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THE ROLE OF TWO HOMOLOGOUS E3 LIGASES IN MUSCLE PHYSIOLOGY

by

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THE ROLE OF TWO HOMOLOGOUS E3 LIGASES IN MUSCLE PHYSIOLOGY

A

Thesis

Presented to the Faculty of

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In Partial Fulfillment

of the Requirements

of the Degree of

MASTER OF SCIENCE

By

Gabrielle Faith Gloston, B.S.

Houston, Texas

December, 2016

Dedication

I would like to dedicate this thesis to the three women that are responsible for all of my successes thus far: Jannifer, Ruby, and Tammy Landry. You've all made sacrifices for me & for that, I'm forever indebted to you. You've all taught me to be the tough, spunky woman that I am today.

I am also dedicating this thesis to my best friend, Oluwadolapo. Everyone needs a cheerleader & unfortunately for everyone else, I got the best one. Thank you, Dolly, you're truly a Godsend.

Thank you again & I love you all!

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THE ROLE OF TWO HOMOLOGOUS E3 LIGASES IN MUSCLE PHYSIOLOGY

Gabrielle Gloston, B.S.

Advisory Professor: Zheng Chen, Ph.D.

Ubiquitin-mediated proteasomal degradation is an essential cellular function that is coordinated by three key components: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligases. There are an estimated 600 E3 ligases, some of which share high sequence homology; however, the functional significance often remains unknown. FBXL3 and FBXL21 are two homologous E3 ligases that have previously been reported to dictate circadian periodicity, with FBXL3 being the dominant E3 ligase and FBXL21 playing a regulatory role. A recent Yeast Two-Hybrid screen revealed a new shared target of FBXL3 and FBXL21: Telethonin (also known as TCAP). TCAP is a sarcomeric z-disc protein expressed in cardiac and skeletal muscle that is critical to proper structure and function of muscles. Through preliminary experiments, we identified TCAP as a novel shared target substrate of FBXL3 and FBXL21. Here we report that FBXL3 and FBXL21 both accelerate TCAP degradation; however, FBXL21 is the more potent E3 ligase. This novel finding underlines the importance of substrate specificity and serves as a paradigm for future mechanistic studies of E3 ligase homologous pairs. Additionally, the findings reported here will facilitate further studies investigating the role of FBXL3/21 in muscle physiology.

Table of Contents

Acknowledgements iii

Abstract iv

Table of Contents v

List of Illustrations vi

List of Tables vii

Chapter 1: Introduction

1.1 Ubiquitin-mediated protein degradation 2

1.2 The mammalian circadian molecular oscillator 4

1.3 The role of FBXL3 & FBXL21 in the circadian clock 7

1.4 Circadian influence on muscle physiology 12

1.5 Telethonin, a novel target substrate of both FBXL3 and FBXL21 13

1.6 Central hypothesis & specific aims 16

Chapter 2: Materials & Methods 18

2.1 Mouse strains 19

2.2 DNA extractions & *Past-time genotyping* 19

2.3 Tissue collection 20

2.4 Protein extractions 20

2.5 Immunoblotting 21

2.6 Cell Culture & Transfection 21

2.7 Plasmids 22

2.8 TCAP degradation assay 22

2.9 Lentiviral construction 23

2.10 Statistical Analysis 23

Chapter 3: Results 25

3.1 Circadian timing of TCAP protein expression in skeletal muscle 24

3.2 Circadian timing of TCAP protein expression in cardiac muscle 29

3.3 TCAP degradation by FBXL3 or FBXL21 29

3.4 Lentiviral constructs expressing FBXL3 and FBXL21 are functional *in vitro* 34

Chapter 4: Discussion & Future Directions 36

4. Discussion 37

4.1 Future Directions 42

Bibliography 45

Vita 50

List of Illustrations

Figure 1 The mammalian circadian molecular oscillator……………………………..6

Figure 2 The role of FBXL3 & FBXL21 in the circadian clock………………………...10

Figure 3 Schematic of Telethonin (TCAP)……………………………………………13

Figure 4 Circadian timing of TCAP protein expression in skeletal muscle…………..26

Figure 5 Circadian timing of TCAP protein expression in cardiac muscle…………..28

Figure 6 Two homologous E3 ligases expression increases TCAP degradation in C2C12 myoblasts…………………………………………………………………......30

Figure 7 FBXL-expressing lentiviral constructs are functional *in vitro*.……………….32

Figure 8 The role of FBXL3 & FBXL21 in muscle physiology……………………..….37

List of Tables

Table 1 Skeletal muscle-specific clock-controlled genes……………………………12

CHAPTER 1: INTRODUCTION

1. Introduction

There are over 600 E3 ligases in the human genome, many of which share high sequence homology with a small subset of other E3 ligases; however, their functional relationship often remains unknown. Additionally, each of these ligases are capable of having multiple target substrates, creating a variety of E3 ligase-substrate combinations (Harper et al 2012). FBXL3 and FBXL21 are two homologous E3 ligases that were previously reported to control circadian periodicity through antagonistic activity on their shared target substrates, CRYPTOCHROME (CRY) 1 and 2. In relation to CRYs, FBXL3 is the predominant E3 ligase that promotes degradation in the nucleus and FBXL21 plays a regulatory role by protecting CRYs in the cytoplasm (Yoo et al 2013).

It is well known that circadian clocks exist within a majority of cells throughout the body, including the muscular system (Harfmann et al 2015). In fact, skeletal muscles heavily rely on the positive regulators of the circadian oscillator, *Bmal1* and *Clock*, which have been shown to affect both structure and function of skeletal muscle when genetically disrupted in mice (Andrews et al 2010). Furthermore, multiple independent studies now suggest that muscle physiology is tightly clock-regulated, making skeletal muscle a suitable candidate to screen for novel protein targets of FBXL3 and FBXL21 (Harfmann et al 2015; Podobed et al 2014; Mayeuf-Louchart et al 2015).

In a Yeast Two-Hybrid screen, we identified TCAP (also known as Telethonin) as a shared target substrate of FBXL3 and FBXL21. TCAP is a cytoplasmic protein that is integral to sarcomeric Z -disc and -line structure and function in both cardiac

and skeletal muscle cells, respectively (Valle et al 1997). Interestingly, TCAP has been shown to be circadian regulated at the mRNA level in cardiac muscle and mutations in TCAP have been associated with Limb Girdle Muscle Dystrophy (Podobed et al 2014; Moreira et al 2000). Our lab found that FBXL3 and FBXL21 tightly and specifically bind to TCAP, with E3 ligase activity on TCAP that opposed the previous paradigm discovered with the CRY proteins. When ectopically expressed, FBXL21 potently accelerated TCAP degradation, whereas FBXL3 showed a much more diminished effect. Therefore, FBXL3 and FBXL21 play distinct roles in the degradation of CRYs and TCAP. Given the crucial role that TCAP plays in the structure and function of skeletal muscle, we suspect that these homologous E3 ligases serve as important regulators of muscle physiology, just as they govern periodicity in the circadian clock.

1.1 Ubiquitin-mediated protein degradation

Ubiquitin-mediated protein degradation is a serial, enzymatic reaction that targets proteins for degradation and is essential to proper cellular functioning (Harper et al 2012). In fact, disruption of the ubiquitin-proteasome system has been associated with serious physiological consequences, such as cancers and neurodegeneration (Welcker and Clurman 2008; Frescas and Pagano 2008; Jin and Youle 2012). The study of ubiquitin signaling has only been recently advanced within the last decade. It is currently known that eukaryotes have 2 E1 enzymes, 40 E2 enzymes, and about 600 E3 enzymes. In order to facilitate the proper execution of protein turnover (and several other ubiquitin-regulated cellular functions), ubiquitin networks are organized in a finely tuned spatiotemporal fashion (Grabbe et 2011).

The three major components that are responsible for orchestrating the ubiquitylation process are: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase) (Harper et al 2012). Ubiquitin (Ub) is first activated by the E1 enzyme (Ub activating enzyme) in an ATP-dependent manner and then becomes Ub-adenylate (ADP). Once activated, Ub is able to form a thioester bond with one of the 40 E2 enzymes (conjugating enzymes). E2 conjugating enzymes play an important role in determining the number of Ub molecules attached to a protein as well as the chain linkage type associated with proteins that are poly-ubiquitinated (Ye and Rape 2009). There are three major groups of E3 ligases (HECT, RING, or RBR), but regardless of the type, Ub is transferred from the E2 conjugating enzyme to the substrate. Ultimately, proteins are either monoubiquitylated, multi-monoubiquitylated or poly-ubiquitylated; these ubiquitylation modifications are a result of the combination of specific E2 and E3 enzymes.

Despite the importance of ubiquitin-mediated protein degradation, a large number of E3 ligases remain poorly characterized. Substrates have been difficult to identify partly because of the transient nature of degradative ubiquitylation targets, in addition to the weak interactions that typically occur between an E3 ligase and its substrate (Harper et al 2012). Before advancements in proteomic technology, substrates were identified by screening for conserved targeting sequences in proteins throughout the human genome that matched the targeting sequences in already identified E3 ligase substrates (Koepp et al 1999; Zachariae and Nasmyth 1999; Harper et al 2002; Willems et al 1999). According to the most recent

predictions (based on E3 functional domains), there are over 600 E3 ligases in the human genome, a number of which remain completely unstudied (Deshaies and Joazeiro 2009).

1.2 The mammalian circadian molecular oscillator

The circadian system has evolved to help the body anticipate predictable environmental changes, allowing the body to optimize its biological processes throughout Earth’s 24-hour cycles of light and dark. Circadian rhythms are generated by the activity of core clock circadian genes in what is known as the mammalian circadian molecular oscillator. This oscillator is ubiquitously expressed in individual cells throughout most organs in the body and functions autonomously. The most important circadian clock is located in the suprachiasmatic nucleus (SCN), which contains around 20,000 neurons with individual molecular oscillators that “tick” in synchrony throughout the entire SCN. A simplified schematic of the mammalian circadian molecular oscillator is pictured below in Figure 1. The core molecular oscillator consists of both positive and negative components. CLOCK and BMAL1 are basic helix-loop-helix/PAS (bHLH/PAS) transcription factors that heterodimerize and translocate to the nucleus, where they bind to the E- (CACGTG or CACGTT) box *cis*-regulatory elements of several circadian genes, including the two other core circadian genes, *Per* and *Cry*. Once bound, these genes are transcriptionally activated, produce proteins that heterodimerize and then translocate from the cytoplasm to the nucleus. Once in the nucleus, this PER/CRY complex transcriptionally represses CLOCK/BMAL1, indirectly repressing their own transcription. This negative transcriptional regulation serves as the end of a full

circadian period and through additional posttranscriptional and posttranslational steps, transcriptional repression is relieved via proteasomal degradation of PER/CRY (Lowery and Takahashi 2011).

