for an average of 15 years (Spalding et al. 2005). The skeletal muscle stem cells, known as satellite cells, rarely enter the cell cycle (Chakkalakal et al. 2012; Grounds and McGeachie 1987; McGeachie and Grounds 1987; Schultz 1976; Snow 1977).

Interestingly, the percentage of cycling satellite cells varies among muscle groups. For example, ~11% of satellite cells are cycling in the plantaris muscle, as measured by BrdU incorporation (Dangott, Schultz, and Mozdziak 2000). In contrast, only ~1% of satellite cells are cycling in the soleus muscle (Mozdziak et al. 2000; Smith et al. 2001). Furthermore, muscle groups have varying numbers of resident satellite cells (Crameri et al. 2004, 2007; Gibson and Schultz 1983; Kadi, Charifi, et al. 2004; Kadi, Schjerling, et al. 2004; Mikkelsen et al. 2009; Murphy et al. 2011; Renault et al. 2002; Shefer et al. 2006; Snow 1977; Umnova and Seene 1991). These data suggests that different muscle groups have distinct requirements for maintenance and repair. To date, no comprehensive studies have determined when satellite cells acquire quiescence or whether satellite cells in different muscles acquire quiescence at distinct times.

In Chapter 2, I examine satellite cell acquisition of quiescence in multiple hindlimb muscles as well at the extraocular muscles (EOM). I demonstrate that satellite cells extensively contribute to both growing and adult muscle. Furthermore, I show that satellite cell contribution to muscle is age and muscle group dependent. I find that satellite cell contribution in hindlimb muscles drops precipitously from 8 to 12 weeks of age and remains constant out to 27 weeks. This indicates that satellite cells have attained adult homeostatic levels of quiescence by 12 weeks. Frequently, 6 -12 week old mice are used for adult regeneration and satellite cell studies. These data suggest that using mice younger than 12 weeks old is inappropriate for studying adult regeneration as satellite cells contribution to muscle is above adult levels.

Despite the overall trend in establishing satellite cell quiescence, there are notable differences among the hindlimb muscles. At 8 weeks, approximately 30% of EDL, soleus, and gastrocnemius myofibers have received satellite cell contribution, in contrast to 63% of TA

myofibers. Some studies have reported that satellite cells preferentially associate with type I muscle fibers and may receive more maintenance than type II fibers (Mustafa 2014). In agreement with this hypothesis, type II fibers atrophy and lose satellite cells to a greater extent than type I fibers in sarcopenic and dystrophic muscle (D'Antona et al. 2007; Lassche et al. 2013; Pedemonte et al. 1999; Verdijk et al. 2007). However, I did not observe a correlation between muscle fiber-type composition and the extent of satellite cell contribution to hindlimb muscles. In dystrophic muscle, the TA is damaged to a greater degree than other hindlimb muscle, including the soleus and gastrocnemius (Gabellini et al. 2006). This suggests that the TA may be more susceptible to damage and may require additional repair to maintain function in healthy muscle. I plan to quantify satellite cell contribution to type I and type II fibers to directly ask whether satellite cells 1) more frequently incorporate into a specific myofiber type and 2) more extensively incorporate into a specific myofiber type.

In contrast to hindlimb, EOM satellite cells contribute to the majority of myofibers through 27 weeks. Although satellite cell contribution to the EOM declines with age, there is no evidence that contribution has plateaued. Therefore, satellite cells in the EOM either do not become quiescent or that quiescence is attained after 27 weeks of age. These observations are consistent with several studies reporting more cycling satellite cells in EOM compared to hindlimb muscle (McLoon and J. D. Wirtschafter 2002; McLoon and J. Wirtschafter 2002; McLoon and Wirtschafter 2003; Rodgers et al. 2014). Additionally, EOM likely has a higher capacity for regeneration than limb muscle. EOM satellite cells have enhanced proliferation *in vitro*, which may lead to a greater amplification of the myoblast pool and contribution to a greater number of myofibers (Pacheco-Pinedo et al. 2009).

