

# Post-Transcriptional Regulation of Cardiac Sarcomere Protein Titin Through 3' Untranslated Regions

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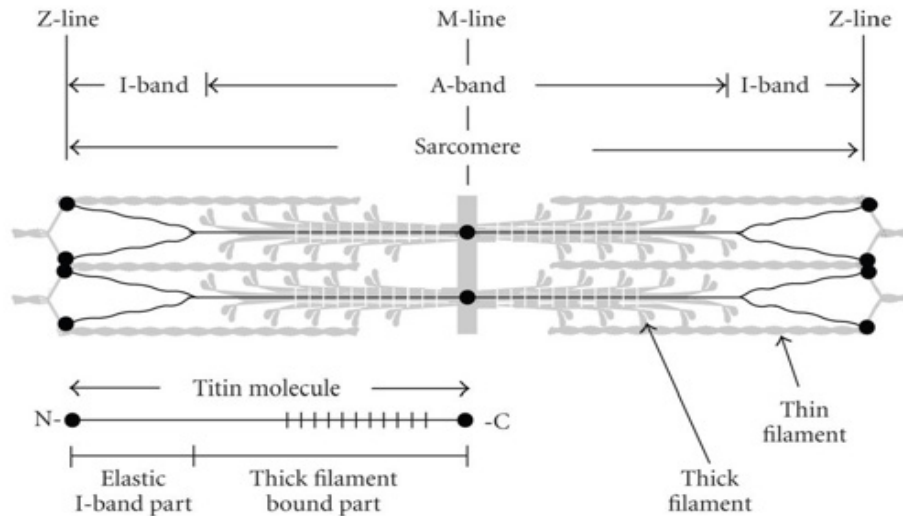
**Titin is the largest known protein in the human body, and forms the backbone of all striated muscle sarcomeres. The elastic nature of titin within each sarcomere is an important component of muscle compliance and functionality. Due to its size, a significant amount of energy is expended to synthesize titin. Thus, we postulate that titin gene expression is under strict regulatory control in order to conserve cellular resources. In general, gene expression is mediated in part by post-transcriptional control elements located within the 5' and 3' untranslated regions (UTRs) of mature mRNA. The 3'UTR in particular contains structural features that affect binding capacity to other RNA components, such as MicroRNA, which control mRNA localization, translation, and degradation. The degree and significance of the regulatory effects mediated by two determined variants of titin's 3' UTR were evaluated in Neonatal Rat Ventricular Myocyte and Human Embryonic Kidney cell lines. Recombinant plasmids to transfect these cell lines were engineered by insertion of the variant titin 3'UTR 431- and 1047-base pairs sequences into luciferase reporter vectors. Quantitative changes in luciferase activity due to the recombinants proportionally represented the effect titin's respective 3'UTR conferred on downstream post-transcriptional expression relative to the control. The effect due to titin's shorter 3'UTR sequence was inconclusive; however, results illustrated that titin's longer 3'UTR sequence caused a 35 percent decrease in luciferase activity. Secondary structural analysis of the two sequences revealed differential folding patterns that could affect the stability and degree of MicroRNA-binding within titin's variant 3'UTR sequences. Such potential changes in titin regulation, via 3'UTRs, impose significant implications for cardiovascular care. In order to maintain striated cardiac muscle under varying disease conditions, titin abundance and turnover must adapt. Although evidence of different titin 3'UTR lengths have not yet been linked to certain disease states, theories are postulated in this study, and catalyze further research to better understand how the cardiac sarcomere protein titin is regulated.**

## INTRODUCTION

In order to better understand how the contractile structure within cardiomyocytes is regulated and turned-over throughout a lifetime, the expression of sarcomeric protein titin was specifically analyzed in this study. Titin, also known as connectin, is the largest identified protein in the human body (Lewinter et al., 2010). Much research shows that titin not only functions as a molecular spring component within the sarcomere of cardiac and skeletal muscle cells, but also serves as a crucial scaffolding protein for normal sarcomere development and maturation (Krüger et al., 2011; Tskhovrebova and Trinick.,2006). As illustrated in Figure 1, titin spans from its N-terminal I-band at the Z-line to its C-terminal A-band at the M-line within the sarcomere structure. Titin's extensive prevalence throughout the sarcomere also allows it to serve as a biomechanical sensor for the maintenance, or turnover, of the myofilament assembly by serving as a template for newly synthesized myosin and actin

filaments (Lim CC and Sawyer DB., 2005).

Although the sarcomere consists of several components, the principle rationale for studying titin expression is not only due to the protein's role in sarcomerogenesis, but more specifically due to this protein's large size (Musa et al., 2006). With a molecular weight of more than three million Daltons, a significant amount of cellular energy is expended to generate and degrade such a protein (Bang et al., 2001). Turnover of titin is therefore likely under more stringent regulation than relatively smaller proteins. Accumulating evidence shows that post-transcriptional regulation of mature mRNA significantly affects the degree of final protein expression (Hofacker, IL., 2007; Mignone et al., 2002). All mRNA transcripts contain three distinct regions as illustrated in Figure 2: a 5' Untranslated Region, the protein-coding sequence, and a 3'Untranslated Region. Translation, degradation, and localization of these mRNA transcripts is in part mediated by control elements located