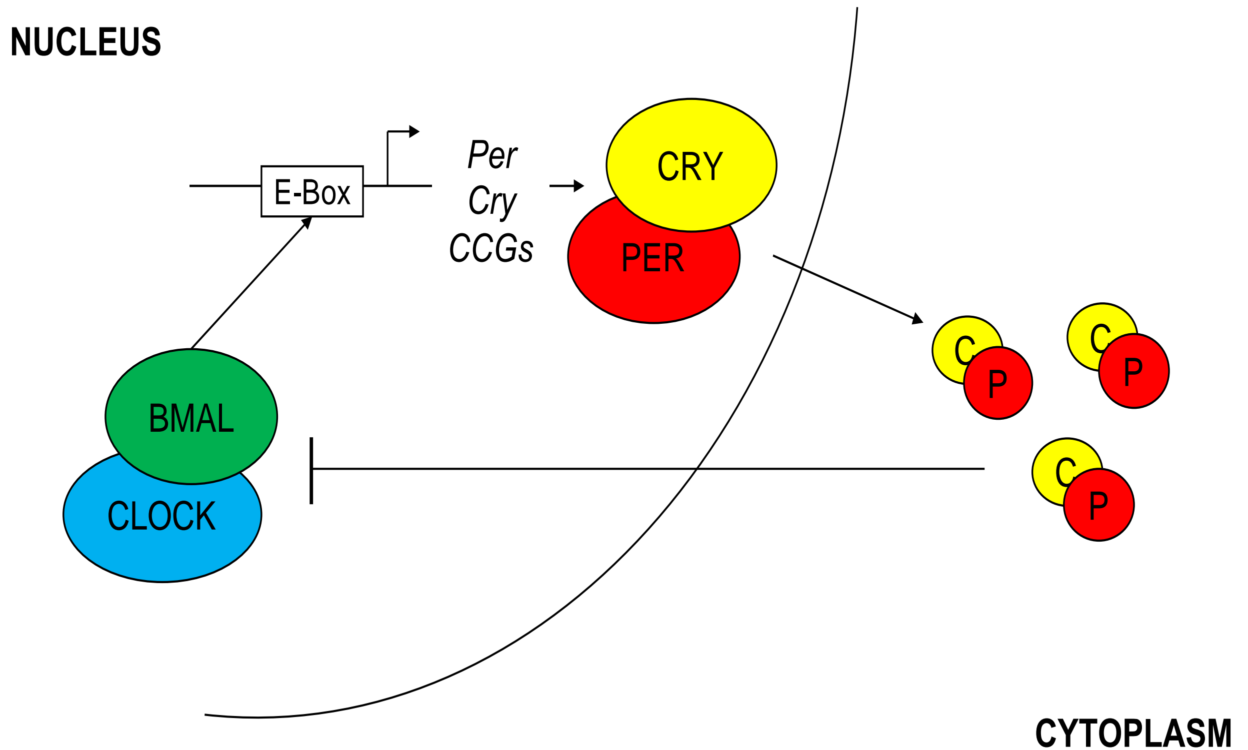

Figure 1: The mammalian circadian molecular oscillator. The molecular oscillator is ubiquitously expressed throughout most cells in the body and the fluctuating activity of its protein products results in circadian rhythms. The core feedback loop consists of BMAL, CLOCK, PER and CRY. BMAL & CLOCK heterodimerize & transcriptionally activate clock genes like *Per* and *Cry*, serving as the positive arm of this feedback loop. Near the end of a circadian period, PER & CRY bind to BMAL & CLOCK, serving as a transcriptional repressor of both the positive and negative arm of this core loop.

1.3 The role of FBXL3 & FBXL21 in the circadian clock

The mammalian SCF ubiquitin ligase is a multi-protein complex that is crucial to post-translational regulation of cellular timing via proteasomal degradation. It is characteristically composed of Skp1, Cul1, and an F-box protein (Cardoza and Pagano 2004). The F-box protein family is the substrate recognition component of the SCF ligase and is typically named according to their substrate-binding domain (Cenciarelli et al 1999; Winston et al 1999). Currently, the F-box family includes FBWs, FBLs, and FBXs. The FBW family includes F-box proteins featuring substrate-binding domain with WD-40 repeats. FBL proteins have substrate-binding domains with leucine-rich repeats (LRRs). The last category of F-box proteins are the FBXs, which have neither WD-40 repeats nor LRRs in their substrate-binding domains (Cardoza and Pagano 2004). The full spectrum of protein substrates are still unknown for many F-box proteins; therefore, the cellular function of many E3 ligases remains unknown. It is expected that these proteins target diverse substrates and have many different cellular functions, given the variety of structural compositions currently known (Kipreos and Pagano 2000).

An FBXL3 mutant mouse line (*Overtime*, *Ovtm*) was derived from a large-scale forward ENU mutagenesis screen and was found to have a circadian period of 25.8 hours. Through positional cloning and genetic complementation, a point mutation in *Fbxl3* was revealed to convert isoleucine to threonine at residue 364. This mutation is mapped to a region between two leucine rich repeat (LRR) domains (LRR 10 and LRR 11) located in the C terminus of *Fbxl3*. *Ovtm* mice showed a

change in circadian gene and protein expression. PER protein levels were significantly reduced in liver tissue, while CRY2 protein levels showed significant elevation. All other core clock machinery, such as CLOCK, BMAL1, and CRY1 showed no significant response to the semidominant, autosomal *Ovtm* mutation. This mouse model served as genetic evidence that FBXL3 may act as an E3 ubiquitin ligase that targets CRY proteins (Cardoza and Pagano 2004). Cellular studies showed that while FBXL3 typically promote proteasomal degradation of CRY, FBXL3^{OVTM} stabilized CRY, resulting in an overall elevation of CRY protein levels. Siepka et al (2007) concluded that the lengthened circadian period and complementary phenotypic alterations induced by the *Ovtm* mutation was caused by an inefficient FBXL3 protein and transcriptional repression via CLOCK and BMAL1 (Siepka et al 2007).

Recently, another ENU mutagenesis-derived mouse lined called *Past-time* was found to have a short circadian period (22.91 hours). Researchers mapped the mutation to the *Fbxl21* gene, more specifically to a point mutation at nucleotide 787 in the 5th exon. Moreover, this mutation causes a conversion of glycine to glutamic acid at residue 149 in the amino acid sequence, which is expected to destabilize the LRR domain of FBXL21 protein (Yoo et al 2013). Although small, this mutation is significant for the functionality of FBXL21 as an E3 ligase because LRR domains typically facilitate protein-protein interactions via substrate recognition (Kobe and Kajava 2001). *In vivo* work revealed that the *Psttm* mutation affected clock gene expression by elevating *Per1/2* and *Cry1/2* mRNA levels in the cerebellum and moderately so in mouse liver. Clock-controlled genes like *Rev-erbα*, *Dec2*, and *Dbp*

were also elevated in liver tissue. Interestingly, CRY protein levels were not elevated as expected and CRY2 levels were actually reduced, suggesting that the *Psttm* mutation affected protein stability leading to a destabilization of CRY2 protein levels (Yoo et al 2013).

FBXL3 and FBXL21 are two homologous E3 ubiquitin ligases that work in direct opposition to tightly regulate circadian periodicity in mammals. The circadian clock is driven by a molecular oscillator that consists of two interlocked loops (Ko and Takahashi 2006). The core loop is composed of a positive (CLOCK and BMAL1) and negative (PER1/2 and CRY1/2) arm. Over the course of a single circadian period, *Per* and *Cry* products are translocated to the cytoplasm where they heterodimerize, return to the nucleus, and eventually repress CLOCK/BMAL1 transcriptional activation, thus turning off their own transcription. When mutated, FBXL3 and FBXL21 can either lengthen or shorten the circadian period by modulating CRY protein levels. Further molecular studies based on the initial *in vivo* findings revealed that FBXL3 is the active E3 ligase for CRY in the nucleus and FBXL21 has dual roles (pictured in Figure 2). In the nucleus, FBXL21 is a regulatory E3 ligase that antagonizes FBXL3 and protects CRY. In the cytoplasm, FBXL21 is a weak active E3 ligase for CRY (Yoo et al 2013). This was the first study to elucidate such a unique homologous E3 ligase paradigm. Discovering one substrate of an F-box protein does not reveal the full gamut of its cellular functions; instead, an E3 ligase typically has multiple target substrates. It is important to note that FBXL21 is subject to circadian regulation and is a paralogue of FBXL3, with ~85% conserved amino acid residues between the two (Yoo et al 2013). It is therefore plausible that

FBXL3 and FBXL21 could share a circadian-regulated target substrate in a similar paradigm as the one mentioned here with CRY.

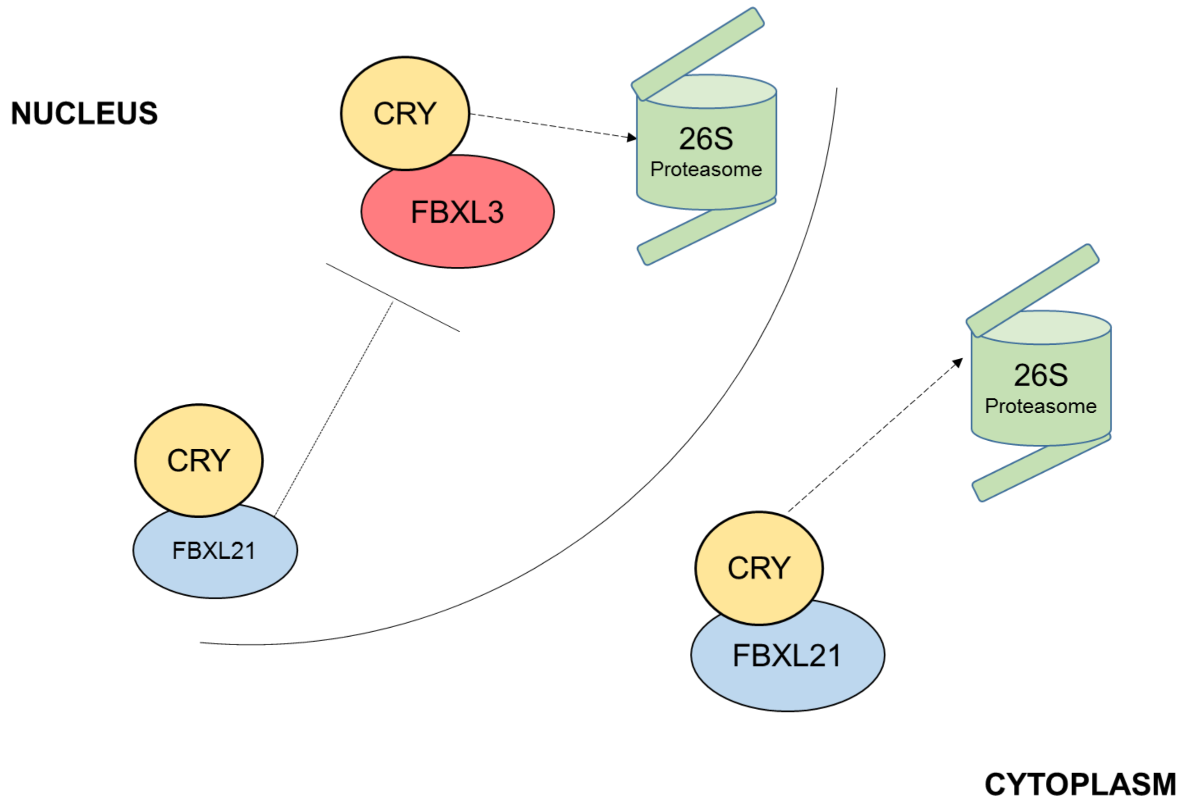

Figure 2: The role of FBXL3 & FBXL21 in the circadian clock. FBXL3 acts as the active E3 ligase, promoting CRY ubiquitin-mediated protein degradation. FBXL21 is present in both the nucleus & cytoplasm & therefore plays two roles with respect to its target substrate CRYs. FBXL21 in the nucleus antagonizes FBXL3, thereby protecting CRYs from degradation; however in the cytoplasm, FBXL21 promotes CRY ubiquitin-mediated protein degradation.