A large percentage of myofibers receive satellite cell contribution in adult tissues. In 8-week-old hindlimb muscles, satellite cells contribute to 30-60% of myofibers within a two-week period, while EOM receives contribution from satellite cells in 80% of myofibers. This observation was unexpected as only a small subset of satellite cells are cycling in adult muscle (Chakkalakal et al. 2012; Grounds and McGeachie 1987; McGeachie and Grounds 1987; Schultz 1976; Snow 1977). This emphasizes how a few satellite cells will produce a large population of transit-amplifying myoblasts capable of significant contribution to the surrounding myofibers. Additionally, these data highlight the role of satellite cells in maintaining skeletal muscle in uninjured conditions.

In the gastrocnemius, satellite cells preferentially contribute to myofibers near the fibula and presumptive blood vessels. Recent studies demonstrate that systemic signals from an injury can trigger an "alert" G_0 phase in uninjured, contralateral satellite cells, enhancing the speed of cell cycle entry and extent of proliferation (Rodgers et al. 2014). It is likely that satellite cells in uninjured organisms also respond to systemic signals to modulate muscle maintenance. If these signals form a concentration gradient while diffusing through the muscle that would account for gradient of satellite cell contribution observed in the gastrocnemius. Alternatively, satellite cells may by unevenly distributed throughout the muscle and, at a similar frequency of activation, this would result in higher contribution to myofibers in areas with more satellite cells. I plan to test this hypothesis prior to submitting Chapter 2 for publication. As the muscle is likely experience fairly uniform stress and damage, the functional significance of asymmetrical satellite cell contribution is unclear.

These observations illustrate the heterogeneity among satellite cells from different muscle groups. Gene expression profiles examining satellite cells from EOM and pharyngeal muscle,

EDL and masseter muscle, and multiple head muscles revealed distinct genetic programs for satellite cells derived from different muscles (Harel et al. 2009; Ono et al. 2010; Sambasivan et al. 2009). Additionally, satellite cells from different muscles are functionally heterogeneous. Satellite cells derived from the EDL, soleus, masseter, and TA have different transplantation efficiencies as well as proliferation and differentiation capabilities *in vitro* (Collins et al. 2005; Yusuke Ono et al. 2010).

This is the first comprehensive study of satellite cell homeostasis in multiple uninjured muscles. These data support the hypothesis that satellite cells are a functionally heterogeneous population among muscles of both similar and distinct developmental origins. Furthermore, this work addresses a gap in knowledge by identifying the age at which satellite cells establish quiescence in multiple skeletal muscle groups and demonstrating variability based on fiber type. Additionally, I expect this work to have an immediate impact on the satellite cell research field as there is currently a widespread inappropriate use of mice younger than 12 weeks old for quiescent satellite cells experiments on the unfounded presumption that adult satellite cell homeostasis has been established.

Satellite cell quiescence is actively maintained by post-transcriptional mechanisms.

Morphological characteristics of quiescent satellite cells such as heterochromatin, few organelles and a low cytoplasm volume to nuclear volume ratio suggest limited metabolic and transcriptional activity (Hanzlíková et al. 1975; Lu et al. 1997; Ontell 1975; Sakai 1977; Schultz 1976). Indeed, quiescent cells typically have reduced metabolic activity and transcription rates compared to their proliferating counterparts (Anon n.d.; Choder 1991). However, recent studies have demonstrated that satellite cells can exist in two distinct quiescent states: a typically G0

state with low metabolic activity and an 'alert' G0 state with elevated metabolic activity (Rocheteau et al. 2012; Rodgers et al. 2014). The increase in cell size, mobility and delay through the initial cell cycle suggest that satellite cells would require transcriptional upregulation of an extensive group of genes. However, studies of serum-starved fibroblasts suggest that transcriptional upregulation alone fails to account for the increased expression of multiple cell cycle regulators (Cortner and Farnham 1991; Bork et al. 1992), suggesting that post-transcriptional mechanisms are involved in the transition to a mitotically active fibroblast. Similarly, others and we have hypothesized that satellite cells are not transcriptionally silent and that, instead, satellite cells actively maintain quiescence while awaiting signals to trigger cell cycle re-entry (Pallafacchina et al. 2010; Rodgers et al. 2014; Hausburg, et. al. in revision).

