

Engineering of a Cellular Model of Freeman-Sheldon Skeletal Muscle Myopathy

Kristen Kokkonen

University of Colorado Boulder, Spring 2013

Defense Date: April 5th, 2013

Department of Molecular, Cellular, and Developmental Biology

Thesis Advisor: Dr. Leslie Leinwand, Department of Molecular, Cellular, and Developmental Biology

Defense Committee:

Dr. Jennifer Martin, Department of Molecular, Cellular, and Developmental Biology

Dr. Kristen Roy, Department of Chemistry and Biochemistry

Abstract:

Freeman-Sheldon Syndrome (FSS) is a distal arthrogryposis syndrome causing muscle contractures of the hands, feet, and face, as well as a small oral opening and an H-shaped dimpling of the chin. In addition, complications such as severe scoliosis, strabismus, and hearing loss are often present in patients. The syndrome has been linked to mutations in the embryonic myosin heavy chain gene (Myh3), which encodes the first molecular motor to be expressed during development. However, while the genetic basis of the disease is known, the mechanism by which mutations in Myh3 lead to the physical symptoms of FSS is poorly understood. Here the generation of an inducible skeletal muscle cell system that allows for precise studies of embryonic myosin's role in developing sarcomeres is described. Using this system, it will be possible to gain a better understanding of the pathogenesis of FSS as caused by mutant embryonic myosin in developing skeletal muscle.

Table of Contents

Abstract.	2
Introduction	4-10
I. Freeman-Sheldon Syndrome and Embryonic Myosin	4-7
II. The Cell System	8-10
Materials and Methods	11-15
Results	15-22
1. Generation of the C ₂ C ₁₂ reporter cell line	15-20
1.1. Isolation of single dox-responsive cells using FACS	16-17
1.2. Quantification of expression in single C ₂ C ₁₂ clones using luciferase assays	18
1.3. Differentiation tests of clone 18-8 luc/GFP	19-20
2. Verification of cassette exchange of luc/GFP for luc/Myh3 in reporter cell line 18-8	20
3. Localization of mutant R672H embryonic myosin in C ₂ C ₁₂ sarcomeres	21-22
Discussion	23-25
Conclusions	25-26
Acknowledgements	26
References	27-28
Supplementary Materials	29-34
I. Plasmid Maps	29-32
II. Primer Sequences	33
III. Multiple Cloning Site Sequence	34
IV. PCR Conditions for Genomic DNA PCR	34

Introduction:

I. Freeman-Sheldon Syndrome and Embryonic Myosin

Freeman-Sheldon Syndrome (FSS) is a severe congenital skeletal muscle syndrome whose mechanism of pathogenesis is currently poorly understood. FSS belongs to the distal arthrogryposis syndromes, and is known specifically as type DA2A (Bamshad et al., 2009). The disease causes muscle contractures of the hands (camptodactyly), feet (clubfeet or talipes equinovarus), and parts of the face, a small oral opening (microstomia), and an H-shaped dimpling of the chin (Figure 1). In addition to these classical characteristics of FSS, 85% of patients have severe scoliosis, 42% have strabismus, and 30% have hearing loss (Stevenson et al., 2006). FSS has been recognized as the most severe distal arthrogryposis syndrome due to the many complications associated with it, and is most typically passed on in a familial dominant pattern (Toydemir et al., 2006). However, cases of recessive inheritance have also been recorded (Alves and Azevedo, 1977; Wang et al., 1987) as well as *de novo* cases (Sánchez et al., 1986; Toydemir et al., 2006).



Figure 1: A father and son with classical FSS characteristics. The H-shaped dimpling of the chin and microstomia, as well as the camptodactyly can be seen (Stevenson et al., 2006).

Mutations in the embryonic myosin heavy chain gene (*Myh3*) have been strongly implicated in this syndrome. Myosins are the motor proteins involved in muscle contraction, and work in a subunit of the muscle known as the sarcomere. Myosins are the most abundant protein in the sarcomere, and they are essential for muscle contraction. ATP binds myosins and is used as an energy source for the movement of the myosins along actin filaments in the sarcomere during muscle contraction. Embryonic myosin is one of three myosin isoforms expressed during fetal development (Lu et al., 1999) (Figure 2). Expression of embryonic myosin is turned off shortly after birth, and is reactivated during adult muscle regeneration. During development, embryonic myosin is expressed throughout the skeletal muscle of embryos (Lu et al., 1999). It has also been shown that embryonic myosin is expressed in the developing heart of chicks (Rutland et al., 2011). Embryonic myosin is part of a gene cluster that also contains the *Ila*, *Ilb*, *Ild/x*, extraocular, and perinatal myosin genes.