1.4 Circadian influence on muscle physiology

The human body is composed of almost 50% skeletal muscle (Goodpaster et al 2000; Hoppeler and Fluck 2002). Aside from enabling movement, skeletal muscle plays important roles in maintaining overall health. Skeletal muscle serves as an amino acid reservoir, a major site for postprandial glucose storage, and also an endocrine tissue (Ripperger et al 1995; Defronzo et al 1981; Ferrannini et al 1988; Febbraio and Pederson 2002). Recently, research groups have discovered circadian gene expression via expression profiling in skeletal muscle tissue, suggesting that up to 2,300 genes are circadian-regulated (McCarthy et al 2007; Pizarro et al 2013). The role of the circadian system is still not completely known; however, it is plausible that the circadian system plays multiple roles in the overall structure and function of skeletal muscle, given the number of muscle-specific clock-controlled genes (Harfmann et al 2015). Table 1 shows more examples of muscle-specific clock-controlled genes that have physiological implications in several aspects of the proper functioning of skeletal muscle. These genes are typically transcriptionally regulated by core clock genes *Clock* and *Bmal1*.

Table 1 Skeletal muscle-specific clock-controlled genes		
Clock-controlled gene	**Physiological Implication**	**Reference**
MyoD1	Glucose metabolism	Andrews et al 2010
Myogenin	Morphology, myogenesis	Shavlakadze et al 2013
Telethonin, Titin-cap (TCAP)	Muscular dystrophy	Podobed et al 2014
Ucp3	Fatty acid oxidation in skeletal muscle cells	MacLellan et al 2005

1.5 Telethonin, a novel target substrate of both FBXL3 and FBXL21

Skeletal and cardiac muscle both require actin and myosin proteins for proper contractile movements. However, it is only in the cardiac and skeletal muscle that organized patterns of actin and myosin are bundled together into sarcomeres, which form the basic contractile unit in striated muscle. The sliding motion of thick (myosin) and thin (actin, tropomyosin, and troponin) filaments within sarcomeres allows muscle contraction to occur, with each sarcomere being divided by z-discs (in skeletal muscle, z-line in cardiac muscle). This is also where thin (actin, tropomyosin, and troponin) filaments are anchored. These z-discs are highly protein-enriched areas of striated muscle, which is thought to be composed of z-disc protein complexes (Faulkner et al 2001). Although some advances have been made, the overall molecular organization of these z-disc proteins is still uncertain.

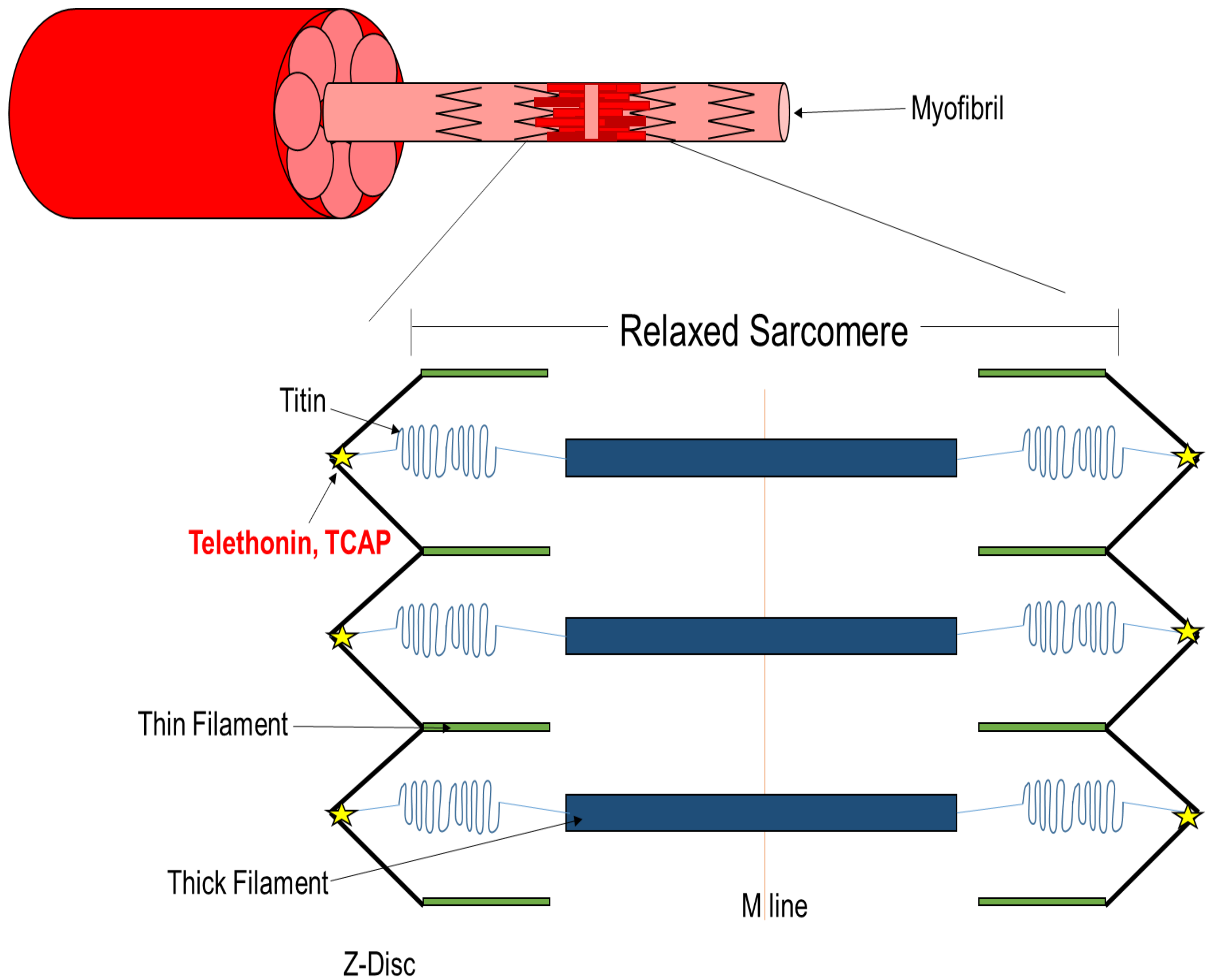

Figure 3: Schematic of Telethonin (TCAP). TCAP is a sarcomeric Z-disc protein that is 19kDa and sits at the end of TITIN protein. It is a critical regulator of both z-disc structure & function.

Our lab recently identified Telethonin, also known as Titin cap or “Tcap”, as a shared target substrate of FBXL3 and FBXL21. Tcap is a small (19kDa), cytoplasmic protein that is expressed exclusively in the heart and skeletal muscle & is one of the most abundant transcripts in striated muscle (Valle et al 1997). TCAP protein is developmentally-regulated, as it has been shown to be expressed at lower levels in neonatal animals than in adult animals. This gradual increase in expression was also observed in C2C12 cells during myofibrillogenesis, which validated the *in vivo* data (Mason et al 1999). Tcap (shown in Figure 3) co-localizes with actin in the thin filaments of the Z-disc (Gregorio et al 1998). Within the sarcomere, Tcap binds to two other z-disc proteins—FATZ and titin. In order to properly bind to titin, the first 140 amino acid residues of the protein must bind with the Z1 and Z2 Ig-like N-terminal repeat region of titin (Mues et al 1998). Interestingly, *Tcap* and its protein product show clear circadian, diurnal expression patterns in mice and blunted cyclic expression in circadian mutant mice (Podobed et al 2014).

Although small, Tcap is essential to proper structure and function of cardiac and skeletal muscle. At least two different mutations in *Tcap* have been associated with a form of autosomal recessive limb-girdle muscular dystrophy (AR LGMD) type 2G. This form of the disease has a milder phenotype than other genetically distinct types of AR LGMD. Clinical features include significantly weakened distal leg muscles, calf hypertrophy, and elevated serum creatine-kinase levels. Many of these patients eventually lose the ability to walk near middle age. Moreira et al 200 showed that these particular mutations affect the carboxy-terminal region of the

Tcap gene, which then has the potential to affect the sarcomeric structure of skeletal muscle (Moreira et al 2000). LGMD2G has been modeled in a TCAP knockout mouse that has a null mutation in the *TCAP* gene. Interestingly, this mouse exhibits a much less severe phenotype compared to LGMD2G patients, with the biggest impact in balance and muscle stiffness (Markert et al 2010). The disparity between the human and mouse phenotypes is most likely due to the mutation type—null (KO mouse) vs dominant negative (patients). Moreover, mutations in the *Titin* gene itself have been associated with dilated cardiomyopathy (DCM), which causes systolic dysfunction and ventricular dilation. (Itoh-Satoh et al 2002). TITIN is a large protein that is also expressed in cardiac and skeletal muscle and has been shown to interact with sarcomeric proteins, including TCAP (Bang et al 2001). Here, researchers found that two of the mutations (Val54Met and Ala743Val) mapped to the Z-line portion of *Titin* decreased TITIN protein's ability to bind to its usual sarcomeric targets, one of them being TCAP; therefore, a subset of *Titin* mutations affect sarcomeric assembly via TCAP (Itoh-Satoh et al 2002). Considering Tcap's vital role in cardiac and skeletal muscle physiology, it is plausible that FBXL3 and FBXL21 regulate skeletal muscle physiology by tightly controlling TCAP protein levels.

1.6 Central hypothesis & specific aims

We hypothesize that FBXL3 and FBXL21 coordinately regulate skeletal muscle physiology via ubiquitin-mediated protein degradation of their shared target substrate TCAP.

Specific Aim 1: To characterize the circadian timing of TCAP expression in wild-type and *Past-time* muscle tissues. We will collect skeletal (*gastrocnemius*) and cardiac muscle tissue from adult wild type and FBXL21 mutant (*Psttm*) mice at CT 0, 4, 8, 12, 16, and 20 to investigate endogenous protein expression of TCAP over a full circadian cycle. *We hypothesize that TCAP expression in Psttm muscle tissue will display an abnormal (overall increase) expression due to the point mutation in Fbxl21.*

Specific Aim 2: To assess the roles of FBXL3 and FBXL21 in TCAP degradation. We will measure TCAP protein stability in the presence of either E3 ligases to determine how FBXL3 and FBXL21 affect TCAP degradation. *We hypothesize that FBXL21 will decrease TCAP stability and FBXL3 will increase TCAP stability.*

Specific Aim 3: To construct a viral expression vector for the overexpression of FBXL3 and FBXL21 in a skeletal muscle *in vitro* model. We will remove *FBXL3/21* insert from the pCMV10-3XFlag and subclone into the pFUW lentiviral vector to overexpress FBXL3/21 *in vitro*. *We hypothesize that FBXL3/21 will be sufficiently expressed in vitro*.