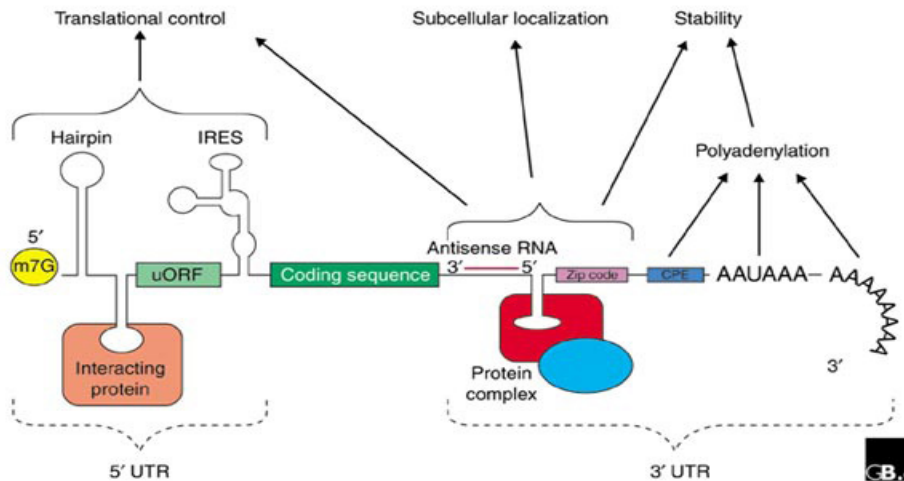
**Figure 1: Schematic representation of titin distribution in the sarcomere (Tsk-hovrebova and Trinick.,2006).**

within the Untranslated Regions (UTRs)(Wilkie et al., 2003).

Studies have illustrated variable effects due to features of the 5'UTR. For example, internal ribosome entry sites (IRES) enhance translation while secondary loops structures and upstream open reading frames (uORFs) have been shown to down-regulate translation of the particular mRNA transcript (Mignone et al., 2002). Analyses of the mRNA regulation due to the 3'UTR, however, are not as comprehensive. It is known that cis-acting elements located in the 3'UTR, allow for binding of microRNAs (miRNA) which regulate mRNA translation. These miRNAs are naturally occurring non-coding RNA sequences of 21-23 nucleotides (nt) that bind to 3'UTR sequences, which have been shown to cause translational repression or mRNA degradation (Mazière P and Enright AJ., 2007; Hofacker, IL., 2007).

Since titin's mRNA is the longest known mature transcript, it is reasonable to infer that several more miRNA binding sites could exist within its 3'UTR to control its stringent regulation (Bear, D., 2012; Mignone et al., 2002). More importantly, substantial differences between the lengths of titin's 3'UTR variants provide insight on this region's role in post-transcriptional regulation of titin and its biological relevance within the cardiac sarcomere.

Given its sheer size, we postulate that titin is a highly regulated protein and that the 3'UTR sequence of its mRNA transcript likely contributes to translational inhibition in order to not produce excess protein under normal conditions. We performed 3' Rapid Amplification of cDNA ends (3'RACE) on *Mus musculus* (mouse) cardiac mRNA (Figure 4) and were able to identify two length variants of 431 and 1047 nucleo-



**Figure 2: Schematic Illustration of mRNA. Differential structural elements and binding sites within the 5'UTR and 3'UTR control post-transcriptional regulation of protein expression (Mignone et al., 2002).**

**Table 1: Outline of Methods and Materials**

	Method	Materials
1	Preparation of reporter vector	pGL3-luciferase reporter vector. NEB restriction enzyme XbaI, buffer 4, and 1X BSA. Promega Quick Blunting Kit (only used for stock to clone Long-3'UTR fragment). Antarctic Phosphatase.
2	Preparation of variant titin 3'UTRs as insert sequences	Short Fragment—Ordered DNA insert sequence of less than 500bp from IDT. Restriction enzyme XbaI, buffer 4, and 1X BSA. Long Fragment—TOPO vector with the cDNA insert sequence for titin's long 3'UTR. NEB restriction enzymes XbaI and EcoRI, buffer 4, and 1X BSA. Promega Quick Blunting Kit.
3	Purification of Vector and insert sequences	Pulsed-Field Electrophoresis machine. 1% agarose gel, ethidium bromide, and 1Kb DNA ladder. Quigen Gel Extraction kit. Nanodrop spectrophotometer.
4	Creation of Recombinant	Promega Quick Ligase Kit. Varying relative concentrations of purified insert and vector.
5	Introduction to host for selection and amplification	DH5alpha electrocompetent cells. SOC medium. LB-agar with ampicillin petri plates. 37°C aerated incubator.
6	PCR screening	Ampicillin-resistant colonies. PCR Machine. Pulsed-Field Electrophoresis machine. 1% agarose gel, ethidium bromide, and 1Kb DNA ladder.
7	Purification of recombinant plasmid	LB-ampicillin media to amplify gel-determined positive clones. 37°C aerated shaking incubator. Quigen Miniprep kit. Nanodrop spectrophotometer. Sanger sequencing facility. Quigen Mediprep kit.
8	Transfection of plasmid to mammalian expression hosts	Purified recombinant plasmid and control pGL3-luciferase reporter vector. Confluent NRVM and HEK cell lines. Lipofectamine. DMEM. Renilla.
9	Measurement of reporter expression	Promega Dual-Luciferase Reporter Assay System. GloMax 96 Microplate Luminometer.

