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The Gsk-3**B**-Fbxl21 Axis Regulates Tcap Via Ubiquitin-Mediated Proteasomal Pathway In The Cytoplasm

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THE GSK-3β-FBXL21 AXIS REGULATES TCAP VIA UBIQUITIN-MEDIATED PROTEASOMAL PATHWAY IN THE CYTOPLASM

by

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THE GSK-3β-FBXL21 AXIS REGULATES TCAP VIA UBIQUITIN-MEDIATED PROTEASOMAL PATHWAY IN THE CYTOPLASM

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Protein turnover is one of the most essential mechanisms controlling circadian rhythms. F-Box and Leucine Rich Repeat Protein21 (FBXL21) is a circadian E3 ligase which shows oscillatory mRNA transcripts and protein levels. It was previously found to perform subcellular compartment-specific E3 ligase activities targeting the core clock proteins CRYPTOCHROME(CRY)1/2. Here we identified a new sarcomeric target substrate, Telethonin(TCAP), which also shows circadian oscillation in its mRNA transcript and protein expression and, importantly, interaction with FBXL21 in an anti-phasic manner. Via computational and pharmacological tests, we identified Glycogen Synthase Kinase-3β(GSK-3β) as a regulator of FBXL21. Biochemical and molecular characterizations demonstrated that GSK-3β interacts with both FBXL21 and TCAP and affects Skp, Cullin1(Cul1), F-box containing (SCF) complex/E3 ligase-Substrate complex formation. Interestingly, GSK-3β knockdown significantly inhibited FBXL21-CUL11 and FBXL21-TCAP complex formation. These results revealed a new circadian regulatory pathway that FBXL21 regulates TCAP via ubiquitin-mediated proteasomal pathway in the cytoplasm coordinatively with GSK-3β.

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Abbreviations

CHX; Cycloheximide

CRY; Cryptochrome

CUL1; Cullin1

FBXL3; F-Box and Leucine Rich Repeat Protein 3

FBXL21; F-Box and Leucine Rich Repeat Protein 21

GSK-3β; Glycogen Synthase Kinase-3β

MDM2; Mouse double minute 2 homolog

MuRF1/2; Muscle RING Finger1/2

PKD; Protein Kinase D

SCF complex; Skp1-Cul1-F-box protein complex

TCAP; Telethonin

CHAPTER 1: INTRODUCTION

1.1 Introduction

The ubiquitin proteasome system (UPS) is involved in post-translational regulatory mechanisms for circadian oscillation by controlling protein turnover throughout the circadian cycle (Stojkovic, Wing et al. 2014). Disruption of this system can cause severe physiological consequences such as neurodegenerative diseases and cancers (Welcker and Clurman 2008; Frescas and Pagano 2008; Jin and Youle 2012). The UPS is comprised of serial enzymatic reactions of E1(ubiquitin-activating enzyme), E2(ubiquitin-conjugating enzyme) and E3(ubiquitin ligase) (Hershko, Heller et al. 1983). E1 activates ubiquitin molecule (Ub) in an ATP-dependent manners and activated Ub can form a thioester bond with E2. E2 plays a major role to determine the number of Ub molecules and chain linkage types which will poly-ubiquitinate target proteins (Ye and Rape 2009). E3 ligases recognize and attach Ub to target proteins to regulate its level and activity. However, there are over 600 E3 ligases in the human genome that are not well studied (Deshaies and Joazeiro 2009), due to the transient nature of degradative ubiquitinated targets and typical weak interactions between E3 ligase and its substrate (Harper et al 2012).

F-box domains mediate the formation of Skp1-Cul1-F-box protein (SCF) ubiquitin-ligase complexes, containing Skp1, CULLIN, and F-box proteins for ubiquitin-mediated proteolysis (Kipreos and Pagano 2000). A catalytic core comprised of Cul1 and Rbx1 binds to F-box protein, which has at least one F-box motif, and Skp1, a substrate recognition (SR) module (Feldman et al., 1997; Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999). There are multiple ways to regulate E3 ligase activity at multiple levels. For example, when ubiquitin-like protein NEDD8 (neural-precursor-cell-expressed developmentally down-regulated 8) conjugated to its target protein (Herrmann J et al. 2007), this needdylation at the c-terminus of Cul1 increases SCF E3 ligase activity by inducing a structural rearrangement in its backbone (Lydeard, Schulman et al. 2013), while F-box proteins serve

as a receptor that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex (Skowyra et al., 1997). In contrast to the molecular mechanisms that regulate F-box protein function, upstream cellular signaling for most F-box proteins in SCF complex formation and E3 ligase activation remains largely unclear.

It was previously reported that the E3 ligase FBXL3 and its paralogue FBXL21 exert spatiotemporal regulation on the degradation of the circadian transcription repressor CRYPTOCHROMEs (CRY). In the nucleus, FBXL3 is an active E3 ligase and degrades CRY, but FBXL21 plays an antagonistic role and protects CRY from degradation by sequestering CRY from FBXL3. However, in the cytoplasm, FBXL21 is the active E3 ligase and degrades CRY. FBXL21 level itself is under circadian control, peaking at subjective night while FBXL3 expresses constitutively (Yoo et al 2013). While regulation of FBXL21 activity is largely unknown, previous studies of E3 ligases have highlighted an important role of phosphorylation of both E3 ligases and substrates in ubiquitin-mediated proteasomal degradation (Gao, Inuzuka et al., 2009, Lin, Wang et al., 2009, Kazlauskaite, Kelly et al. 2014, Filipcik, Curry et al. 2017).

Increasing evidence indicates that muscle structure and physiology are subjected to clock regulation (Podobed et al 2014; Moreira et al 2000). It was reported that there are muscle-specific clock-controlled genes that are regulated by the core clock and perform important physiological functions in skeletal muscle. For example, Myogenic Differentiation 1(MyoD1) is regulated by Clock and Bmal1, genetic disruption of each gene, namely Clock (Δ19) and Bmal1(-/-), leads to defects in structure as well as function of skeletal muscle in mice (Andrews et al 2010).