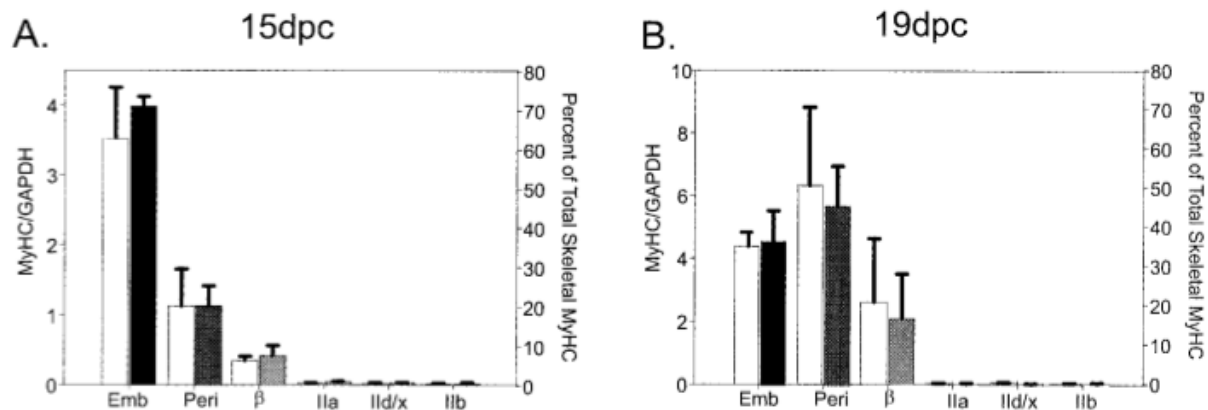


Figure 2: Expression profile of six myosin isoforms in mouse embryos. It can be seen that embryonic myosin (denoted “Emb”) is highly expressed on day 15 of fetal development, and its expression decreases by day 19. “dpc” denotes days post-coitum (Lu et al., 1999).

Several point mutations in the Myh3 gene resulting in single amino acid changes have been directly linked to FSS (Toydemir et al., 2006). The mechanism by which mutations in Myh3 lead to the physical symptoms of FSS is not fully understood. However, most of these mutations are located near the ATP-binding pocket in the motor domain of embryonic myosin (Figure 3), which implies that the mutations interfere with ATP binding, hence decreasing the effectiveness of embryonic myosin during fetal muscle contraction.

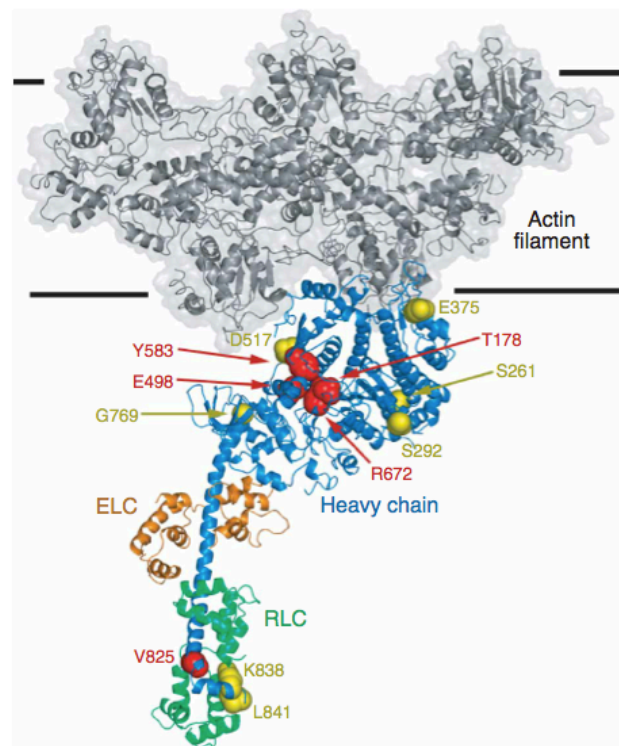


Figure 3: Protein structure of embryonic myosin. Mutations causing FSS are denoted in red. (Toydemir et al., 2006)

The most common of the point mutations in Myh3 that have been linked to FSS are R672C and R672H. These two mutations account for approximately 72% of FSS cases (Toydemir et al., 2006). Both these mutations are located at the same amino acid position, indicating this is a mutational hot spot in the Myh3 gene. Given the high frequency of these two

mutations within the FSS population, this project has concentrated on these two mutants in order to help understand the pathogenesis of FSS.