tides (nt) for titin's 3'UTR. Evidence suggests that variable 3'UTR sequences from the same mRNA transcripts causes differences in transcriptional regulation. For example, one study demonstrated that deletions in the 3'UTR of a specific protein's mRNA transcript significantly correlated with overexpression of that protein in mice (Ideka et al., 2011). Similar results were obtained by another study, which found that proliferating cells express mRNAs with shortened 3'UTRs that specifically have fewer miRNA binding sites (Sandberg et al., 2008). Alternatively, longer 3'UTRs could confer greater stability for the mRNA transcript by increased polyadenylation, increasing the probability of expression (Colgan DF, Manley JL., 1997). Secondary structural intra-folding within longer 3'UTR sequences could also cover or sterically hinder miRNA-binding sites that might otherwise be accessible in shorter 3'UTR versions. These hypotheses may explain the results found in another study in which a longer 3'UTR conferred "robust activation of translation from a reporter," whereas this did not occur due to the shorter 3'UTR sequence (Lau et al., 2010).

Recently, potential biological consequences of differences in titin's short and long 3'UTR presence have been investigated (Ji and Tian, 2009; Yang and Kaye, 2009). One study found that "3'UTRs of mRNAs

were generally shortened," during disease conditions such as cardiac hypertrophy (Park et al., 2011). Such a switch in 3'UTRs to a shorter version may affect protein expression through a decrease in miRNA binding sites, as supported by the fact found in the same study that "microRNA target genes were generally depressed" (ibid). These findings suggest that a coordinated mechanism exists to "increase mRNA stability and protein production during hypertrophy," possibly as a compensatory mechanism (ibid).

The functional relevance of the two different titin's 3'UTR sequence lengths on protein expression was investigated in this study through molecular cloning of the sequences into a reporter vector. This allowed for determination if titin's 3'UTRs resulted in altered gene expression, and if this regulatory effect is directional due to the shift in titin 3'UTR length. We hypothesized that titin synthesis is regulated, and specifically repressed, by both length variants of the 3'UTR. Given that the 3'UTR is typically associated with miRNA, which results in translational silencing, we also hypothesized that the shift from the long to short 3'UTR eliminates miRNA binding sites and therefore causes less translational repression.

## METHODS AND MATERIALS

*Mus musculus* (mouse) titin mRNA transcripts from several biological samples were previously sequenced, and subjected to Rapid Amplification of 3' cDNA ends (3'RACE) and anchored-PCR. This reaction was carried out as previously described (Borson et al., 1992). The cDNA products of this reaction on titin's 3'UTR sequences were run alongside a 1Kb ladder on a 1% SDS-agarose gel with ethidium bromide. Separation of the titin 3'UTR variant sequences was shown by UV visualization. Two distinct bands, representing the short and long titin 3'UTR variants, were visualized, and can be seen as the representative image in Figure 3 below in the Results section. These bands were cut from the agarose gel and the cDNA of each band was extracted and purified through use of the Qiagen Gel Extraction Kit. Purified DNA concentrations were measured by a Nanodrop spectrophotometer. Samples were sent to a sequencing facility that utilized the Sanger sequencing methods previously described (Sanger et al., 1997). The cDNA of titin's 3'UTR sequences were cloned into separate TOPO vectors for storage at -80°C.

The molecular cloning process of this project involved insertion of the isolated cDNA fragments from titin's 3'UTR sequences into a reporter vector, and successive expression in mammalian hosts. These steps were outlined in Table 1, and described in further detail below.

### *Preparation of the pGL3-Luciferase Reporter Vector*

A pGL3 reporter vector was ordered from Promega, and linearized by a single restriction enzyme digestion at the XbaI site for one hour at 37°C. This digestion followed the standard Promega protocol (Promega, Restriction Enzyme Protocol., 2012). The XbaI enzyme was then heat inactivated by incubation of the digested product at 65°C for twenty minutes. Reaction of one stock of the linearized vector with a Promega Quick Blunting Polymerase then filled in the 3' and 5' nucleotide overhangs; however, another stock of this purified linearized vector was not treated with Quick Blunting kit and therefore left the linearized vector with cohesive ends due to the XbaI restriction enzyme digest. Both pGL3 vector stocks were treated with Antarctic Phosphatase by following the New England Biolabs (NEB) protocol (NEB, Vector Dephosphorylation Protocol) The prepared linearized pGL3 vector, a control uncut pGL3 vector, and a 1Kb DNA ladder was then subjected to SDS-1% agarose electrophoresis at 90V for 45 minutes. UV-visualization allowed interpretation of the relative DNA sizes and properties. Less-shifted

linear vector was cut from the agarose gel, purified by the Qiagen Gel Extraction Kit with elution in 40uL of double-distilled(dd) water. (Qiagen, QuiQuick Gel Extraction). Final concentration of the purified pGL3 vector was determined by a Nanodrop spectrophotometer.