Through yeast 2-hybrid screening using a mouse muscle expression library, our lab identified a sarcomere protein Telethonin, also known as Titin cap or TCAP as a new substrate for FBXL21. TCAP is 19kDa cytoplasmic protein expressed exclusively in the cardiac and skeletal muscle. TCAP interacts with titin through its N-terminal β-sheet to anchor titin to the Z-disc (Gregorio, Trombitas et al. 1998, Mues, van der Ven et al. 1998, Pinotsis, Petoukhov et al. 2006). It is one of the most abundant transcripts in striated muscle (Valle, Faulkner et al. 1997). Interestingly, previous studies reported that TCAP is transcriptionally regulated by the circadian clock (Podobed et al 2014). Clinically it is reported when the c-terminal of Tcap was affected by particular mutations, sarcomeric structure of skeletal muscle is potentially affected then induces physiological defects such as weakened muscle, muscle hypertrophy as well as elevated serum creatine-kinase levels (Moreira et al 2000).

With TCAP as a newly identified substrate of FBXL21, we are expecting to elucidate the mechanism of cytoplasm specific E3 ligase activity of FBXL21 as well as a new circadian regulatory pathway which controls TCAP degradation through SCF complex formation.

1.2 Central hypothesis and specific aims

We hypothesize that FBXL21 is the main E3 ligase for cytoplasmic TCAP degradation in a circadian manner through a phosphorylation-dependent regulatory mechanism.

Specific Aim 1: To determine the circadian interaction between FBXL21 and TCAP *in vivo*. Published and preliminary results strongly suggest a circadian pattern of FBXL21-TCAP interaction. We will collect skeletal muscle tissues throughout the Zeitgeber time course (ZT 0, 4, 8, 12, 16, 20) from wild-type mice, and perform reciprocal co-immunoprecipitation and immunoblotting with TCAP and FBXL21 antibodies respectively.

Specific Aim 2: To map TCAP ubiquitination sites and investigate whether mutant TCAP will be resistant to FBXL21-mediated degradation. We will mutate 5 TCAP lysine sites to arginine which is structurally inactive for ubiquitination. It is expected that mutation of the ubiquitination sites will result in attenuated protein degradation by FBXL21, and this can be confirmed from protein half-life measurement using cycloheximide (CHX).

Specific Aim 3: To screen candidate kinases involved in regulatory phosphorylation and investigate its function in the FBXL21-TCAP interaction. Using a pharmacological approach, we will screen 5 candidate kinases. We will employ half-life and bimolecular fluorescence complementation assays to assess kinase function in TCAP degradation, FBXL21-SCF complex formation, and FBXL21-TCAP interaction.

Our study will demonstrate a regulatory pathway underlying circadian TCAP degradation by FBXL21. These experiments will elucidate a novel mechanism delineate the circadian machinery which can modulate muscle structure and function.

CHAPTER 2: MATERIALS & METHODS

2.1 Mouse strains and tissue collection

C57BL/6J (Stock # 000664) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were group-housed and maintained under Light-Dark: LD 12:12 conditions (transferred to constant darkness: DD only if needed) 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.

C57BL/6J mice were maintained in LD 12:12 and then moved to constant darkness (DD) 36 hours prior to tissue collection. Mice were euthanized by cervical dislocation under infrared lights at ZT (Zeitgeber Time) 0, 4, 8, 12, 16 and 20. Gastrocnemius, tibialis anterior, and quadriceps were harvested under normal white light. Tissues were sectioned, snap-frozen on dry ice, then transferred to pre-chilled 1.5mL micro-centrifuge tubes to prevent proteasomal degradation. Samples were stored in -80°C prior to further experiments.

2.2 Protein extractions

Frozen tissues were immersed in 300uL of cold extraction buffer, chopped with scissors and homogenized with an electric homogenizer for 2 seconds. Then added 200uL of cold extraction buffer and vortexed at 3000rpm for 30 seconds every 5 minutes for a total 20 minutes for full disruption. Samples were then centrifuged at 13000rpm for 20minutes at 4°C. Supernatants were transferred to new pre-chilled 1.5mL tubes. Bradford Assay was performed to quantify as described (Bradford MM. 1976). After measuring optical density of each samples at 595nm on the Tecan plate reader, lysates were diluted with extraction buffer to 300uL with 1.5µg/uL concentration.

2.3 Immunoprecipitations and Immunoblotting

For immunoblotting, 20uL were aliquoted into a new tube with 5x Laemmli loading buffer and stored in -20°C for further processing. For immunoprecipitation, lysates were hybridized with Guinea Pig IgG conjugated with anti-FBXL3(generated as specified in Yoo et al 2013), Rabbit IgG conjugated with anti-FBXL21 (generated as specified in Yoo et al 2013), and Mouse IgG conjugated with anti-TCAP (BD Biosciences) respectively. 10ug of muscle lysates as well as IgG-pulled down samples were resolved by SDS-PAGE and Western Blotting analysis. Antibodies were used from 1:1000 to 1:3000. Relative protein abundance was quantified using ImageJ. Two-way ANOVA was performed to measure differences between TCAP and FBXL21. Bonferroni's multiple comparison test was used to compare among zeitgeber time points using GraphPad Prism (GraphPad Software, La Jolla California USA). Error bars represent the standard error of the mean (SEM) for each time point from three independent replicates.

2.4 Plasmids and Mutagenesis

FBXL3 and FBXL21 cDNA was synthesized as specified in Yoo et al (2013). Full-length Fbxl21 in pCMV10-3XFlag (Sigma) was used to generate mutant forms of Fbxl21. PCR-based site-directed mutagenesis was performed to generate each mutant (Table 1).

2.5 Cell culture and Transfection

293T cell line (ATCC CRL-3216) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin.

For TCAP degradation assay, cells were plated onto 12well plates with 1.5x105 24hours in advance then transfected with indicated constructs using iMFectin (GenDepot).

For bimolecular fluorescence complementation assay, glass covers were coated with poly-L-Lysine prior to cell seeding. 2x105 cells were plated onto 6well plates 24 hours prior to transfection.

2.6 TCAP degradation assay

Media were replaced 16 hours after transfection. 36 hours after transfection, cells were treated with 100μg/mL of cycloheximide (CHX) for 0, 2, 4, and 6 hours. At the end of each indicated treatment time, 293T cells were washed with PBS, pelleted and stored in -80°C until further process. 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).