CHAPTER 2: MATERIALS & METHODS

2.1 Mouse strains

C57BL/6J (Stock # 000664) mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Psttm/Psttm* ("Past-time") mutant mice were cloned by Dr. Seung-Hee Yoo. The *Past-time* mouse line was generated from ENU mutagenesis as previously described (Yoo et al 2013). All mice were group-housed according to genotype and maintained under LD 12:12 conditions with food and water provided *ad libitum*. All animal care and procedures were approved by the Animal Care and Use Committee of the University of Texas Health Science Center in Houston.

2.2 DNA extractions & *Past-time genotyping*

Genotyping was performed using DNA derived from a 2 mm tail tip extracted from each *Past-time* mouse. To extract genomic DNA, tail tips were placed in a 1.5 mL micro-centrifuge tube and heated to 95^0C with 90 uL 50 mM NaOH for 45 minutes. To neutralize the extract, 10 uL 1M Tris (pH 4.7) was added to each sample and the DNA extract was vortexed. To genotype by PCR analysis, the DNA extract was diluted 1:5 with nuclease-free water and stored at 4^0C.

Past-time mice were maintained as homozygote breeding pairs (2 *PSTM* homozygote females, 1 *PSTM* homozygote male), so the resulting progeny did not require genotyping. *Past-time* genomic DNA was amplified during PCR using forward (5'CAGAACTCGGTTGCTGGATT-3') and reverse (5'CAGCAACTCCGTCTTTCCTC-3') primers at 100uM. PCR products are purified using the QIAquick Gel Extract kit (Qiagen) and then submitted to GeneWiz for sequencing to detect single nucleotide polymorphisms. The *PSTM* wild-type and

PSTM homozygous sequences are as follows: CCTTGGGATTG and CCTTGGAATTG.

2.3 Tissue collection

C57BL/6J and *Psttm* mice were maintained in LD 12:12 and then moved to constant darkness (DD) 36 hours prior to tissue collection at 13 weeks of age. Mice were sacrificed by cervical dislocation under infrared lights at CT 0, 4, 8, 12, 16, and 20. *Gastrocnemius* and *tibialis anterior* tissue was harvested under normal white light. Tissue was sectioned into 50-100mg pieces, immediately snap-frozen in dry ice and then placed in a pre-chilled 1.5mL micro-centrifuge tube to prevent proteasomal degradation. Prior to protein extraction, tissue samples were stored at -80^0 Celsius. A one-inch portion of the tail was also collected and frozen to verify the genotype of any given mouse, if necessary, for future experiments.

2.4 Protein extractions

Frozen mouse tissue was immersed in a total of 500uL of cold extraction buffer & then homogenized with an electric homogenizer. To fully disrupt the lysate, samples were vortexed at 3000rpm for 30 seconds every 5 minutes for a total of 15 minutes. Samples were then centrifuged at 13,000 rpm for 20 minutes at 4^0 C and the supernatant was aspirated (avoiding any lipid or other insoluble materials) into a fresh tube and kept on ice for protein quantification. To quantify protein concentrations, we performed the Bradford Assay as previously described Bradford, MM 1976 and measured the optical density (OD) of each sample at 595 nm on the Tecan plate reader. Lysate was diluted with EB first until all samples were 2ug/uL

and then with one-fifth volume 5x Laemmli loading buffer and lastly boiled at 95^0 C for 5 minutes to denature the quarternary structure of the protein samples.

2.5 Immunoblotting

10-12ug of WT and *Psttm* muscle lysate was resolved by SDS-PAGE and Western Blotting analysis. The following antibodies were used: TCAP 1:1000 (BD Biosciences, Abcam), FBXL21 1:2000 (generated as specified in Yoo et al 2013), FBXL3 1:3000 (generated as specified in Yoo et al 2013), GAPDH 1:10000 (Ambion), FLAG 1:1000 (Sigma). Relative protein abundance was quantified using ImageJ (Schneider et al 2012) and two-way ANOVAs were performed to measure differences between WT and *Psttm* expression across circadian time on GraphPad Prism software. Error bars represent ± SEM (n=3).

2.6 Cell Culture & Transfection

The C_2C_{12} cell line (ATCC) is commonly used to model skeletal muscle differentiation. C_2C_{12} myoblasts were thawed from a frozen stock and maintained as myoblasts in high glucose + glutamine Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. To prevent differentiation into myotubes, C_2C_{12} cells were subcultured at 80-90% confluency.

To perform the TCAP protein degradation assay, C_2C_{12} cells were plated into two 6-well plates at 1×10^5 per well 17 hours before transfection. Cells were then transfected with *Tcap* with or without *Fbxl3* or *Fbxl21* constructs using Effectene transfection reagent (Qiagen). 40ug/mL cycloheximide (CHX) was then added to each well 24 hours after transfection. To confirm functionality of the FBXL3/21-

expressing lentivirus constructs, HEK 293T cells were plated at 1×10^5 per well in a 6-well plate, then transfected 17 hours later using Effectene transfection reagent (Qiagen). 24 hours after transfection, FBXL3/21 expression was confirmed via confocal microscopy and immunoblotting. To produce the FBXL3/21 lentiviruses, 293T cells were plated in a polyD lysine-coated 10cm dish at 6.0×10^6 in antibiotic-free 10% FBS media. The 293T cells were transfected with pCMV-deltaR8.91 and pVSV-G packaging vectors with Lipofectamine 2000 transfection reagent (Thermo Fischer Scientific). 12-16 hours after transfection, the Lipofectamine-containing media was replaced with complete media for 293T cells. 48 hours later, the virus-containing supernatant was transferred to a polypropylene tube and centrifuged at 4,000 rpm for 10 minutes to remove the remaining 293T cells. The virus was then filtered using a 0.45 um cellulose acetate filter and stored at 4^0 C for short-term storage and -80^0 C for long-term storage.

2.7 Plasmids

Fbxl3 and *Fbxl21* cDNA was synthesized as specified in Yoo et al (2013).

2.8 TCAP degradation assay

C_2C_{12} cells were treated with 40ug/mL of cycloheximide for 0, 2, 4, or 6 hours. Cycloheximide is a commonly used reagent to inhibit protein synthesis by preventing the elongation step of eukaryotic translation [Schneider-Poetsch et al 2010]. At the end of each indicated treatment time, C_2C_{12} cells were lysed and used for immunoblotting. Half-life parameter K was calculated using one-phase decay.

Statistical difference between half-life parameter K was calculated by one-way ANOVA. Error bars represent ± SEM (n=3).

2.9 Lentiviral construction

To improve transfection efficiency of C2C12 cells, a lentivirus system with GFP markers has been employed for the muscle differentiation experiment. Full-length *Fbxl3* and *Fbxl21* cDNA (tagged with a Flag sequence) was digested from its original vector, pCMV10-3XFlag (Sigma) and subcloned into the pFUW lentivirus vector. To confirm the insert sequence, lentivirus vectors expressing Fbxl3 and Fbxl21 were submitted to GeneWiz. To confirm functionality of these lentivirus vectors *in vitro*, 293T cells were transfected with both vectors using Effectene reagent (Qiagen), lysed 24 hours later, and Fbxl3 and Fbxl21 expression was confirmed via immunoblotting.

2.10 Statistical Analysis

All statistical analysis was performed in GraphPad Prism (Version 5). Two-way ANOVA analysis was performed to compute the differences in protein expression between WT and *Psttm* muscle tissues across six circadian time points. Two-way ANOVA analysis helps to determine how the dependent variable is affected by two variables or factors. In this case, both the genotype of each mouse that the muscle tissue was collected from and circadian time contribute to the response (protein expression). Error bars represent ± SEM (n=3).

Half-life of TCAP protein alone or in the presence of FBXL3/21 was determined using one-phase decay analysis, where K is the half-life parameter. This

statistical test was chosen because cycloheximide treatment results in only one decay phase as opposed to multiple phase decays. One-way ANOVA analysis was performed to compute the statistical difference between half-life parameter, K, for each condition. Error bars represent ± SEM (n=3).

CHAPTER 3: RESULTS

3.1 Circadian timing of TCAP protein expression in skeletal muscle

Preliminary experiments validated our novel finding that TCAP is a shared protein target of E3 ligases FBXL3 and FBXL21. This homologous pair was shown to differentially affect TCAP expression in HEK293T cells. Yoo et al (2013) reported that FBXL3 was the dominant E3 ligase in CRY protein degradation, while FBXL21 was considered to be a regulatory E3 ligase. Unexpectedly, FBXL21 was much more potent in TCAP degradation than FBXL3, suggesting that FBXL21 is the primary E3 ligase in ubiquitin-mediated protein TCAP degradation. These newly identified FBXL3/21-TCAP interactions directly oppose the previous mechanism established in (Yoo et al 2013) in which FBXL3 was the active E3 ligase and FBXL21 was predominantly a regulatory ligase for the CRYPTOCHROME (CRY) substrates. This difference in E3 ligase-substrate activity highlights the importance of substrate specificity in characterizing E3 ligases' cellular function(s). TCAP is ranked as the 12th most abundant transcript in skeletal muscle tissue (Valle et al 1997) and is a circadian-regulated transcriptional target (Podobed et al 2014). We therefore isolated WT and *Psttm gastrocnemius* (skeletal) muscle to characterize the circadian timing of TCAP protein expression. We hypothesize that the instability of *Fbxl21* in the *Psttm* mutant mice will lead to elevated TCAP protein levels throughout the circadian cycle under normal (12:12 LD conditions).

We observed a circadian oscillation of TCAP protein with a peak at CT16 in WT mice (Figure 4), whereas the *Psttm* mutation caused a significant increase in overall TCAP protein expression. This increase in expression is consistent with our sub-hypothesis that TCAP protein expression would be elevated throughout the

circadian cycle due to the lack of stability in *Fbxl21* in *Psttm* mice. We also observed a circadian oscillation in FBXL21 protein, with a slight phase advance in expression compared to TCAP protein expression. FBXL21 protein expression is unstable throughout the circadian cycle in *Psttm* skeletal muscle, as expected (Yoo et al 2013). FBXL3 was constitutively expressed in both WT and *Psttm* tissue, consistent with the literature that shows that FBXL3 is not circadian regulated (Yoo et al 2013). Most importantly, two-way ANOVA analysis shows significant ($p<0.001$) genotype effect on TCAP and FBXL21 protein expression, and TCAP protein expression is elevated throughout the entire circadian cycle in *PSTM* homozygous mice compared to *PSTM* WT mice. Interestingly, when TCAP protein expression is highest (CT16), FBXL21 protein expression was low as shown in Figure 5A. This observation is consistent with our preliminary results, which implies that TCAP expression should be highest when the active E3 ligase (FBXL21) is not present or expressed at relatively low levels.