### *Preparation of cDNA inserts of titin's short and long 3'UTR Recombinants*

The short and long titin cDNA inserts were separately and uniquely prepared. The short 431bp sequence was ordered from Integrated DNA Technologies (IDT) as a "gBlock" (IDT, gBlock Gene Fragments) and subjected to an XbaI restriction enzyme digestion. Varying ratio concentrations of 1:1, 2:1, 3:1, and 6:1 of this digested short 3'UTR "gBlock" insert relative to the prepared stock of non-blunted pGL3 reporter vector were mixed together for the ligation reaction. Ligations proceeded as described by NEB's protocol for its Quick Ligation Kit. Amplification of ligation products was achieved through heat-shock transformation of 50uL of DH5alpha cells. 2uL of the pre-chilled ligation product was added to these electrocompetent cells on ice for 30 minutes. Cells were then subjected to a temperature of 42°C for 30 seconds, and placed on ice for 2 minutes. 450 uL of Super Optimal Broth (SOC media) was then added and cells were placed in a 37°C aerated shaking incubator for one hour. Varying volumes of transformed DH5alpha cells were spread on (LB)-Amp agar plates along with 100uL of SOC media. Plates were then incubated overnight in an aerated incubator at 37°C. Colonies were screened by Polymerase Chain Reaction (PCR) using appropriate flanking primers to amplify the respective titin 3'UTR insert and cycles of the following methods: 7 minutes at 95°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 1 minute at 68°C; then 5 minutes at 58°C. Products were analyzed by Pulsed-Field Electrophoresis and UV-visualization. Positive recombinant clones were purified using Qiagen's MidiPrep Protocol and concentrations were determined by a Nanodrop spectrophotometer.

### *Cell Culture Model*

Neonatal Rat Ventricular myocytes (NRVMs) were harvested from freshly dissected ventricles of 1 to 3 day-old Sprague-Dawley rats. Cells were plated and cultured in Dulbecco's Modified Eagle Media (DMEM), 7% fetal bovine serum (FBS), 1% penicillin and streptomycin). After three days, cells were transferred to low serum media (DMEM + 3.5% FBS). Cell cultures were maintained at 37°C and 5% CO<sub>2</sub> with fresh maintenance media added every 2-3 days. Human



Embryonic Kidney (HEK) cells were also maintained in previously described DMEM cultures and transferred to the low serum media before transfection. All culture media was purchased from Invitrogen, and sera was purchased from Gemini BioProducts. Animal studies were in accordance with Vanderbilt University guidelines. Transfection of the cell lines in 24-well plates followed Invitrogen’s Protocol for Eukaryotic transfection using Lipofectamine2000 (Invitrogen, 2012). In addition, 100uM of Phenylephrine (PE) was introduced a variable condition for both pGL3-Control and pGL3-Recombinant transfections.

After twenty-four hours, media was aspirated from the cultured cells and were rinsed with pre-chilled 1X (PBS). 80uL of 1X Passive Lysis Buffer (PLB) was added to each cell well. This amount of PLB was less than recommended from the Promega Dual-Luciferase Reporter Assay (Promega, Dual-Luciferase Reporter Assay, 2012). Cell incubation and preparation of reagents for the Dual-Luciferase Reporter Assay followed the rest this protocol, and results were quantified using a Promega GlowMax 96 Microplate Luminometer.

**Structural Analysis of Titin 3’UTR Sequences**

Secondary structures of the short and long 3’UTR sequences were analyzed through use of the computer-based program Mfold as previously described (Zuker, M., 2003).

**RESULTS**

Molecular cloning of the titin 3’UTR sequences into the reporter vector allowed analysis of how titin expression is regulated within the two variant parameters. A switch from one of these 3’UTR sequences to

the other could correlate with the biological turnover rate of the cardiac sarcomere. 3’ Rapid Amplification of cDNA ends (3’ RACE) and anchored-Polymerase Chain Reaction (PCR) on *M. musculus* (mouse) titin cardiac mRNA was previously performed in order to effectively amplify and sequentially isolate the 3’ ends of this mRNA. Results identified the two different titin 3’-UTR sequences of 431 and 1047 nucleotides (nt). These results were obtained through ClustalW software, and suggest highly conserved regions of regulatory importance and relevance to a human model. Figure 3 illustrates the 3’RACE-PCR products that were separated by electrophoresis and shown by UV visualization.



Figure 3: 3’UTR Results from Rapid Amplification of cDNA ends (3’ RACE) on *M. musculus* (mouse) cardiac mRNA identified two different titin 3’-UTR sequences of 431 (short) and 1047 (long) nucleotides (nt). These are positioned next to a 1Kb labeled ladder, which was run on the same 1% agarose gel.

The known sequence of the short titin 3’UTR was ordered from IDT as a “gblock.” This insert was digested by XbaI in order to create cohesive ends with the