To date, little in-depth research has been performed on mutations in Myh3 that are associated with FSS. The majority of studies done concerning FSS have been case studies of patients, which detail physical symptoms associated with the syndrome (Stevenson et al., 2006). Immunohistochemical staining of patient muscle biopsies has been rare, and often is done with samples from muscles that are either not affected or only weakly affected by FSS. One larger study by Toydemir et al. involving 28 patients was performed which led to more detailed analysis of the mutations in Myh3 that lead to FSS. In this study, the most common FSS-causing mutations in Myh3 were determined, as well as their relative locations in the embryonic myosin protein. This study has been the only large genetic screen of FSS patients done to date, and has been highly influential as it was the first study to map the mutations in Freeman-Sheldon patients to the embryonic myosin heavy chain gene (Toydemir et al., 2006).

Currently, the only treatment options for FSS patients are postnatal corrective surgeries on the affected distal limbs. However, these surgeries are often complicated, with multiple operations being necessary in order to fully correct the muscle contractures. FSS patients frequently have an increased sensitivity to anesthesia (Laishley and Roy, 1986) making operations more risky than for normal patients. Due to this and the various other complications of living with FSS, insight into the mechanisms of FSS could be invaluable for producing better therapies for this syndrome in the future, as well as gaining a general knowledge of the role of embryonic myosin in human disease.

II. The Cell System

This project involved the generation of a unique cell system via integration of a reporter gene cassette into the genome of cells using a retrovirus. The reporter gene cassette contains the luciferase and GFP genes, which allow for screening of cells both by biochemical luminescence and fluorescence. These two genes are under the control of Tet-On promoter. The salient feature of the cell system is the ability to replace the stably integrated reporter gene cassette with a cassette containing a gene of interest (in this case, the embryonic myosin heavy chain gene, Myh3). This cassette exchange is mediated by Flp-recombinase, and so is known as recombinase mediated cassette exchange (RMCE) (Schlake and Bode, 1994). In order to use Flp-recombinase to mediate cassette exchange, the cassette includes two unique Flp recombination target (FRT) sites at the ends (Figure 4). This allows for direction-specific insertion of cassettes.

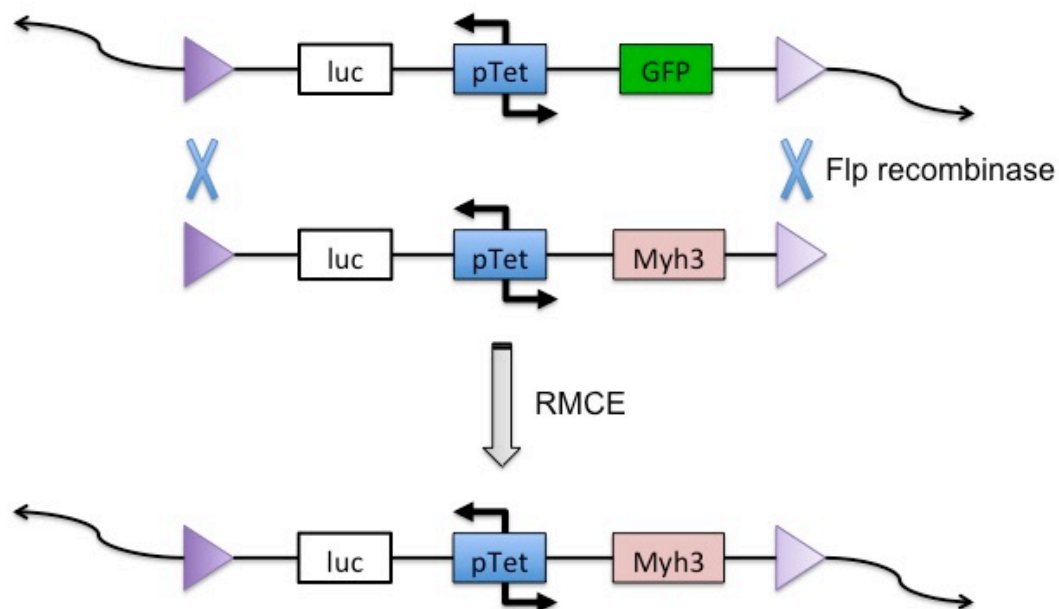


Figure 4: Diagram of recombinase-mediated cassette exchange (RMCE) in the C₂C₁₂ cell line. The luc/GFP reporter gene cassette is shown being exchanged for the luc/Myh3 cassette. The two different purple triangles represent the two different FRT sites flanking the cassette, with light purple being wild-type FRT and dark purple being the variant FRT3.