2.7 Bimolecular Fluorescence Complementation (BiFC) assay

To observe in vitro localization of SCF complex as well as ligase-substrate complex, we employed the bimolecular fluorescence complementation (BiFC) assay as previously described (Kerppola 2006). PCR amplified TCAP cDNA was cloned into KpnI and BamHI sites of VenC. VenN-TCAP or VenN-Fbxls and VenC-Skp1 or VenC-Cullin were transfected into 293T cells using the iMFectin (GenDepot). GSK-3β shRNA (pLKO.1-GSK-3β-1: Addgene 32496, pLKO.1-GSK-3β-2: Addgene 32497) were co-transfected as indicated.

Cells were fixed with 4% paraformaldehyde in PBS for 15 min. Samples were stained with DAPI (1μg/ml), washed twice, immersed with PBS and sealed with top-seal A membrane (Perkin-Elmer). Fluorescence images were acquired on a Axiocam 506 mono (Carl Zeiss) equipped with an inverted 63x 0.45NA UPLFL objective on an Axio Imager M2 Upright Microscope (Carl Zeiss). Seven locations in each slide were selected. Images were taken in the channel sequence of FITC (Ex 493, Em 528), and DAPI (Ex 390, Em 435) filter

sets. For quantification of BiFC results, original image files were imported to ImageJ (NIH) and signal was quantified by. Briefly, cytoplasms were recognized based on FITC fluorescence (regions with no fluorescence considered as background) and corrected total cell fluorescence (CTCF) was calculated by integrated density. About 100 cells were identified and measured in each image, and the average values of cells in one image were obtained for further normalization and statistical analysis. Seven images from each slide were averaged and the experiments were replicated three times.

2.8 Statistical analysis

All statistical analysis was performed in GraphPad Prism (Version 8). Results are presented as mean ± SEM unless otherwise stated. Data were analyzed using student's t-test, one-way ANOVA followed by post-hoc analysis using Dunnett's multiple comparison test or two-way ANOVA followed by post-hoc analysis using Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

Table 1. Site-directed Mutagenesis primers

Flag-TCAP K26R Forward	CTTCTGGGCTGAGTGGAGAGACCTGACTCTGTCTA
Flag-TCAP K26R Reverse	TAGACAGAGTCAGGTCTCTCCACTCAGCCCAGAAG
Flag-TCAP K98R Forward	CATCTTCACGCCCACCAGGGTGGGGGCCTCCAAGG
Flag-TCAP K98R Reverse	CCTTGGAGGCCCCCACCCTGGTGGGCGTGAAGATG
Flag-TCAP K103R Forward	CAAGGTGGGGGCCTCCAGGGAGGAGCGCGAGGAGA
Flag-TCAP K103R Reverse	TCTCCTCGCGCTCCTCCCTGGAGGCCCCCACCTTG
Flag-TCAP K138R Forward	CGTGGCTGAGATCACAAGGCAGCTTCCCCCTGTGG
Flag-TCAP K138R Reverse	CCACAGGGGGAAGCTGCCTTGTGATCTCAGCCACG
Flag-TCAP K148R Forward	GTGCCAGTCAGCAGACCCGGGCCCCTG
Flag-TCAP K148R Reverse	CAGGGGCCCGGGTCTGCTGACTGGCAC

CHAPTER 3: RESULTS

3.1 Circadian time dependent TCAP-FBXL21 interaction with anti-phasic expression

Preliminary experiments with ectopic expressions of FBXL3, FBXL21 and TCAP in 293T cells showed that both FBXL3 and FBXL21 interact with TCAP (Figure 1A). However, FBXL21 dose-dependently destabilized TCAP which suggests that FBXL21 plays as the primary E3 ligase for TCAP (Figure 1B). Considering a strict localization of TCAP (Valle, Faulkner et al. 1997) and cytoplasm-specific E3 ligase function of FBXL21(Hirano, Yumimoto et al. 2013, Yoo, Mohawk et al. 2013) together, we hypothesize that FBXL21 will form a complex with TCAP prior to the degradation of TCAP in a circadian time-dependent manner. Co-immunoprecipitation was performed using gastrocnemius muscle collected from WT C57BL/6 mice during a circadian cycle under 12hr:12hr light:dark (LD) cycle.

We observed oscillatory expressions of FBXL21 and TCAP in an anti-phasic manner (Figure 2A). TCAP peaks at ZT8 and ZT12 while FBXL21 peaks at ZT0,4 and ZT16,20, whereas FBXL3 shows constitutive expression as previously reported (Yoo et al 2013). Proteins expression shows significant statistical difference via Two-way ANOVA ($p < 0.0001$). Bonferroni's multiple comparison test shows significant difference at ZT16 ($p < 0.05$) and ZT20 ($p < 0.01$) (Figure 2B). Interestingly, this anti-phasic expression reflects robust TCAP-FBXL21 circadian interaction which occurs strongly at ZT0 and ZT4, suggest that FBXL21-mediated circadian proteasomal TCAP degradation. Whereas FBXL3 did not show any significant interaction with TCAP throughout the circadian cycle presumably due to the localization of TCAP (strict cytoplasmic) and FBXL3 (dominantly nuclear) (Siepka, Yoo et al. 2007) (Figure 2C). This result suggests that FBXL21-mediated circadian proteasomal degradation of TCAP contributes to the robust TCAP protein oscillation, in addition to the rhythmic *tcap* transcript levels as previously reported (Podobed, Alibhai et al. 2014, Hodge, Zhang et al. 2019).