**PCR Screens of pGL3-Titin Short 3’UTR Construct**

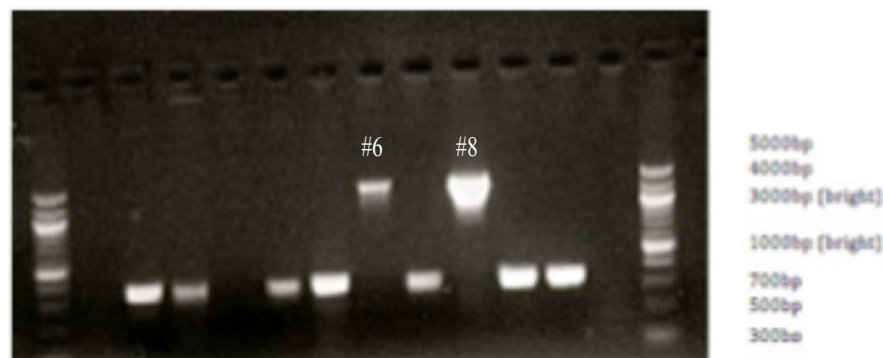
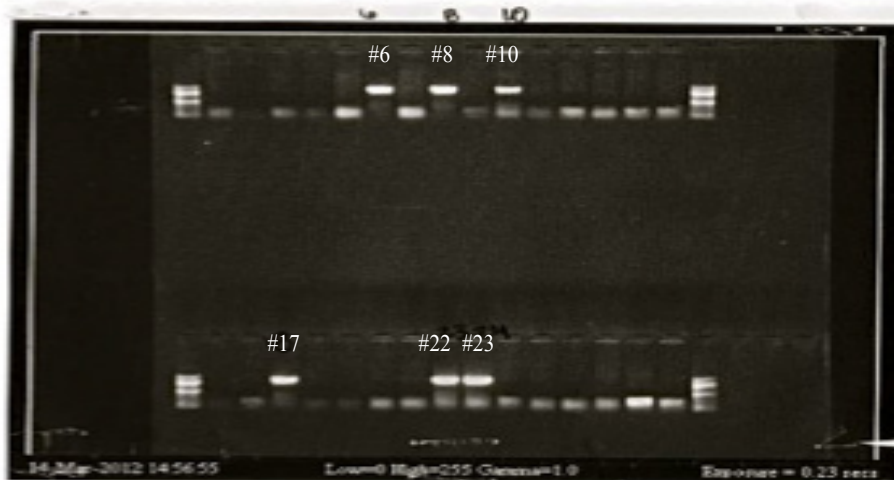


Figure 4: UV-Visualized PCR products of PGL3-short 3’UTR on 1% agarose gel with 1Kb DNA ladder. Gel-determined positive clones (5441bp): 6, 8. Positive clones sequencing results first revealed that the correct titin short 3’UTR insert sequence was detected; however, further sequencing results of clone 6 concluded that the functional luciferase coding sequence of the recombinant plasmid was lost.

## PCR Screens of pGL3-Titin Long 3'UTR Construct



**Figure 5:** UV-Visualized PCR products of PGL3-long 3'UTR on 1% agarose gel with 1Kb DNA ladder. Gel-determined positive clones (6057bp): 6, 8, 10, 18, 23, and 24. Positive clones sent to sequencing and results concluded all clones were the correct pGL3-titin long 3'UTR recombinant plasmid.

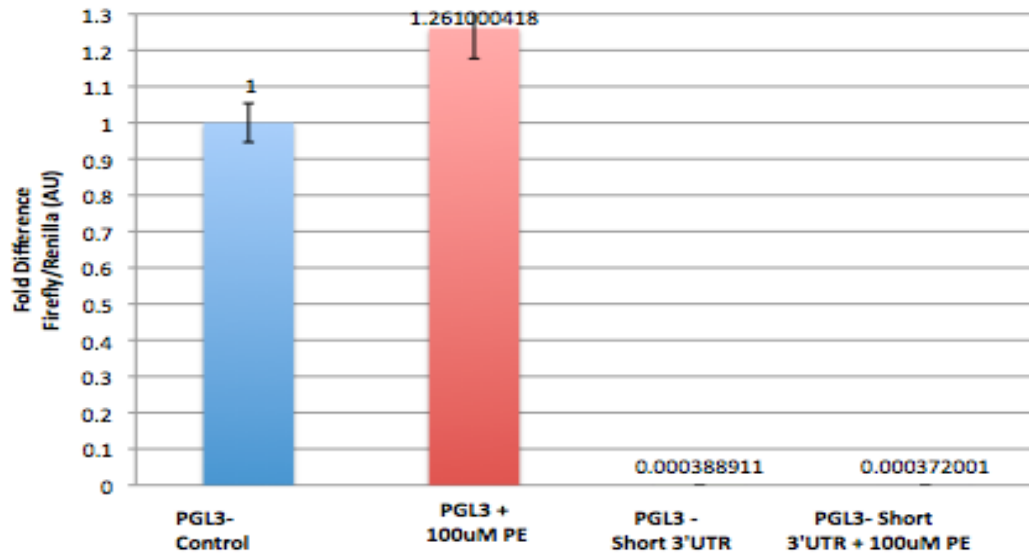
non-blunted pGL3 vector stock, and therefore increase later ligation efficiency. The long titin 3'UTR sequence of 1047bp was too long for generation of a “gBlock” insert, and therefore retrieval of this sequence from a stored TOPO vector was necessary. An XbaI and EcoRI digest was necessary to remove the long 3'UTR. The digested product was analyzed by UV-Visualized on a 1% agarose gel with a 1Kb DNA ladder as a marker. The lower bands of 1047bp were isolated and sequencing confirmed they were the long titin 3'UTR sequence. Blunting of the insert and its vector ends was necessary in order to achieve ligation. Reaction with Antartatic Phosphatase of both pGL3 vector stocks removed 5'phosphates in order to prevent re-ligation of the vector.