The cell system also features the Tet-On promoter, which allows for fine-tuned control of gene expression from the cassette. The Tet-On promoter is controlled by the addition of doxycycline (dox). When dox is added, the rtTA transactivator binds to the Tet-On promoter to induce expression (Gossen and Bujard, 1992) (Figure 5A and B). The Tet-On promoter used in this system is bi-directional, which allows for expression of both the gene of interest and a reporter gene, such as luciferase. Being able to induce expression from the Tet-On promoter, along with the ability to use RMCE to insert cassettes containing genes of interest, makes this cell system an ideal way of studying FSS at the sarcomere level of skeletal muscle.

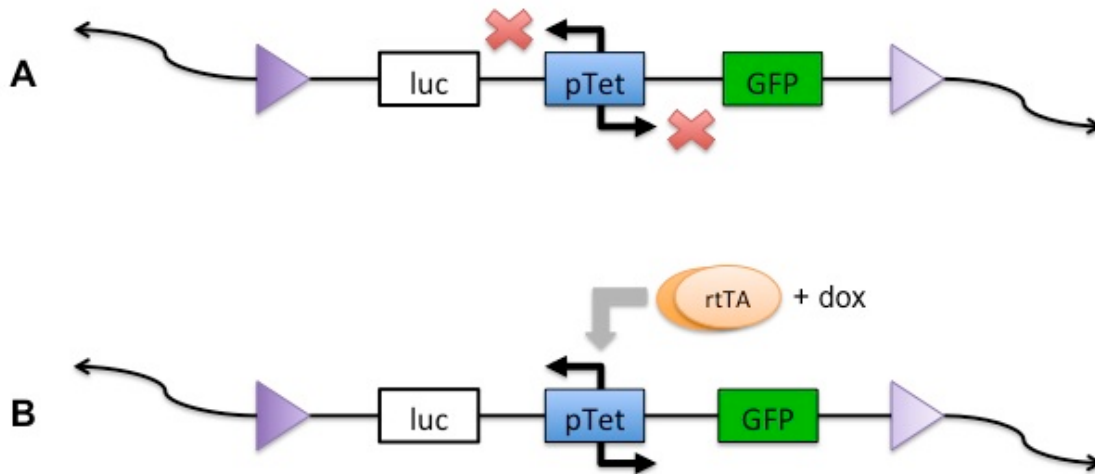


Figure 5: Diagram of how the Tet-On promoter works. A) In the absence of dox, the Tet-On promoter is not active, and gene expression does not occur (indicated by the red “X”). B) In the presence of dox, the transactivator rtTA activates the Tet-On promoter, and gene expression occurs.

The specific cell lines generated in this project were derived from the C₂C₁₂ mouse skeletal muscle cell line. These cells can be differentiated from myoblasts into myotubes, which allows for studies of sarcomere structure and function without the use of a model organism. The cell lines are derived from a isogenic population of cells, making expression from the integrated

cassette uniform throughout the cell population. This eliminates any uncertainty in results due to variation between different cells in their expression profiles, as all cell lines have identical integration sites of the cassette in the genome. The integrated cassette contains the luciferase gene, as well as the mutant or wild-type Myh3 gene. The Myh3 gene has an added Myc tag to allow for the transgene embryonic myosin to be distinguished from the endogenous myosins in the cells.

Ultimately this cell system comprises only one part of the approach towards investigating embryonic myosin's role in FSS, as cell culture cannot address the physiological role of embryonic myosin in an organism. In order to address this, transgenic mouse lines have been generated to facilitate studies of FSS in the setting of a model organism. The mice provide an *in vivo* approach for elucidating the mechanism of FSS, while the cell system model provides an *in vitro* look at the disease. Additionally, biochemical assays are being performed to further understand the behavior of embryonic myosin, both in its wild-type and mutant forms. In this thesis, however, the discussion will be limited to development and studies of the *in vitro* cell system.

Materials and Methods:

Overview of Cell System Generation

The following flowchart provides an overview of the process of generating the cell system as well as the studies done at each step (Figure 6). Detailed methods for the entire process follow this overview.