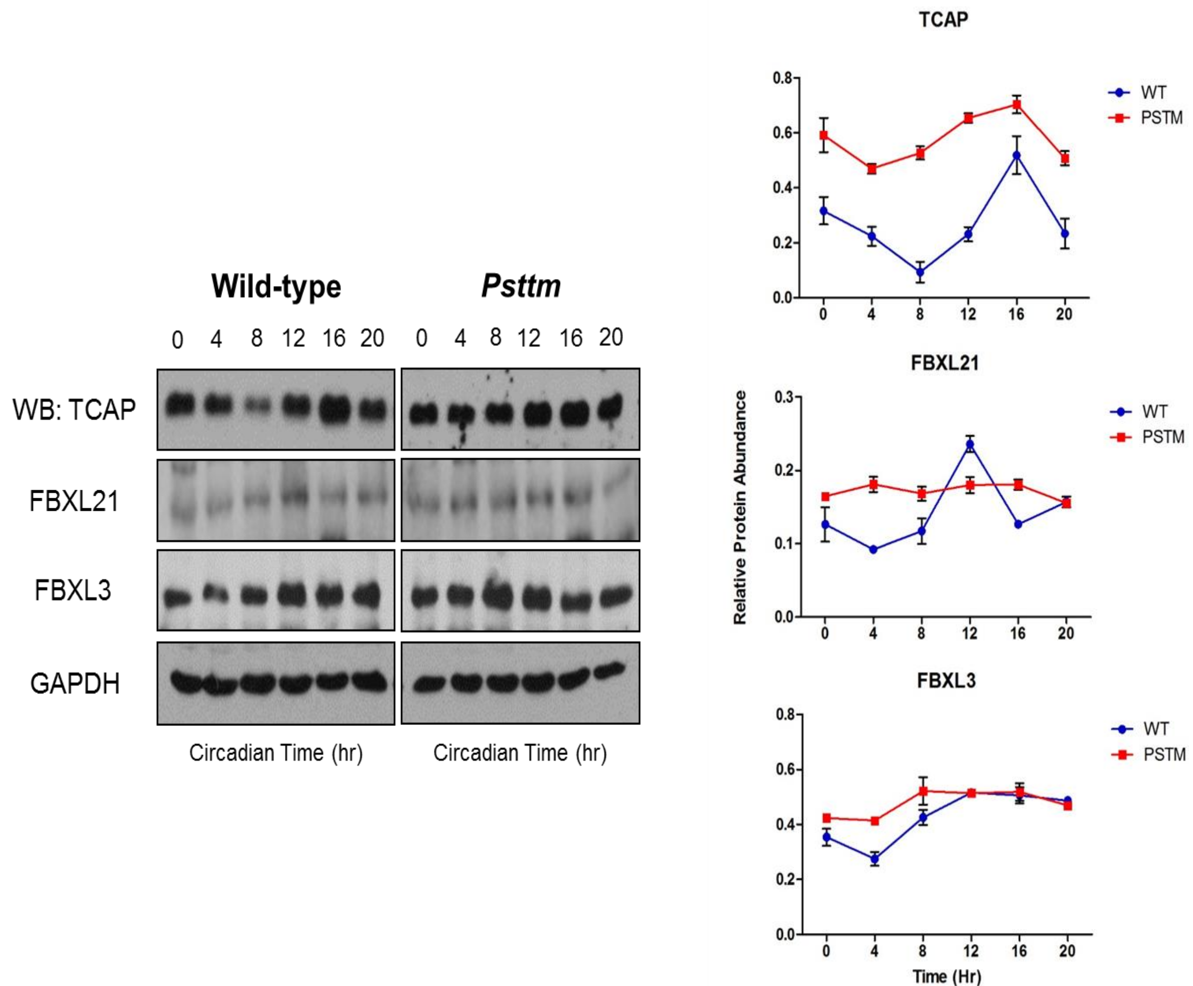

Figure 4: Circadian timing of TCAP protein expression in skeletal muscle. Mice were sacrificed at CT 0, 4, 8, 12, 16, and 20 under infrared lights and Western Blots were performed with whole protein lysate (extracted from *gastrocnemius* muscle) using the indicated antibodies. Representative Western Blots are shown to the left with corresponding quantifications on the right. In the plots, blue data points denote WT mice and red data points represent *PSTM* homozygote protein expression. Two-way ANOVA analysis shows significant ($p<0.001$) differences between WT and *Psttm* protein levels of TCAP and FBXL21. Error bars represent mean ± SEM (n=3).

3.2 Circadian timing of TCAP protein expression in cardiac muscle

TCAP is also a critical regulator of sarcomeric z-disc structure and function in cardiac muscle (heart) (Valle et al 1997). To further characterize TCAP protein expression throughout the circadian cycle, we isolated WT and *Psttm* heart tissue. Again, we observed a circadian oscillation of TCAP protein in WT heart tissue with a peak at CT16 (Figure 6) and significantly elevated levels of TCAP protein throughout the circadian cycle. FBXL21 protein expression displayed a moderate oscillatory pattern across the circadian cycle in WT mice, but was unstable in *Psttm* mice, due to the point mutation in *Fbxl21*. FBXL3 protein showed slight fluctuations in expression throughout the circadian cycle in WT cardiac muscle, but this is not due to circadian regulation. FBXL3 protein expression in *Psttm* cardiac muscle was found to be unstable. Overall, two-way ANOVA analysis showed significant ($p<0.001$) genotype effect on TCAP protein expression, and again TCAP protein expression is elevated throughout the entire circadian cycle in cardiac muscle of *PSTM* homozygous mice compared to *PSTM* WT mice.

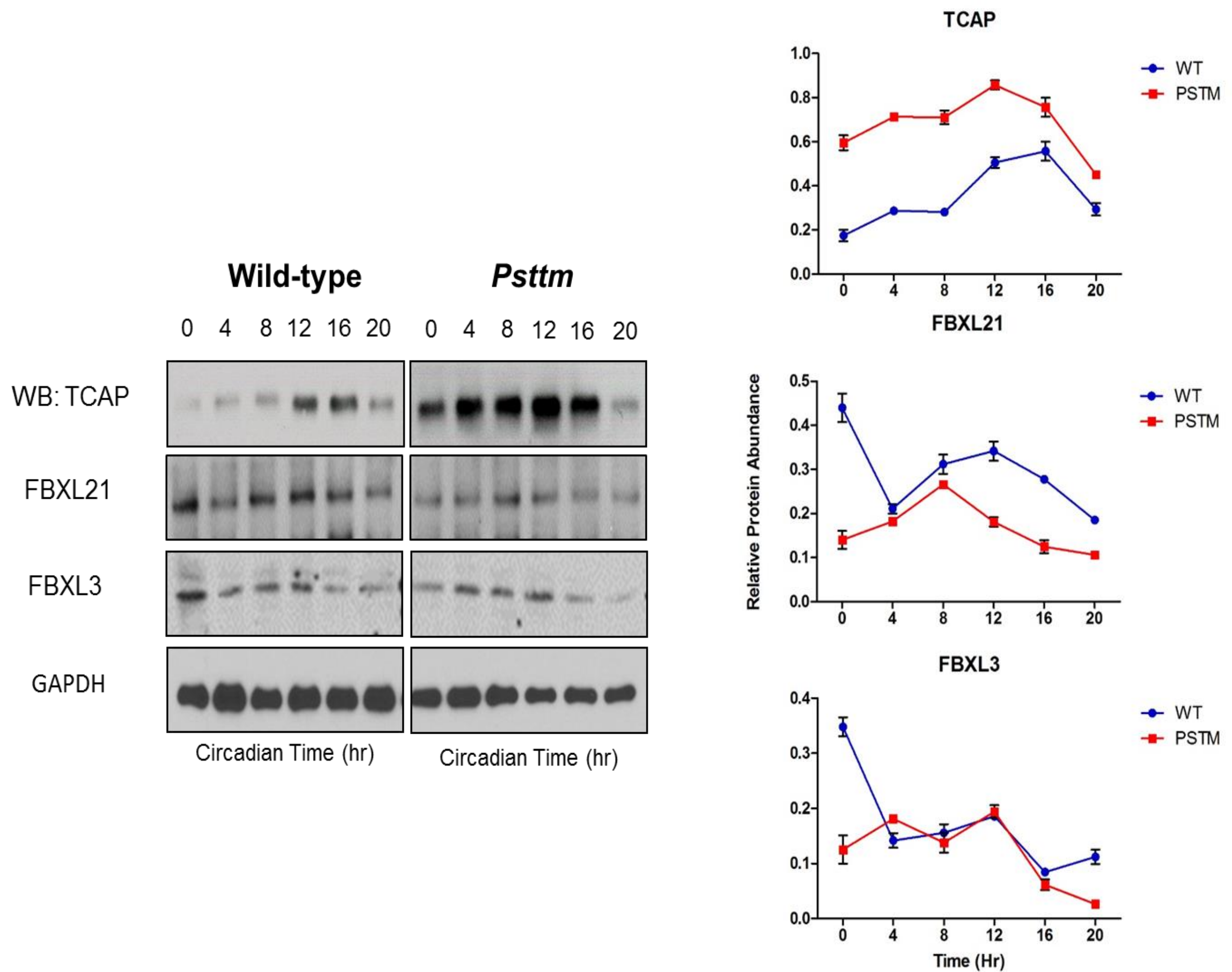

Figure 5: Circadian timing of TCAP protein expression in cardiac muscle. Mice were sacrificed at CT 0, 4, 8, 12, 16, and 20 under infrared lights and Western Blots were performed with whole protein lysate (extracted from heart tissue) using the indicated antibodies. Representative Western Blots are shown to the left with corresponding quantifications on the right. In the plots, blue data points denote WT mice and red data points represent *PSTM* homozygote protein expression. Two-way ANOVA analysis shows significant ($p<0.001$) differences between WT and *Psttm* protein levels of TCAP. Error bars represent mean ± SEM (n=3).

3.3 TCAP degradation by FBXL3 or FBXL21

Based on our Yeast Two Hybrid screening results, we performed a protein half-life experiment to determine the stability of TCAP protein with or without FBXL3 or FBXL21 in HEK293T cells. Unlike the previous study of these two homologous E3 ligases, TCAP was least stable in the presence of FBXL21, suggesting that FBXL21 is the active E3 ligase in TCAP ubiquitin-mediated protein degradation. Although unexpected, this change in E3 ligase activity is reasonable, considering that FBXL21 and TCAP are both either predominantly or completely cytoplasmic proteins, in addition to both being subject to circadian regulation (Yoo et al 2013; Valle et al 1997). Because this experiment was performed in a cell line that lacks physiological relevance to cardiac or skeletal muscle, the half-life experiment was then performed in C2C12 myoblasts. The C2C12 cell line is derived from primary mouse myoblasts and can be differentiated into myotubes, thus making this cell line effective for modeling mammalian myogenesis (Burattini et al 2004).