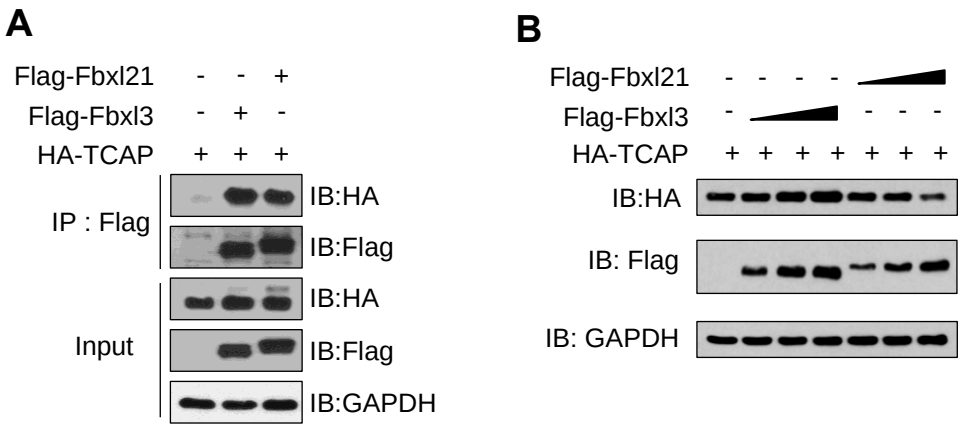

Figure 1. Identification of TCAP as a substrate of FBXL21 *in vitro*

(A) Interaction of FBXL3 and FBXL21 with TCAP. 293T cells were transfected with Flag-Fbxl3, Flag-Fbxl21 and HA-TCAP and immunoprecipitation were performed using anti-Flag antibody (M2). Co-immunoprecipitated proteins were analyzed by Immunoblotting with anti-HA antibody. (B) FBXL21 but not FBXL3 decreases TCAP amount in a dose-dependent manner. 293T cells were co-transfected with indicated constructs. 32 hours after transfection, cells were harvested and subjected to Immunoblotting with indicated antibodies.

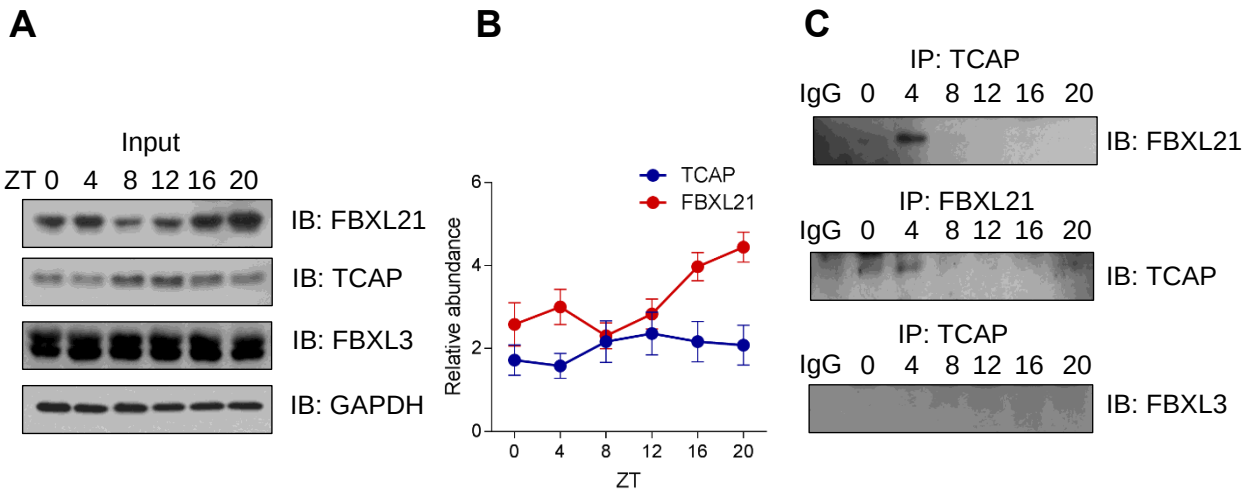

Figure 2. Identification of TCAP as a substrate of FBXL21 *in vivo*

(A) FBXL21 showed anti-phase oscillation with TCAP in skeletal muscle. (B) quantification of TCAP and FBXL21. Error bars represent ±SEM for each time point from three independent replicates. Two-way ANOVA shows significant statistical difference between TCAP and FBXL21 ($p < 0.0001$). Bonferroni's multiple comparison test shows significant difference at ZT16 ($p < 0.05$) and ZT20 ($p < 0.01$). (C) co-immunoprecipitation using FBXL3, FBXL21, TCAP showed circadian time dependent TCAP-FBXL21 interaction.

3.2 Identification of TCAP ubiquitination lysine sites by FBXL21

Preliminary experiments validated that FBXL21 degrades TCAP via ubiquitin-mediated pathway by showing blocked TCAP degradation with MG132(10μg/mL), a proteasome inhibitor treatment (Figure 3A). Through ubiquitination assay with MG132 treatment(10μg/mL), it was observed that co-expression of FBXL21 results to enriched poly-ubiquitinated TCAP compared to TCAP expression itself (Figure 3B) which enabled us to conclude that FBXL21-mediated TCAP degradation is mediated by ubiquitination-dependent proteasomal degradation. With these results, we sought for the ubiquitination sites on TCAP required for its degradation.

There are 5 lysine sites in TCAP (K26, K98, K103, K138, and K148), so first, each of them was converted to arginine individually which is structurally inactive form for ubiquitination (Figure 4A) (Xu and Jaffrey 2014). An endpoint degradation assay was performed with FBXL21 co-expression to see whether mutant TCAPs show attenuated degradation compare to WT. From a primary screen, K26R and K98R mutations conferred relative resistance to FBXL21-mediated TCAP degradation (Figure 4B). Then we generated K26R/K98R mutant and perform CHX chasing assay with FBXL21 co-expression and found this mutant form shows a significant reduced degradation rate (Half-life: TCAP: 5.549, TCAP/FBXL21: 2.299, TCAPK26R/K98R: 19.45, TCAPK26R/K98R /FBXL21: 9.408 hours) compare to WT TCAP (Figure 4C). This result indicates that FBXL21 targets K26 and K98 on TCAP for the ubiquitin-mediated proteasomal degradation.