Ligation of the insert sequences and appropriate pGL3 reporter vector was set up by varying concentrations of the insert to vector ratio to increase probability of a reaction efficiency. Results concluded that a 3:1 ratio produced the most positive colonies. Ligation products were amplified in DH5alpha electrocompetent cells and PCR of colonies allowed detection of recombinants. Figures 5 and 6 illustrate the UV-Visualized PCR Products of the short and long titin 3'UTR-pGL3 recombinants, respectively. Sequencing of positive long titin 3'UTR recombinants, as seen as “#6” and “#8” in Figure 4, revealed that they were fully correct. Sequencing of positive clones of the short 3'UTR recombinants, as seen as “#6,” “#8,” “#10,” “#17,” “#22,” and “#23” in Figure 5, revealed that the correct titin short 3'UTR insert sequence was detected; however,

further sequencing results concluded that the functional luciferase coding sequence of the recombinant clone number “#6” was lost.

Transfection with recombinant reporter plasmids utilized Neonatal Rat Ventricular Myocytes (NRVMs) as a mammalian model because titin is only found in skeletal and cardiac muscle cells, and therefore specific control elements such as miRNA that bind to titin's 3'UTR are likely localized in this cell line (LeWinter, Granzier., 2010; Hofacker., 2007). Human Embryonic Kidney (HEK) cells were also used a model to determine if titin's 3'UTR sequences affected expression in non-muscular cell lines. Results illustrated that the short titin 3'UTR-pGL3 reporter construct significantly expressed less luciferase relative to the control in both cell lines. Due to the significant degree of inhibition caused by this construct, it was re-sequenced. Significant inhibition of pGL3 Luciferase Expression resulted from insertion of titin's short 3'UTR Sequence encouraged re-sequencing. Results confirmed that the luciferase coding sequence of the short titin 3'UTR construct was disrupted, which was used for transfection of NRVMs and HEK cells. Quantitative results from the pGL3-short titin 3'UTR construct transfection are illustrated in Figures 6 and 7. Figure 6 illustrates results from NRVMs treated under four conditions. The first condition was detection of the Firefly/Renilla expression in the unaltered pGL3 construct, and serves as our control. Other conditions were set as a fold difference from the control's expression level. The second condition is illustrated as the red bar, representing the

### Transfection of NRVMs With Short 3'UTR Construct



**Figure 6: Transfection of NRVMs with pGL3 Control and pGL3-3' Short UTR Construct under conditions of with or without Phenylephrine(PE) (N= 3). Complete inhibition of pGL3 Luciferase Expression with insertion of titin's Short 3'UTR Sequence encouraged re-sequencing. Results confirmed that the luciferase coding sequence of short titin 3'UTR construct used for transfection was disrupted and therefore results from above data are inconclusive.**

increased expression level due to addition of phenylephrine (PE), which increases cellular activity through stimulation of  $\alpha$ -adrenergic receptors on the NRVMs. The third condition introduces the recombinant short 3'UTR pGL3 construct. As shown, significant inhibition of gene expression occurs with this introduction. Even with the fourth condition of NRVMs transfected with the recombinant short 3'UTR and PE, gene expression is not recovered. Although these results support previous studies and our hypothesis that 3'UTRs confer gene expression inhibition, conclusions for the effect that this short 3'UTR construct induces cannot be drawn because re-sequencing of the construct showed that a slight disruption in the coding sequence of the luciferase reporter gene occurred. Although the luciferase gene may still have expressed luciferase properly despite the disruption, this study cannot conclusively determine what effects occurred.

As illustrated in Figure 7, transfection of HEK cells with the sequenced pGL3-titin long 3' UTR construct revealed an average 35% decrease in relative luciferase expression when compared to the control. These results were obtained from two biological samples, and infer that the UTR imposes restricted titin expression. Comparison results with the short 3'UTR construct cannot be compared due to the coding sequence disruption described previously. Only HEK cells were used for the pGL3-titin long 3' UTR construct trans-

fection because isolation of NRVMs for transfection of the same construct failed, and thus preparation of cultured cells was not possible. Plotted error bars in this Figure represent the standard deviation within the data sets. Values of these error bars were 0.03 for the pGL3 "pGL3- Short 3' control samples and 0.02 for the pGL3-long titin 3'UTR construct samples. Not only do these values convey significant results, but also the error bars do so visually. According to Cumming et al., Lack of overlap of the error bars conveys visually that a P value of  $< 0.05$  was achieved, as further determined by the values given above. This fact that space exist between the error bars for the pGL3 control construct results and the pGL3-long 3'UTR results also infers that there is strong evidence that little variance occurs within each data set, and that actual differences between the two data sets exist. (Cumming et al., 2007).

Structural analysis results demonstrate the stability and potential binding capacity of the sequences. MFold determined the most thermodynamically stable secondary structure of titin's two 3'UTR sequences as measured by changes in free energy ( $\Delta G$ ). The most stable structure of titin's short 3'UTR sequence of 431bp had a  $\Delta G$  of -91 kcal/mol, while that of titin's long 3'UTR sequence of 1045bp had a structure with  $\Delta G$  of -272 kcal.mol. Structural results are presented in Figure 8, of which titin's short 3'UTR sequence (A) is also highlighted within the longer 3'UTR structure (B)