To measure the half-life of TCAP protein, C2C12 cells were co-transfected with TCAP and either FBXL3 or FBXL21. 24 hours after transfection, cells were treated with cycloheximide (CHX), a protein synthesis inhibitor, which effectively facilitates measurement of protein degradation of pre-existing proteins. Interestingly, our TCAP degradation assay results differed from our preliminary results (in HEK293T cells), which emphasizes the importance of context in characterizing E3 ligase activity paradigms. In 293T cells, FBXL21 was found to be the active E3 ligase, whereas FBXL3 increased TCAP stability increased TCAP protein expression. TCAP protein stability was still negatively affected by FBXL21 co-expression in C2C12 cells; however, FBXL3 was unexpectedly found to also be a

weaker, but still active E3 ligase in TCAP ubiquitin-mediated protein degradation (Figure 7). TCAP, when co-expressed with either FBXL3 or FBXL21, exhibited a decrease in protein stability and therefore a shorter half-life. This differential degradation pattern was noted as early as the 2-hour CHX treatment time point. TCAP was nearly diminished after 6 hours of CHX treatment, so half-life quantification is based on 4 hours of CHX treatment. Here, we observed that both FBXL3 and FBXL21 were active E3 ligases, although FBXL21 was the stronger, more efficient ligase.

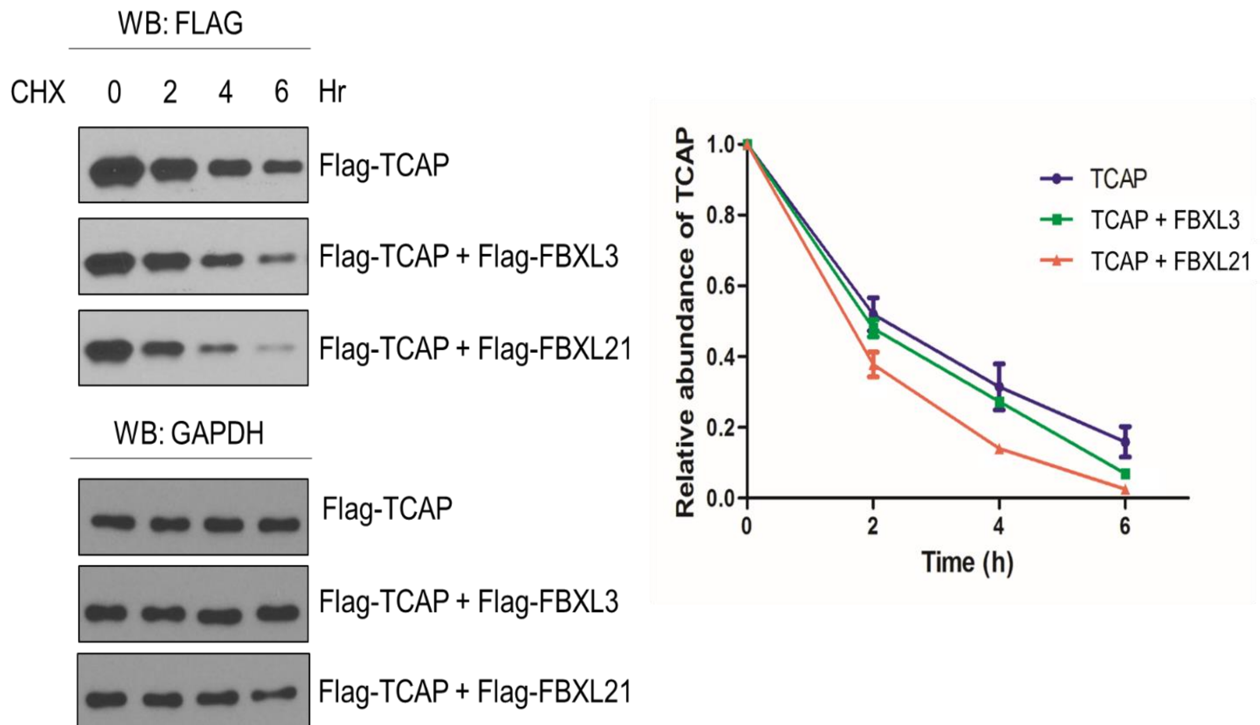

Figure 6: Two homologous E3 ligases expression increases TCAP degradation in C2C12 myoblasts. C2C12 myoblasts were co-transfected with the constructs indicated above. 24 hours later, these cells were treated with 40ug/mL cycloheximide and harvested at either 0, 2, 4, or 6 hours post-treatment. Western Blotting was performed to detect TCAP protein levels under three conditions in addition to GAPDH for loading control. Left: representative Western Blots of TCAP protein expression, bottom: quantification of representative Western Blots. Half-life of TCAP protein was determined by non-linear, one-phase exponential decay analysis. TCAP half-life alone: 2.277 hr, TCAP + FBXL3: 1.914 hr, TCAP + FBXL21: 1.392. One-way ANOVA shows no significant difference in half-lives, but there is a noticeable difference at time points 2 and 4 hr.

3.4 Lentiviral constructs expressing FBXL3 and FBXL21 are functional *in vitro*

To achieve sufficient overexpression in C2C12 cells, we chose a lentiviral system. These lentiviral constructs will be used for future experiments investigating the cellular function and biochemical mechanism of FBXL3/21 in *in vitro* models of skeletal muscle. I have created two lentiviral constructs expressing FBXL3 and FBXL21 by first removing the FBXL3/21 inserts from their original vector, pCMV10-3XFlag (Sigma) and subcloning them into a pFUW lentiviral vector that allows for lentiviral transduction when introduced into HEK293T cells with two packaging vectors, pCMV-deltaR8.91 and pVSV-G. This final aim of creating the lentiviral constructs expressing FBXL3/21 was necessary for further *in vitro* studies.

After subcloning was complete, small aliquots of each plasmid were submitted to GeneWiz for sequencing. Once sequencing was confirmed, plasmids were transduced in HEK293T cells and cells were harvested 24 hours post-transduction. As shown in Figure 8, both FBXL3/21 were successfully subcloned and expressed *in vitro*. Adequate protein levels were detectable via both Western blotting analysis and confocal microscopy.

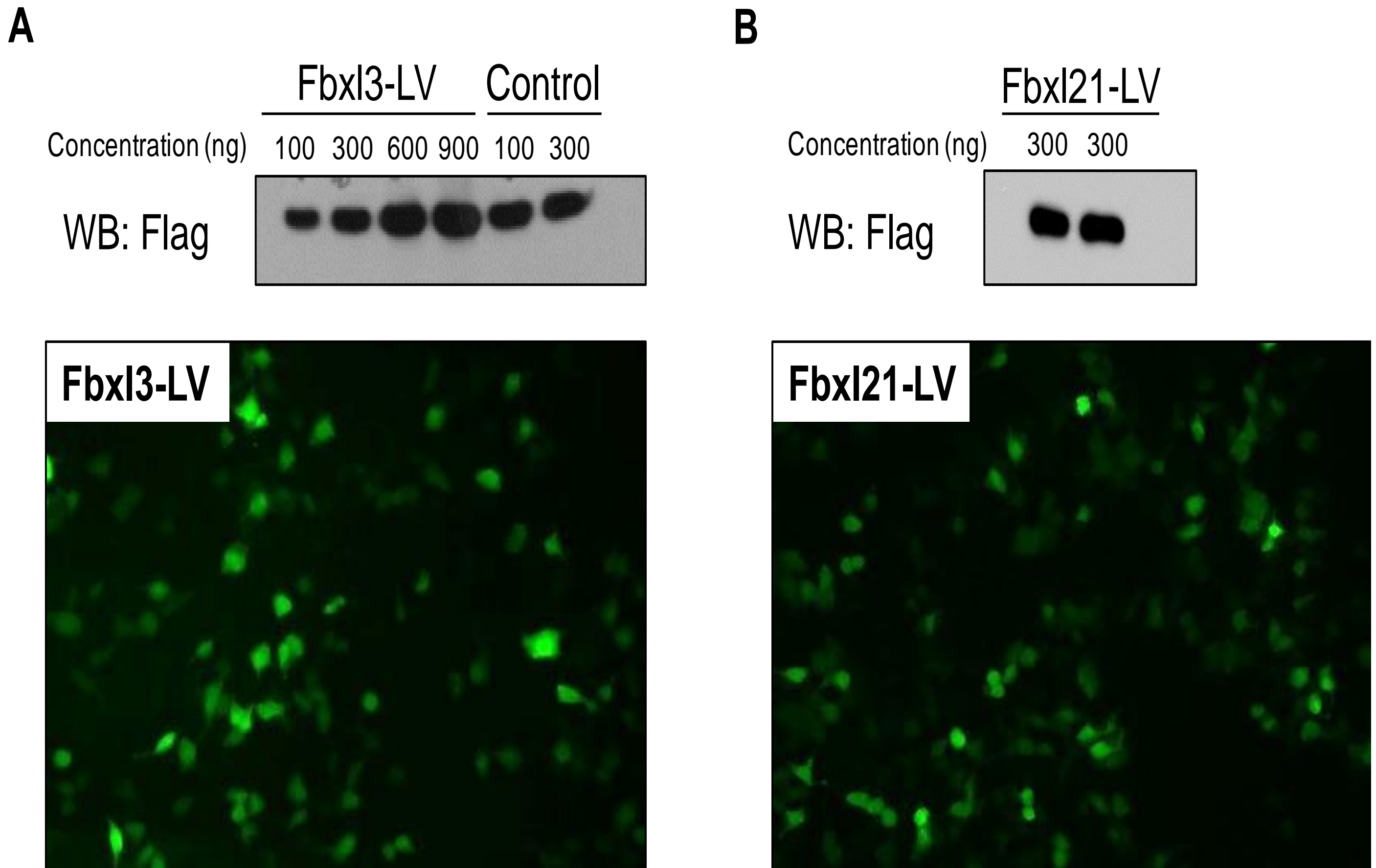

Figure 5: FBXL-expressing lentiviral constructs are functional *in vitro*. (A) top: representative Western Blot of FBXL3, bottom: representative GFP image expressing *Fbxl3* cDNA. (B) top: representative Western Blot of FBXL21, bottom: representative GFP image expressing *Fbxl21* cDNA. Lentiviral constructs expressing FBXL3/21 were transduced in HEK293T cells 17 hours later. 24 hours post-transduction, GFP images were taken and cells were harvested for Western Blotting analysis.

CHAPTER 4: DISCUSSION & FUTURE DIRECTIONS

4. Discussion

There are an estimated 600 E3 ubiquitin ligases in the human genome and the majority of them are understudied (Grabbe et al 2011; Harper et al 2012). Subsets of E3s share high sequence homology, thus allowing them to share one or more protein targets for proteasomal degradation. Yoo et al (2013) found that CRY proteins are substrates of both FBXL3 and FBXL21, two homologous F-box-containing E3 ligases. Localization studies showed that FBXL3 predominantly resides in the nucleus, whereas FBXL21 resides in both the nucleus and the cytoplasm. When present with its substrate, FBXL3 acts as an active E3 ligase, targeting CRY for degradation; however; FBXL21 antagonizes this activity when present in the nucleus and actually protects CRY from degradation. On the other hand, FBXL21 is capable of targeting CRY for proteasomal degradation in the cytoplasm, although less efficiently than FBXL3. This discovery lead to the elucidation of a previously unknown paradigm for homologous mammalian E3 ligases. To further understand the cellular function(s) of this pair, we conducted a Yeast 2-Hybrid screen in skeletal muscle to find additional shared substrates and we identified TCAP as a novel shared target substrate of FBXL3 and FBXL21. We then aimed to elucidate the role of FBXL3 and FBXL21 in regulating muscle physiology via TCAP ubiquitin-mediated protein degradation. Here, we show that the circadian-regulated FBXL21 is the more efficient E3 ligase in TCAP protein degradation, with implications for myogenesis in skeletal muscle.