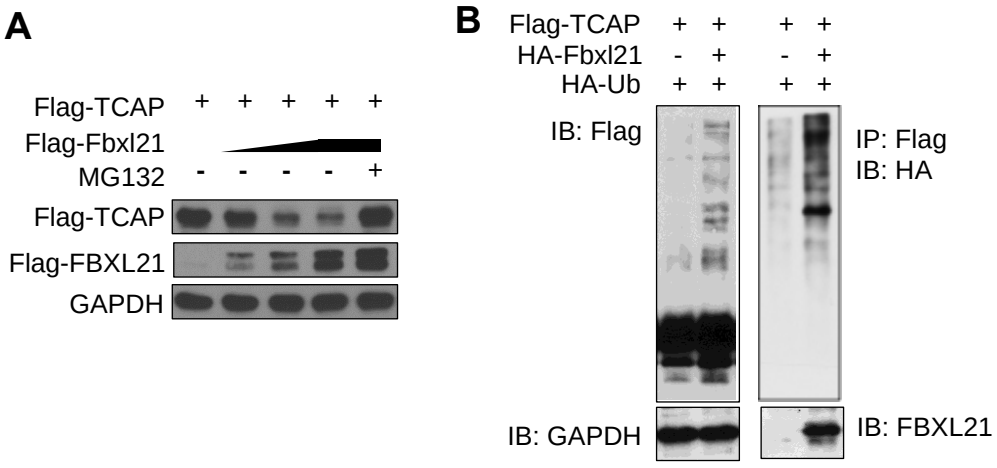

Figure 3. TCAP degradation via ubiquitin-mediated proteasomal pathway

(A) MG132 inhibited TCAP degradation by FBXL21. 293T cells were co-transfected with indicated constructs, 30 hours later cells were treated with MG132 (10μg/ml) for 6 hr. Cells were harvested and subjected to Immunoblotting with indicated antibodies. Right panel: Real-time RT-PCR analysis of hFbxl21 mRNA expression (*, $p < 0.05$) (B) 293T cells were transfected with indicated constructs. Cells were treated with MG132 (10μg/ml) 6 hours before harvest. Cell lysate were immunoprecipitated with Flag antibody and Immunoblotting were performed by indicated antibodies.

Table 2. Lysine sites on TCAP

Telethonin, *Mus musculus*

26
MATSELSCQVSEENQERREAFWAEW**K**DLTLSTRPEEGCSLHEEDTQRHETYHRQ
98 103
GQCQAVVQRSPWLVMRLGILGRGLQEYQLPYQRVLPLPIFTPT**K**VGAS**K**EEREETP
138 148
IQLRELLALETALGGQCVERQDVAEIT**K**QLPPVVPVS**K**PGPLRRTLSRSMSQEAQR

G

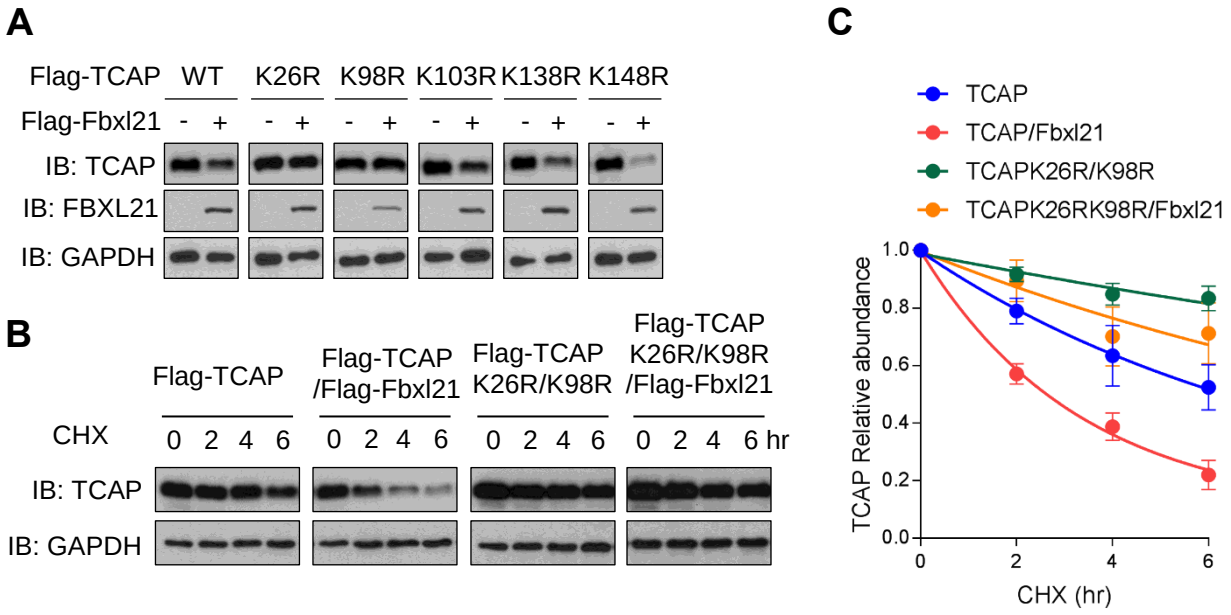

Figure 4. K26R/K98R are ubiquitinated sites on TCAP

(A) The five lysine sites in TCAP were individually mutated to arginine and stability of each K to R mutant was examined by co-transfecting Fbxl21 expression construct into 293T cells. (B) 293T cells were co-transfected with indicated constructs. 32 hours after transfection, cells were treated with 40 μg/ml CHX and incubated for the indicated time before harvest. Immunoblotting was performed to detect TCAP, FBXLs levels using anti-Flag antibody. (C) Quantification of the effect of FBXL21 on wild type TCAP and K26R/K98R stability. Error bars represent mean ±SEM (n=3). Half-life was determined by using nonlinear, one-phase decay analysis (Half-life: TCAP: 5.549, TCAP/FBXL21: 2.299, TCAPK26R/K98R: 19.45, TCAPK26R/K98R /FBXL21: 9.408 hr).

3.3 GSK-3β regulates FBXL21-TCAP and FBXL21-SCF complex formation

We identified that a sacromere protein TCAP is regulated via FBXL21-mediated ubiquitin proteasomal pathway, and TCAP has 2 lysine sites which can be regulated by ubiquitin. However, upstream signaling for F-box proteins, such as FBXL21, remains unstudied. As there are previous studies revealed regulatory phosphorylation in both substrates and E3 ligase (Gao, Inuzuka et al. 2009, Lin, Wang et al. 2009, Kazlauskaite, Kelly et al. 2014, Filipcik, Curry et al. 2017), we were trying to examine the upstream regulatory mechanism for FBXL21.