### Transfection of HEK Cells With Short and Long 3'UTR Constructs

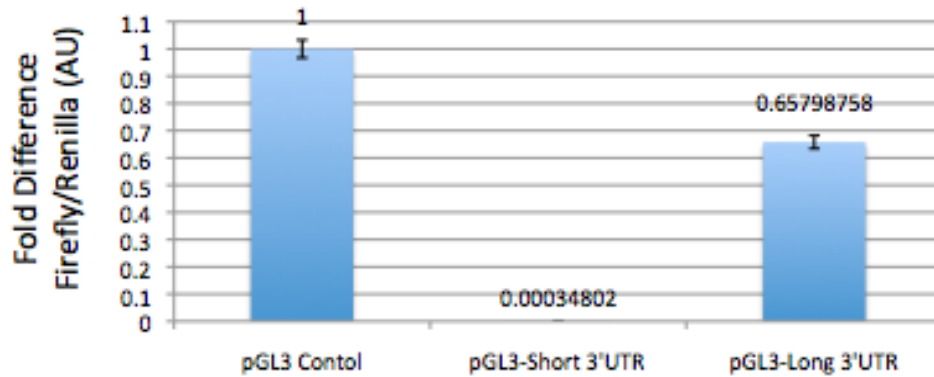


Figure 7: Transfection of HEK Cells with pGL3-Control and Short and Long 3'UTR Constructs. (N=2). StDev of 0.03 and 0.02 for the pGL3 Control and pGL3-Long 3'UTR, respectively. Results from transfection of pGL3-Short 3'UTR construct must be disregarded due to disrupted luciferase coding sequence.

(Mfold Web Server, 2012).

## DISCUSSION

### Interpretation of Results

In order to better understand how the contractile structure within cardiomyocytes is regulated throughout a lifetime, the expression of sarcomeric protein titin due to its 3'UTR was specifically analyzed in this study. We hypothesized that titin synthesis is regulated, and specifically repressed, by both known length variants of its 3'UTR. The two known length variants of *M. musculus* mRNA transcripts were specifically studied not only due to convenience, but more importantly because sequence alignment of titin's 3'UTR for mice, rats, and humans revealed 85% sequence conservation between titin 3'UTR sequences. Thus, *M. musculus* transcripts served as appropriate mammalian representatives to study potential human biology. Given that the 3'UTR is typically associated with miRNA, which results in translational silencing, we also hypothesized that the shift from the long to short 3'UTR eliminates miRNA binding sites and therefore causes less translational repression.

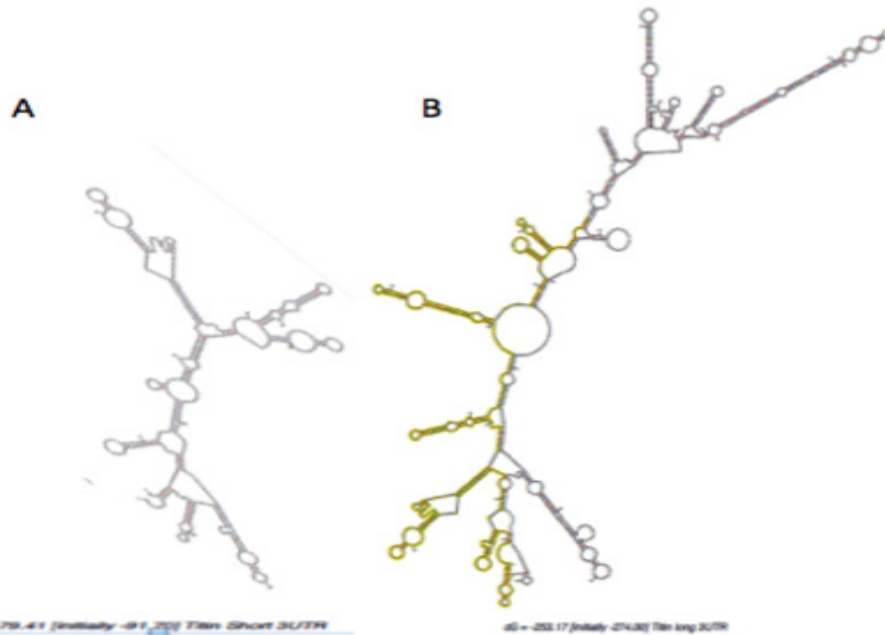
In order to determine how these respective sequences affect expression, a reporter vector and appropriate 3'UTR insert sequences were prepared. A pGL3-luciferase reporter vector was specifically used because it has an easily manipulated XbaI restriction site immediately downstream of the reporter luciferase gene. This serves as the only XbaI site within the vector, allowing it to be linearized by the single-digestion. Purified 3'UTR insert sequences were cloned into prepared linearized pGL3 reporter vectors at the XbaI

site, placing the respective titin 3'UTR sequence with potential microRNA binding sites downstream of the luciferase coding sequence. This technique, and site of insertion in the pGL3 reporter vector, has successfully been used by several studies to determine the effect that insert sequences confer on luciferase expression (Dou et al., 2011). Figure 3 illustrates the structure of the pGL3 control vector, which also contains an SV40 promoter to drive expression in almost all mammalian systems (Promega, pGL3 Luciferase Reporter Vector). The pGL3 reporter vector also contains an antibiotic-resistance gene for ampicillin (Amp), and resulted in selection of clones on the LB-amp plates.