TCAP protein showed an oscillatory expression pattern, suggesting that it is circadian regulated in both murine skeletal and cardiac muscle. TCAP has been

previously reported as a transcriptional target of circadian machinery, with both protein and gene expression peaking near the early dark phase in murine cardiac muscle (Podobed et al 2014), so our data is consistent with the literature. In both instances, peak expression was observed in the dark phase of the circadian cycle (CT 16), which is also the active phase for mice. Circadian rhythms influence genes that are responsible for transcription, metabolism, and myogenic capacity in skeletal muscle, allowing all of these processes to anticipate environmental changes, which is highly beneficial from an evolutionary perspective (Andrews et al 2010; Chatterjee et al 2011; Zhang et al 2012). In the heart, TCAP is vital to both mechanical and signaling processes (Zou et al 2006) and can lead to contractile and mechanical sensing defects when genetically ablated (Ibrahim et al 2013). Additionally, contractile performance in the heart has been reported to vary over time (Young et al 2001), providing even more evidence that the circadian system heavily influences the muscular system. Taken together, it can be deduced that the circadian system functions to maintain normal cardiac functioning. Based on our differentiation experiments, we assume the same is true for circadian regulation of TCAP protein in skeletal muscle. In *gastrocnemius* muscle, TCAP protein expression peaks at the time that FBXL21 protein expression is lowest, while FBXL3 protein expression remains relatively constant. This finding suggests that TCAP undergoes double circadian regulation—transcriptionally and post-translationally (protein degradation).

The phenotype of the *Psttm* mouse might be interesting to investigate given the proposed relationship of TCAP and FBXL21 in muscle tissue. As previously stated, the *Psttm* mouse has a semidominant, hypomorphic mutation in the *Fbxl21*

gene. This mutation is expected to affect the LRR domain of FBXL21 protein, which is likely to affect substrate recognition and thus, protein-protein interactions (Yoo et al 2013). One striking feature of the *Psttm* mutant mouse is the size difference that is evident from birth. The weaning date is typically extended up to 7 days for these mice because they are smaller than normal pups. Again, skeletal muscle accounts for about 45% of the body (Goodpaster et al 2000; Hoppeler and Fluck 2002), so if there is a deficiency in skeletal muscle development (and possibly function) due to ineffective E3 ligase regulation of TCAP by FBXL21, this could potentially contribute to the low bodyweight observed in *Psttm* mice. Preliminary weight measurements of *Psttm* WT and homozygote littermates (data not shown) are consistent with this observation; further detailed investigation of the skeletal muscle phenotype in *Psttm* mice will be conducted in the future.

Another important finding is that both FBXL3 and FBXL21 appear to act as active E3 ligases in C2C12 myoblasts, although FBXL21 is the more potent of the two (pictured in Figure 8). This was unexpected on two fronts: 1) FBXL3 was the only known active E3 ligase in CRY-targeted proteasomal degradation and 2) our preliminary experiments in 293T cells lead us to believe that only FBXL21 would be the active E3 ligase for TCAP proteasomal degradation. Given the current literature about TCAP's role in cardiac and skeletal muscle, this paradigm is most likely to affect myogenesis & subsequent muscle functioning. Upon further scrutiny of these results, it is quite possible that FBXL3 and FBXL21 are both degrading TCAP protein in these cells because we analyzed the whole cell lysate. TCAP is known to be a cytoplasmic protein (Valle et al 1997); however, we have yet to directly check where

this protein localizes when overexpressed. Limb girdle muscular dystrophy type 2G (LGMD2G) patients have been found to lack TCAP protein in the sarcomeric regions in type 1 and 2 fibers obtained from muscle biopsies, but instead some anti-telethonin fluorescence labeling was present in nuclei. It is, however, important to note that these patients all have autosomal recessive mutations in Telethonin (TCAP) (Vainzof et al 2002). If overexpressed TCAP does translocate to the nucleus at some point, FBXL3 might be a less efficient E3 ligase that only functions in the nucleus, causing us to observe both E3 ligases activities at once. Nevertheless, this finding is interesting and emphasizes the importance of substrate specificity for characterizing E3 ligases and their substrate-ligase paradigm.

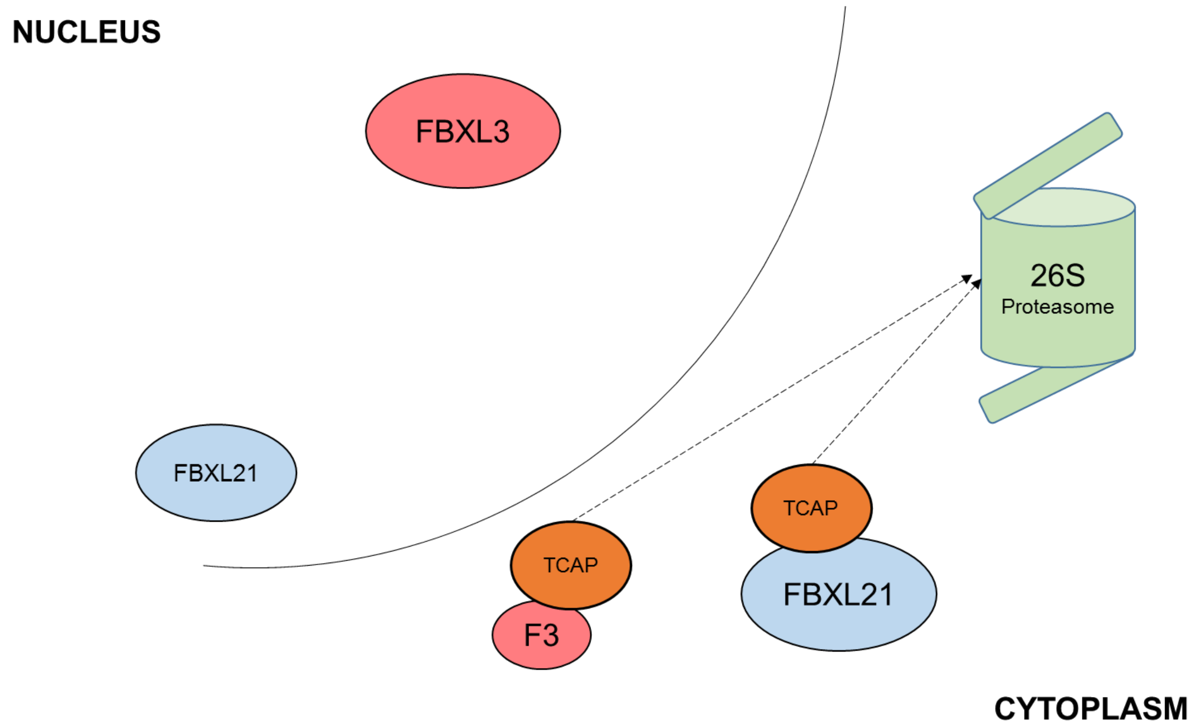

Figure 8: The role of FBXL3 & FBXL21 in muscle physiology. Our data suggests that FBXL3 and FBXL21 both accelerate TCAP protein degradation. Moreover, TCAP is more rapidly degraded in the presence of FBXL21, a protein that is circadian regulated and primarily cytoplasmic. FBXL3 is also capable of promoting proteasomal degradation of TCAP, but to a lesser extent than FBXL21.

Broadly speaking, there are multiple lines of evidence suggesting that skeletal muscle is circadian regulated; however, there is still no definitive answer to the question, “Why is muscle circadian regulated?” Some studies have, however, provided some insight into this phenomenon. CLOCK, an important component of the circadian molecular oscillator, has been shown to play an important role in mitochondrial maintenance in skeletal muscle. Moreover, mice with a genetic disruption in the *Clock* gene exhibited a diminished ability to recover from chronic exercise. Interestingly, this inability to adapt to chronic exercise was reduced after endurance training (Pastore and Hood 1985). Since then, exercise has been shown to be a non-photic entrainment cue of the circadian system as evidenced in Schroeder et al (2012). Scheduled exercise had various effects on wildtype and VIP-deficient mice, but notably it enhanced the phase and amplitude of PER2::LUCIFERASE expression rhythms in both the core and peripheral clocks of the *Per2::Luc* mice. Considering these findings and the many functions of skeletal muscle throughout the body, it is possible that circadian regulation of skeletal muscle has implications for metabolic functioning, circadian rhythm synchrony, locomotor activity, and mitochondrial functioning. The specific role of TCAP in relation to circadian regulation still remains elusive, but it most likely has the largest influence in mechanical stability or mechano-sensing.

4.1 Future Directions

Based on these findings, there are several directions to explore. Although we revealed the role that FBXL3 and FBXL21 play in TCAP protein degradation, the spatiotemporal mechanism has yet to be fully elucidated. Where do the SCF^{TCAP},

SCF^{FBXL3}, and SCF^{FBXL21} complexes localize and at what times do these complexes form? Because both FBXL3 and FBXL21 were found to facilitate degradation of TCAP to similar degrees, we assume that both E3 ligases form SCF complexes; however, this has not been confirmed. We expect that FBXL3 is predominantly present in the nucleus and forms an SCF complex with Skp1 and Cul1, but is a weaker E3 ligase because TCAP is a cytoplasmic protein (Valle et al 1997). We also expect FBXL21 to form an SCF complex in the cytoplasm because it has been found to do so in the past (Yoo et al 2013). In light of the *in vitro* results, a closer examination of the *Past-time* mouse model is also warranted to better understand the physiological impact of TCAP post-translational regulation via FBXL3 and FBXL21 E3 ligase activity. How does the skeletal muscle develop in young and adult *Past-time* (*Fbxl21* mutant) and *Overtime* (*Fbxl3* mutant) mice compared to WT mice? Other potential avenues of investigation include ubiquitin chain linkage type and other shared substrates based on the Yeast 2-Hybrid screen.

Our next experimental aim is to determine whether FBXL3 and/or FBXL21 affects muscle differentiation *in vitro*. We will infect C2C12 myoblast cells with a lentivirus expressing either FBXL3 or FBXL21 and visualize the subsequent effects on myotube differentiation. I have created two lentiviral constructs expressing FBXL3 and FBXL21, so the next step is to transfect these plasmids with two packaging vectors (pCMV-deltaR8.91 and pVSV-G) into 293T cells, harvest the virus and concentrate it with the Lenti-X Concentrator (ClonTech). To objectively measure the effect of overexpressing these E3 ligases, we will harvest muscle cells and measure the expression levels of differentiation markers and endogenous TCAP over the

course of the differentiation process. We hypothesize that FBXL21 overexpression will promote rapid TCAP degradation & therefore hinder muscle differentiation.