First, we screened putative phosphorylation sites of FBXL21 using phosphoScan, NetPhos3.1(Blom, Sicheritz-Ponten et al. 2004) which resulted to 5 candidate kinases with relatively high scores (> 0.45); GSK-3; DNA-PK; CDK; PKC; and CK1. We performed a pharmacological assay using specific inhibitors for each kinase (CHIR99021: GSK-3 inhibitor, NU7441: DNA-PK inhibitor, Ro3306: CDK inhibitor, Sotrastaurin: PKC inhibitor, IC261: CK1 inhibitor) to figure out protein degradation effect and found CHIR99021, a pan-GSK-3 inhibitor, shows strongly repressed FBXL21 mediated TCAP degradation (Figure 5A). However, since GSK-3β, not GSK-3α, is known for an essential role in muscle (van der Velden, Schols et al. 2008, Verhees, Schols et al. 2011, Beurel, Grieco et al. 2015), we focused on GSK-3β for sarcomere protein, TCAP degradation.

Second, we performed co-immunoprecipitation to see whether GSK-3β can interact with both FBXL21 and/or TCAP. With co-expression of GSK-3β, both FBXL21 and TCAP can be pulled down (Figure 5B), suggesting they both can be substrates of GSK-3β.

Therefore, we sought for a GSK-3β regulation role on FBXL21-TCAP and FBXL21-SCF complex formation so employed the bimolecular fluorescence complementation (BiFC)

assay as previously described (Kerppola 2006). Split N-, C-terminal venus fluorescence constructs were respectively fused to Fbxl21 (results to VenN-Fbxl21) and Tcap (results to VenC-Tcap). Split form of Venus itself cannot emit fluorescence, however, expression of constructs was confirmed through Western Blotting assay (Figure 7A, 7B). VenN-FBXL21 and VenC-TCAP interacts in the cytoplasm measured at the Venus emission wavelength (528nm). With GSK-3β shRNA co-expression, we were able to observe significantly decreased fluorescence intensity which suggests GSK-3β knockdown inhibits FBXL21-TCAP complex formation (Figure 6A).

To investigate the upstream regulatory mechanism of FBXL21, next we utilized components of SCF complex to examine a role of GSK-3β. First, VenC-CUL1 was used as previously reported (Yoo, Mohawk et al. 2013) whose expression was validated through Western Blotting assay (Figure 7B). FBXL21 and CUL1 forms complex in the cytoplasm primarily and this interaction was significantly impaired with GSK-3β shRNA co-expression (Figure 6B) which suggests GSK-3β might mediate FBXL21 phosphorylation that leads to FBXL21 activation and SCF complex formation regulation. VenC-SKP1 was used to seek whether a default state of SCF complex can also be regulated by GSK-3β. VenC-SKP1 expression was also confirmed through Western Blotting assay (Figure 7C). Interestingly, FBXL21-SKP1 complex formation was not affected by GSK-3β knockdown (Figure 6C). Together, it was able to deduce that the regulatory role of GSK-3β applies to FBXL21-CUL1 complex formation, which is an example of active stage of SCF complex (F-box protein-CUL1), but not to FBXL21-SKP1 complex formation, an example of default state of SCF complex (F-box protein-SKP1) (Reitsma, Liu et al. 2017).

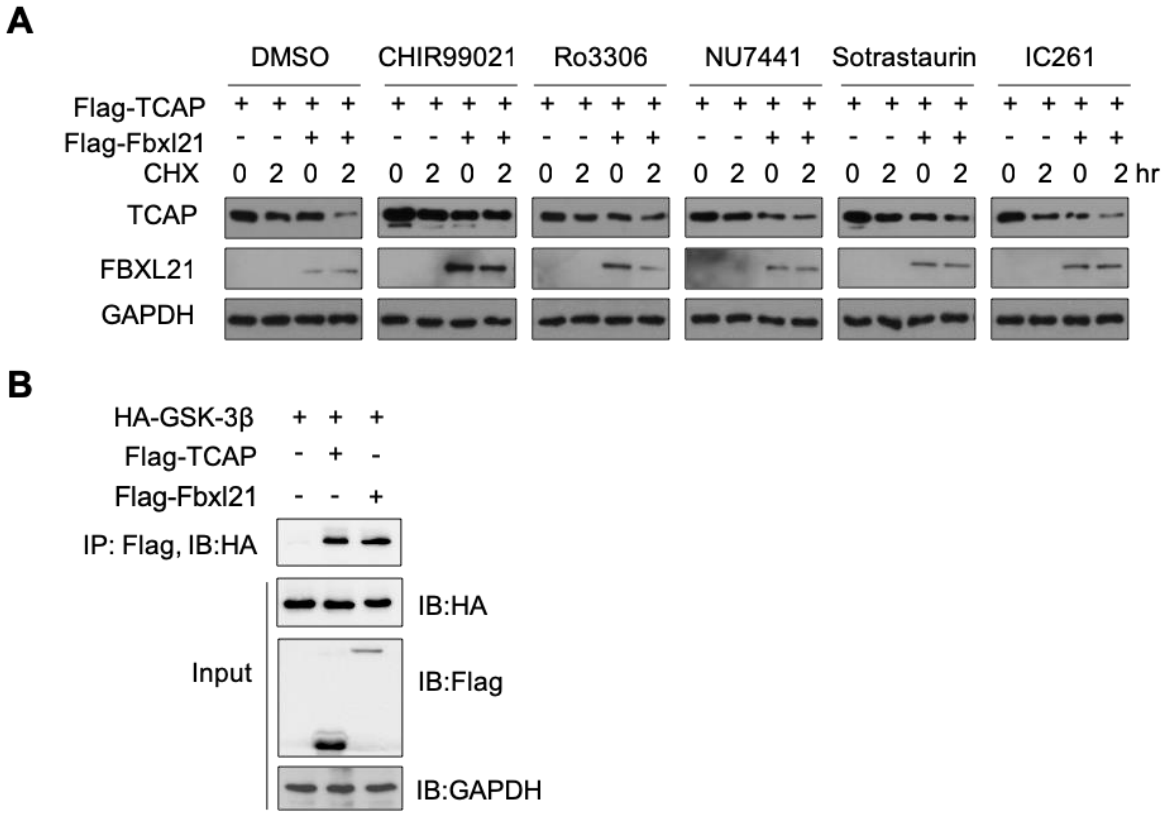

Figure 5. GSK-3β regulates FBXL21-mediated TCAP degradation

(A) 293T cells were co-transfected with indicated constructs. 12 hours after transfection, cells were treated with indicated kinase inhibitors for 24 hours before CHX treatment. Immunoblotting were performed to detect TCAP and FBXL21. (B) Interaction of TCAP and FBXL21 with GSK-3β. 293T cells were transfected with Flag-TCAP, Flag-Fbxl21 and HA-GSK-3β and immunoprecipitation were performed using anti-Flag antibody (M2). Co-immunoprecipitated proteins were analyzed with immunoblotting with anti-HA antibody.