Results from transfection experiments confirmed that the longer titin 3'UTR sequence conferred inhibition of expression, with an average 35% decrease in relative luciferase expression when compared to the control. Only two biological samples of this transfection were obtained, and therefore statistical analysis of these results could illustrate misleading data. In addition, comparative analysis of the short and long titin 3'UTR sequences due to expression in NRVMs and HEK cells was inconclusive. Structural analysis of the two titin 3'UTR variants, however, confirms that although both structures are very stable, the long titin 3'UTR sequence has a more negative change in free energy and is thus more structurally stable.

Due to the fact that sequence alignment of the titin's long 3'UTR in mouse, rat, and human revealed 85 percent conservation between these species, this suggests conservation of important regulatory sequences. It is widely confirmed that miRNA serves as a regulatory element by binding to 3'UTR sequences and inhibiting





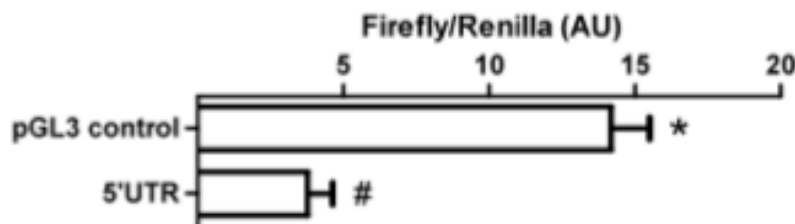
**Figure 8: Most thermodynamically –favored structures as determined by Mfold programming. A—Short titin 3’UTR(431bp) with  $-\Delta G= -91$  kcal/mol. B—Long titin 3’UTR (1047bp) with  $-\Delta G=-272$  kcal/mol. The Short 3’UTR sequence is highlighted within the longer 3’UTR sequence (Mfold Web Server, 2012).**

relative protein expression. More inhibition, therefore, is proportionally related to the amount of functional miRNA binding sites. Whether or not the length of the binding site is related to the overall length titin 3’UTRs is not yet conclusive. The expression and prevalence of specific miRNAs is different for certain types of tissue. Effects of the titin 3’UTR constructs should therefore have different effects on the NRVM and HEK cell lines. Due to disruption of luciferase-coding sequence of the short 3’UTR reporter vector, these effects were not studied. This disruption of the coding sequence within the backbone of the pGL3 reporter vector is likely due to sheering events in the earlier stages of molecular clon-

ing. Before ligations, the linearized pGL3 reporter vector was relatively unstable, and considering the restriction site that opened the vector was immediately after luciferase coding sequence, leaving its coding sequence more vulnerable to degradation. Further plans include engineering of a functionally correct pGL3-titin short 3’UTR construct.

Structural analysis of the titin 3’UTR sequences infers relative stabilities and binding capacity. Previous reports conclude that very stable 5’UTR structures with free energy changes below 50 kcal/mol directly cause a significant decrease in translation (Mignone et al., 2002). The longer titin 3’UTR sequence had a

### Transfection of HL-1 Cells with pGL3 Control Reporter Vector and pGL3-5’UTR Construct



**Figure 9: Previous Transfection of HL-1 cells with pGL3 Control and pGL3-5’UTR Construct. \* pGL3 Control  $p<0.05$  vs. 5’UTR; # $p<0.05$  vs. pGL3 Control. (n=5-6 for each group) (Lim CC et al., unpublished data as of 2012).**

lower free energy change. This offers evidence that the longer 3'UTR may comparatively further inhibit post-transcriptional expression. Both the long and short titin UTRs, however, illustrate very stable and structured sequences. The 3'UTR sequences did illustrate differential binding capacity because the short 3'UTR sequence is covered by the additional nucleotides of titin's long 3'UTR. This effect may cover potential miRNA binding sites and reduce the relative degree of inhibition in expression.

### ***Future Directions***

Given that miRNA binding to 3'UTR sequences of titin causes a degree of inhibition in expression, further studies with up-regulation and knock-out of candidate miRNAs for the two titin 3'UTR sequences could provide more detail to how post-transcriptional regulation of titin is specifically controlled. Targetscan software was used to identify potential miRNA target sites in both the long and short 3'UTR sequences of titin. Overexpression of potential miRNA candidates was successively used in previous studies of miRNA interaction with 3'UTR regions (Dou et al., 2011). If assays reveal that overexpression of a specific miRNA significantly reduces reporter activity, then it is likely that it binds within the 3'UTR and thus inhibits translation of the downstream reporter coding sequence. Furthermore, mutant 3'UTR's can be generated by an overlap extension PCR method. If the activity of a mutated 3'UTR construct is consequently not affected when compared to the control construct, then it is likely that a miRNA binding site was disrupted. These techniques have been used in many studies to determine the relationship between a miRNAs and gene expression (Dou et al., 2011)

A number of studies have demonstrated interactions between 5' and 3' UTR sequences (Doran, G., 2008; Wilkie et al., 2003). Some models illustrate circularized mRNAs in which the 3' UTR binds and modulates events, such as ribosome initiation at the 5'UTR end of the mRNA (Doran, G., 2008). Previous research also demonstrates repression of luciferase activity due to the 5'-UTR sequence when compared to the control SV40-pGL3 reporter (Mignone et al., 2002). Figure 9 illustrates these findings (Lim et al., unpublished data as of 2012). Further studies and creation of a recombinant construct that contains both titin's 3' and 5'UTR sequences may address the possibility that the regulation of titin turnover is in part controlled by the UTRs in concert.

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