TCAP is a newly identified shared target substrate of FBXL3 and FBXL21. It's importance to the anatomy and physiology of skeletal muscle implies that E3 ligase regulation of TCAP degradation serves an important physiological purpose. Interestingly, TCAP protein expression is highest when FBXL21 protein expression is low, which we reason that TCAP accumulation can occur in the absence of its E3 ligase, FBXL21. When co-transfected with TCAP, FBXL3 and FBXL21 both decrease TCAP protein half-life, suggesting that they affect TCAP protein stability via ubiquitin-mediated protein degradation. These results were initially surprising because FBXL3 was the primary, active E3 ligase in CRY ubiquitin-mediated protein degradation. More importantly, the reversal of roles highlights the importance of substrate specificity in characterizing homologous E3 ligase paradigms. These two paradigms may serve as a guide for future mechanistic studies of other understudied E3 ligases.

BIBLIOGRAPHY

Andrews JL, Zhang X, McCarthy JJ, McDearmon EL, Hornberger TA, Russell B, Campbell JS, Arbogast S, Reid MB, Walker JR, Hogenesch JB, Takahashi JS, Esser KA. 2010. CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proc National Academy Science USA* 107, 19090-19095.

Bang, M. L., Centner, T., Fornoff, F., Geach, A. J., Gotthardt, M., McNabb, M., Witt, C. C., Labeit, D., Gregorio, C. C., Granzier, H., et al. 2001. The complete gene sequence of titin, expression of an unusual -700 kDa titin isoform and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ. Res*. 89, 1065–1071.

Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Burratini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. 2004. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *European Journal of Histochemistry*. 48(3)L223-234.

Cardozo, T., and Pagano, M. 2004. The SCF ubiquitin ligase: insights into a molecular machine. Nat. Rev. Mol. Cell Biol. 5, 739–751.

Chatterjee S, Barquero NP, Li L, Ma K. 2011. Circadian clock gene, Bmal1, regulates skeletal muscle metabolism and development. *Endocr Rev.*; 32:3–434.

Deshaies, R. J., and Joazeiro, C. A. 2009. RING domain E3 ubiquitin ligases. *Annu. Rev. Biochem*. 78, 399 – 434

Defronzo RA, Simonson D, Ferrannini E, Barrett E. 1981. Insulin resistance: a universal finding in diabetic states. *Bull Schweiz Akad Med Wiss*.81:223–238.

Febbraio MA, Pedersen BK. 2002. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J.*; 16:1335–1347.

Ferrannini E, Simonson DC, Katz LD, Reichard G Jr, Bevilacqua S, Barrett EJ, Olsson M, DeFronzo RA. 1988. The disposal of an oral glucose load in patients with non-insulin-dependent diabetes. *Metabolism*; 37:79–85.

Frescas D and Pagano M. 2008. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nature Reviews. Cancer.* 8, 438-449.

Goodpaster BH, Kelley DE, Thaete FL, He J, Ross R. 2000. Skeletal muscle attenuation determined by computed tomography is associated with skeletal muscle lipid content. *J Appl Physiol.* 89:104–110.

Grabbe C, Husnjak K, Dikic I. 2011. The spatial and temporal organization of ubiquitin networks. *Nature reviews. Molecular Cell Biology.* 12, 295.

Gregorio, C. C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Susuki, K., Obermayr, F., Herrmann, B., Granzier, H., Sorimachi, H., and Labeit, S. 1998. The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J. Cell Biol.* 143, 1013–1027.

Harfmann BD, Schroder EA, Esser KA 2015. Circadian Rhythms, the Molecular Clock, and Skeletal Muscle. *Journal of Biological Rhythms* 30: 84-94. Doi:10.1177/0748730414561638.

Harper, J. W., Burton, J. L., and Solomon, M. J. 2002. The anaphasepromoting complex: it's not just for mitosis any more. *Genes Dev.* 16, 2179 –2206.

Harper JW, Tan MK. 2012. Understanding cullin-RING E3 biology through proteomics-based substrate identification. *Molecular & Cellular Proteomics: MCP*. 11, 1541.

Hoppeler H, Fluck M. Normal mammalian skeletal muscle and its phenotypic plasticity. J Exp Biol. 2002; 205:2143–2152.

Ibrahim M, Siedlecka U, Buyandelger B, Harada M, Rao C, Moshkov A, Bhargava A, Schneider M, Yacoub MH, Gorelik J, Knoll R, and Terracciano CM. 2013. A critical role for Telethonin in regulating t-tubule structure and function in the mammalian heart. Hum Mol Genet 22: 372–383.

Itoh-Satoh M, Hayashi T, Nishi H, Koga Y, Arimura T, Koyanagi T, Takahashi M, Hohda S, Ueda K, Nouchi T, Hiroe M, Marumo F, Imaizumi T, Yasunami M, and Kimura A. 2002. Titin Mutations as the Molecular Basis for Dilated Cardiomyopathy. *Biochemical and Biophysical Research Communications*. 29,, 385-393.

Jin SM and Youle RJ. 2012. PINK- and Parkin-mediated mitophagy at a glance. *Journal of Cell Science*. 125, 795-799.

Kipreos ET and Pagano M 2000. The F-box protein family. *Genome Biology*

Ko CH J. S. Takahashi. 2006. Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15 Spec No 2, R271

Kobe B, Kajava AV 2001. The leucine-rich repeat as a protein recognition motif. *Curr*

Opin Struct Biol 11:725–732

Koepp, D. M., Harper, J. W., and Elledge, S. J. 1999. How the cyclin became a cyclin: Regulated proteolysis in the cell cycle. *Cell* 97, 431– 434

Lowrey PL, and Takahashi JS 2011. Genetics of circadian rhythms in mammalian model organisms. *Adv. Genet. 74*, 175-230.

Markert CD, Meaney MP, Voelker KA, Grange RW, Dalley HW, Cann JK, Ahmed M, Bishwokarma B, Walker SJ, Yu SX, Brown M, Lawlor MW, Beggs AH, Childers MK. 2010. Functional analysis of the Tcap knockout mouse. *Human Molecular Genetics* 19:2268-83.

Mason P, Bayol S, and Loughna PT. 1999. The novel sarcomeric protein telethonin exhibits developmental and functional regulation. *Biochemical and Biopsychical Research Communications* 257, 699-703.

McCarthy JJ, Andrews JL, McDearmon EL, Campbell KS, Barber BK, Miller BH, Walker JR, Hogenesch JB, Takahashi JS, Esser KA. 2007. Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genom*. 31:86–95.

Moreira ES, Wiltshire TJ, Faulkner G, Nilforoushan A, Vainzof M, Suzuki OT, Valle G, Reeves R, Zatz M, Passos-Bueno MR, Jenne DE. 2000. Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. *Nature Genetics*. 24, - .

Mues, A., van der Ven, P. F. M., Young, P., Furst, D. O., and Gautel, M. 1998. Two immunoglobulin-like domains of the Z-disc portion of titin interact in a conformation-dependen t way with telethonin. *FEBS Lett*. 428, 111–114.

Pastore S and Hood DA. (1985). Endurance training ameliorates the metabolic and performance characteristics of circadian Clock mutant mice. *J Appl Physiol.* 114: 1076-84.

Pizarro A, Hayer K, Lahens NF, Hogenesch JB. 2013. CircaDB: a database of mammalian circadian gene expression profiles. Nucl Acids Res.; 41:D1009–D1013.

Podobed PS, Alibhai FJ, Chow C-W, Martino TA 2014. Circadian Regulation of Myocardial Sarcomeric Titin-cap (Tcap, Telethonin): Identification of Cardiac Clock-Controlled Genes Using Open Access Bioinformatics Data. *PLoS ONE* 9(8): e104907. doi:10.1371/journal.pone.0104907

Ripperger JA, Fritz S, Richter K, Hocke GM, Lottspeich F, Fey GH. 1995. Transcription factors Stat3 and Stat5b are present in rat liver nuclei late in an acute phase response and bind interleukin-6 response elements. J Biol Chem. 270:29998–30006.

Siepka SM, Yoo S-H, Park J, Song W, Kumar V, Hu Y, Lee C, Takahashi JS 2007. Circadian Mutant Overtime Reveals F-box Protein FBXL3 Regulation of Cryptochrome and Period Gene Expression. *Cell* 129, 1011-1023.

Schneider-Poetsch T; Ju J; Eyler DE; Dang Y; Bhat S; Merrick WC; Green R; Shen B; Liu J. 2010. Inhibition of Eukaryotic Translation Elongation by Cycloheximide and Lactimidomycin. *Nature Chemical Biology* 6(3), 209–217. *http://doi.org/10.1038/nchembio.304*

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. "NIH Image to ImageJ: 25 years of image analysis". *Nature Methods* 9, 671-675, 2012.

Schroeder AM, Truong D, Loh DH, Jordan MC, Roos KP, Colwell CS. 2012. Voluntary scheduled exercise alters diurnal rhythms of behavior, physiology and gene expression in wild-type and vasoactive intestinal peptide-deficient mice. *Journal of Physiology* 590: 6213-26.

Valle G, Faulkner G,De Antoni A,Pacchioni B,Pallavicini A,Pandolfo D,Tiso N,Toppo S,Trevisan S and Lanfranchi G 1997. Telethonin, a novel sarcomeric protein of heart and skeletal muscle, *FEBS Letters*, 415, doi: 10.1016/S0014-5793(97)01108-3

Vainzof M, Moreira ES, Suzuki OT, Faulkner G, Valle G, Beggs AH, Carpen O, Ribeiro AF, Zanoteli E, Gurgel-Gianneti J, Tsanasclis AM, Silva HCA, Passos-Bueno MR, Zatz M. 2002. Telethonin protein expression in neuromuscular disorders. *Biochimica et Biophysica Acta (BBA) Molecular Basis of Disease*. 9, 33-40.

Welcker M and Clurman BE. 2008. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nature Reviews. Cancer.* 8, 83-93.

Willems, A. R., Goh, T., Taylor, L., Chernushevich, I., Shevchenko, A., and Tyers, M. (1999) SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 354, 1533–1550

Ye Y and Rape M. 2009. Building ubiquitin chains: E2 enzymes at work. *Nature Reviews. Molecular Cell Biology*. 10, 755-764.

Yoo SH; Mohawk JA; Siepka SM; Shan Y; Huh SK; Hong HK; Kornblum I; Kumar V; Koike N; Xu M; Nussbaum J; Liu X; Chen Z; Chen ZJ; Green CB; Takahashi JS. 2013. Competing E3 Ubiquitin Ligases Govern Circadian Periodicity by Degradation of CRY in Nucleus and Cytoplasm. *Cell* 152(5):1091-105.

Young ME, Razeghi P, Cedars AM, Guthrie PH, Taegtmeyer H 2001. Intrinsic diurnal variations in cardiac metabolism and contractile function. Circ Res 89: 1199–1208.

Zachariae, W., and Nasmyth, K. 1999. Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev*. 13, 2039 –2058

Zhang X, Patel SP, McCarthy JJ, Rabchevsky AG, Goldhamer DJ, Esser KA. 2012. A non-canonical E-box within the MyoD core enhancer is necessary for circadian expression in skeletal muscle. *Nucl Acids Res*. 40:3419–3430.

Zou P, Pinotsis N, Lange S, Song YH, Popov A, et al. 2006. Palindromic assembly of the giant muscle protein titin in the sarcomeric Z-disk. Nature 439: 229–233.

Vita

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