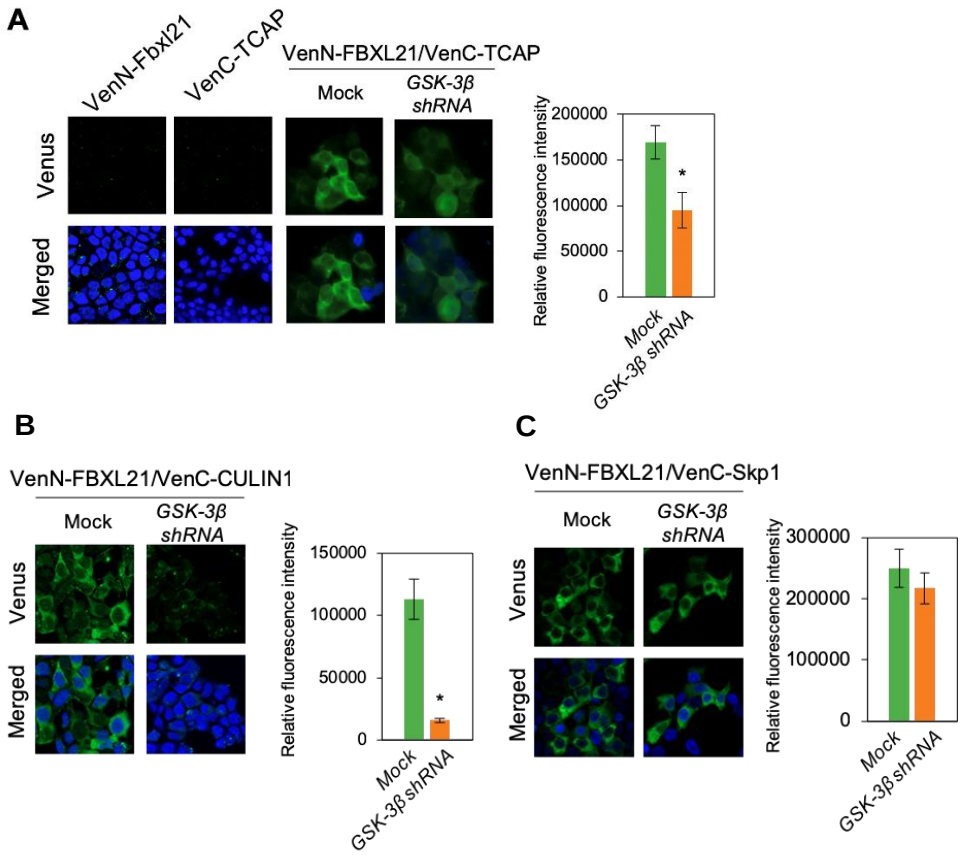

Figure 6. FBXL21-TCAP and SCF complex formation regulated by GSK-3β

(A) TCAP and FBXL21 form complex in the cytoplasm and shGSK-3β co-transfection inhibited complex formation. Right panel: bar graph shows the mean ±SEM of quantification of BiFC signal from three replicate experiments. One-way ANOVA shows the relative fluorescence intensity shows significant statistical difference between mock and GSK-3β shRNA. (***, $p < 0.0001$). (B) FBXL21-CULLIN1 complex formation was significantly inhibited by shGSK-3β co-transfection. Right panel: bar graph show the mean ±SEM of quantification of BiFC signal from three replicate experiments. One-way ANOVA shows significant statistical difference between mock and GSK-3β shRNA (****, $p < 0.0001$). (C) FBXL21-SKP1 complex formation was not affected by shGSK-3β co-transfection. Right panel: bar graph shows the mean ±SEM of quantification of BiFC signal from three replicate experiments. (One-way ANOVA shows the relative fluorescence intensity is statistically not different).

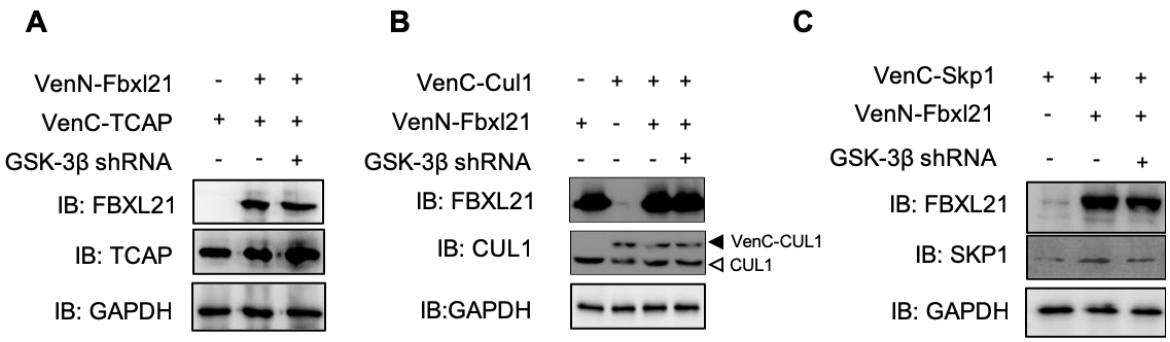

Figure 7. Expression validation of Venus-fused constructs

293T cells were co-transfected with indicated constructs. (A) VenC-TCAP expression validation. 32 hours after transfection, MG132(10μg/mL) was treated 5hours prior to cell harvest. Immunoblotting was performed to detect TCAP (B) VenN-FBXL21 and VenC-CUL1 expression validation. 32 hours after transfection, cells were harvested. Western blotting was performed to detect CUL1 and FBXL21. (C) VenC-SKP1 expression. 32 hours after transfection, cells were harvested. Immunoblotting was performed to detect SKP1