In Chapter 3, I explored the role of post-transcriptional regulation of satellite cell quiescence and activation in both adult and aged satellite cells. I compared genome-wide expression profiles of satellite cells from uninjured and injured muscle to identify a cohort of genes up- or downregulated during activation. The majority of transcripts expressed in satellite cells decrease steady-state level upon activation. Additionally, Gene Ontology analysis revealed enrichment for mRNA post-transcriptional regulation in the activation dataset and no enrichment for transcription. These data support our hypothesis that satellite cells actively maintain quiescence and regulate the transition to a transit-amplifying myoblast through post-transcriptional mechanism. Furthermore, the majority of RNA binding proteins in the activation dataset have known roles in muscle function, disease and stem cells, validating our approach.

Among the RNA binding proteins with changing expression during satellite cell activation are CELFs 1, 2 and 4. Dysregulation of CELF proteins contributes to DM1 pathogenesis. Satellite cell homeostasis is disrupted in DM1. Satellite cell numbers are increased

in the more affected, distal muscle of DM1 patients, while cultured DM1 satellite cells have significantly reduced proliferation and differentiation capacity (Beaulieu et al. 2012; Beffy et al. 2010; Bigot et al. 2009; Furling, Coiffier, et al. 2001; Pelletier et al. 2009; Thornell et al. 2009). These phenotypes have been linked to reduced *Myod1* expression (Amack, Reagan and Mahadevan 2002; Beffy et al. 2010). Recently, CELF1 knockdown in C2C12 myoblasts stabilized *Myod1* transcript (Lee et al. 2010). This raises the hypothesis that increased CELF1 protein in DM1 satellite cells destabilizes *Myod1*, impairing satellite cell function and contributing to the progressive muscle wasting phenotype characteristic of DM1; however, the role of the CELF family in regulating satellite cell function, in normal or diseased muscle, remain largely uninvestigated.

In Chapter 3, I also show that transcripts downregulated during activation are enriched for CELF1/2/4 targets, including members of the TGF-β and p38 MAPK pathways. The TGF-β superfamily contains several regulators of satellite cell function, the best known is the negative myogenic regulator myostatin. As I discuss in the review *Dysregulation of activation pathways in aged satellite cells,* p38α/β MAPK is required for satellite cell activation and elevated p38α/β MAPK signaling contributes to impaired proliferation and self renewal of aged satellite cells.

Comparisons of the satellite cell activation dataset with gene-expression profiles of aged satellite cells revealed dysregulation of FGF, ERK1/2 and p38 MAPK signaling pathways. *Celf1, 2* and *4* expression is dysregulated with age. Furthermore, CELF1/2/4 targets members in each of the FGF, ERK1/2 and p38 MAPK pathways that are dysregulated with age. Together, this places the CELF family in signaling pathways that regulate satellite cell function and suggests that CELF4 has a role in regulating the satellite cell fate decision between proliferation and self-renewal.

In comparing the aged satellite cell expression profiles to the activation dataset, I found a general upregulation of genes downregulated upon activation. Upregulation of 'quiescence genes' agrees with the impaired self-renewal and enhanced premature cell-cycle exit and differentiation behaviors of aged satellite cells. Notably, genes involved with RNA processing and splicing are elevated with age. This implicates post-transcriptional dysregulation of gene expression in the impaired functions of aged satellite cells. Future work examining the dysregulation of 'quiescence genes' may provide mechanistic insight into how aged satellite cells are functionally impaired.

This work highlights the importance of post-transcriptional fine-tuning of gene expression to maintain satellite cells in a quiescent state, poised to quickly activate in response to muscle injury, and to appropriately determine cell fate between proliferation and self-renewal. Future work examining targets of Celf1, 2 and 4 in satellite cells may identify novel activation and cell fate regulatory pathways.

Additionally, this work established a novel role for the CELF family of RNA binding proteins in regulating satellite cell activation and cell fate. CELF proteins are dysregulated in myotonic dystrophy type 1 (DM1), resulting in multiple splicing and translation defects. To date, DM1 research has identified Celf1 dysregulation as contributing to progressive muscle wasting but has failed to identify the mechanisms mediating this phenotype. I propose that dysregulation of Celf1, 2 and 4 in DM1 satellite cells alters activation, proliferation and self-renewal, thereby inefficiently regenerating muscle and resulting in progressive muscle wasting. Future work to test this hypothesis should focus on characterizing DM1 satellite cell function (kinetics of activation and proliferation, and ability to self-renew), altered Celf1, 2 and 4 function, expression, localization and modifications in DM1 satellite cells, and global identification of

altered expression or processing of CELF targets during activation, proliferation and self-renewal.

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