CHAPTER 4: DISCUSSION

4. Discussion

Ubiquitin-mediated proteasomal degradation plays an essential role in regulation at the protein level (Stojkovic, Wing et al. 2014). Previously, two paralogous E3 ligases, FBXL3 and FBXL21, were reported to play antagonistic roles depend on localization onto core clock protein CRY. We focused on FBXL21 due to its interesting localization-specific function; specifically, FBXL21 protects CRY in the nucleus but degrades CRY in the cytoplasm (Yoo, Mohawk et al. 2013). The current project aims to further investigate cytoplasm-specific regulatory function of FBXL21.

Through yeast 2-hybrid assays, TCAP was identified as a new target substrate for FBXL21. Muscle structure and physiology have been studied in terms of clock regulation (Podobed et al 2014; Moreira et al 2000) with evidence showing correlation between muscle-specific clock controlled genes and physiological impairments structurally and functionally (MacLellan et al 2005; Andrews et al 2010; Shavlakadze et al 2013; Podobed et al 2014; Harfmann et al 2015; Mayeuf-Louchart et al 2015). As TCAP strictly exists in the cytoplasm and both FBXL21 and TCAP transcripts levels oscillate throughout the circadian cycle, TCAP was thought to be a good molecule to study localization specific FBXL21 activity.

Here we showed that the circadian E3 ligase FBXL21 interacts with TCAP in an anti-phasic manner consistent with their protein expression patterns. We further demonstrated that the degradation of TCAP was conferred by ubiquitin-mediated proteasomal pathway. Using TCAP mutant forms, we were able to pinpoint sites responsible for ubiquitination. Among the 5 lysine sites, K26 and K98 were found to be responsible for TCAP ubiquitination and TCAP with a missense mutation on each of these sites showed attenuated degradation even in the presence of FBXL21.

We sought to understand regulatory mechanisms of FBXL21 activity and examined a possible crosstalk between ubiquitination and phosphorylation (Gao and Karin et al 2005). Phosphorylation can regulate ubiquitination of protein via multiple mechanisms; in particular, it has been reported that phosphorylation can influence ubiquitination by regulating substrate/ligase interaction at the level of subcellular compartmentalization (Hunter 2007). Through multiple steps of screening, we identified GSK-3β as a kinase interacting with both FBXL21 and TCAP. Previously, protein kinase D(PKD), thymidine kinase(TK), and Ca^{2+}-Calmodulin-dependent Kinase II(CaMK) was reported to phosphor-regulate TCAP in cardiac myocytes (Gautel 2011, Candasamy et al. 2013) while none of kinases have been reported for FBXL21. By employing BiFC assays to directly visualize protein complexes within cells, we determined that not only substrate/ligase complex formation but also SCF complex formation itself can be regulated by GSK-3β. Taken together, experiments aforementioned support our hypothesis by showing a novel mechanism involving GSK-3β mediated co-phosphorylation of a circadian E3 ligase and its cytoplasmic target substrate. Our future experiments will aim to elucidate deeper regulatory mechanisms as well as physiological functions of FBXL21 regulation by GSK-3β in muscle physiology.

TCAP has been studied in relevance to diseases such as Limb-girdle muscular dystrophy and dilated cardiomyopathy (Knoll, Hoshijima et al. 2002, Hershberger, Parks et al. 2008, Markert, Meaney et al. 2010, Hirtle-Lewis, Desbiens et al. 2013, Francis, Sunitha et al. 2014, Chamova, Bichev et al. 2018). Previously, other E3 ligases such as MDM2(Mouse double minute 2 homolog) and MuRF1/2(Muscle RING Finger1) have been found to interact with TCAP. However, MDM2 and MuRF1/2 showed indirect or unsubstantiated degradation mechanisms related to TCAP (Witt, Granzier et al. 2005, Tian, Li et al. 2006, Witt, Witt et al. 2008). In comparison, our study shows FBXL21 as a direct E3 ligase responsible for TCAP proteasomal degradation. On-going experiments will reveal

further regulatory post-translational modification on TCAP to understand its stability for proper structure and function maintenance. Cardiac TCAP has been shown to interact with PKD in terms of maintaining transverse tubule organization and intracellular Ca^{2+} transients in myocytes (Mayans, van der Ven et al. 1998, Candasamy, Haworth et al. 2014); however, PKD selective inhibitor did not affect TCAP phosphorylation (Candasamy, Haworth et al. 2014). This suggests that there may be additional kinases, potentially GSK-3β, involved in TCAP phosphorylation.

According to previous studies, GSK-3β has been shown to phosphorylate five core clock proteins including PER2, CRY2, CLOCK, BMAL1 and REV-ERBα (Yin, Wang et al. 2006, Kaladchibachi, Doble et al. 2007, Spengler, Kuropatwinski et al. 2009, Kurabayashi, Hirota et al. 2010, Sahar, Zocchi et al. 2010). Previous studies emphasized an importance of the UPS system in GSK-3β regulated muscle protein turnover. However, GSK-3 regulated E3 ligase activity and the molecular mechanism of myofibrillar protein degradation have not been reported. As our results unveiled an involvement of GSK-3β mediated regulation of a circadian E3 ligase important for SCF complex formation and FBXL21-substrate complex formation, the FBXL21-GSK-3β axis represents a novel circadian regulatory pathway with a broad physiological function and cellular targets.

In conclusion, my work has demonstrated that TCAP is a bona fide cytoplasmic substrate of the circadian E3 ligase FBXL21. GSK-3β was found to phosphorylate both FBXL21 and TCAP to regulate SCF and FBXL21-TCAP complex formation. Our findings therefore establish FBXL21-GSK-3β as a novel circadian regulatory axis that controls accumulation of a key sarcomere component in skeletal muscle and suggest a broad physiological relevance in other GSK-3β signaling pathways.

Bibilography

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