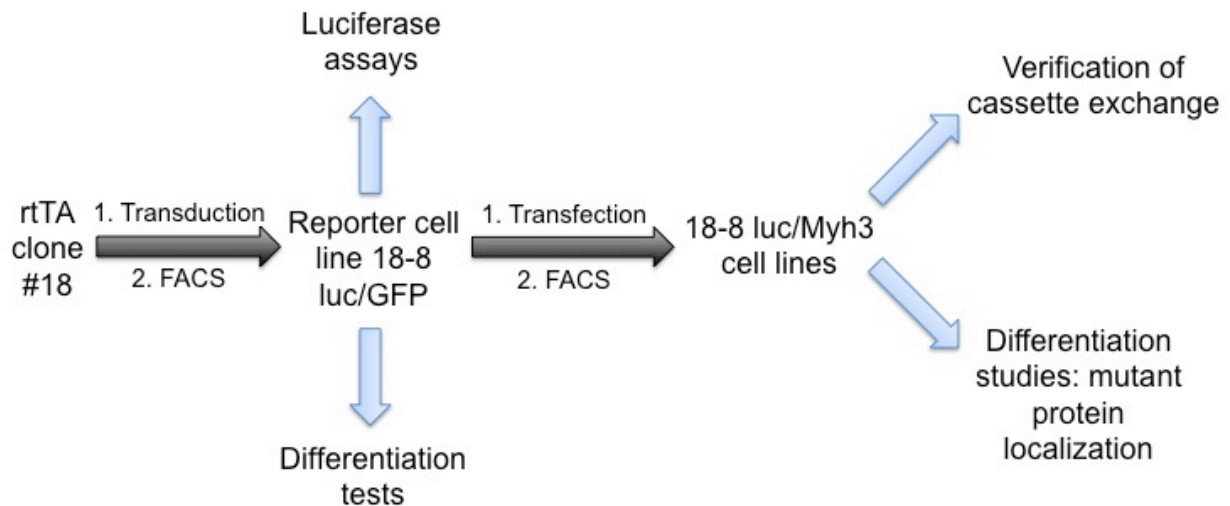


Figure 6: A flowchart overview of the generation of the cell system. Black arrows represent major procedural steps; blue arrows represent studies done to characterize the indicated cell lines.

Cell Culture

For normal growth, C₂C₁₂ myoblasts were cultured in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) containing 20% FBS, 100U penicillin/streptomycin, 4 mM L-glutamine, and 1 mM sodium pyruvate (overall, referred to as "growth media"). For differentiation, C₂C₁₂ myotubes were cultured in DMEM containing 2% horse serum, 100U penicillin/streptomycin, 4 mM L-glutamine, and 1 mM sodium pyruvate (overall, referred to as "differentiation media").

During retroviral transduction, HEK293T cells were cultured in DMEM containing 10% heat-inactivated FBS, 100U penicillin/streptomycin, 4 mM L-glutamine, and 1 mM sodium pyruvate.

All cells were kept in incubators set to 37° C and 5% CO₂.

Plasmid Constructs

The constructs pVPack-vsug, pVPack-gagpol, SF2cLM2CGFRT3, pCAGGS-Flpe-IRES-Puro, and pMefc2Myo-mouseMyh3 (WT, RC, or RH) were graciously provided by Ina Weidenfeld. The constructs SF2-luc/Myh3 (WT, R672C, or R672H) FRT3 were derived from the parent construct SF2cLM2CgFRT3 via EcoRI/NotI digestion to remove the GFP gene. A multiple cloning site containing the restriction sites AgeI and MluI was ligated into the resulting plasmid via EcoRI and NotI overhangs, and reopened via AgeI/MluI digestion. The Myh3 gene (either WT, R672C, or R672H) was then ligated into the gap. The Myh3 gene was derived from the parent construct pMef-Myog-Myh3 (WT, RC, or RH) via AgeI/MluI digestion. Sequencing to verify correct gene sequence was performed by ACGT, Inc. with primers that covered the entire Myh3 gene sequence.

Plasmid maps, the multiple cloning site sequence, and primer sequences can be found in the Supplementary Materials.

C₂C₁₂ Reporter Cell Line Generation

HEK293T cells were cultured in DMEM media containing 10% heat-inactivated FBS. FBS was inactivated at 55° C. Virus production was carried out according to the protocol in Loew et al., 2006. The construct SF2cLM2CGFRT3 was co-transfected along with the viral packaging vectors pVPack-gagpol and pVPack-vsug. A previously generated C₂C₁₂ cell line

constitutively expressing the rtTA gene, designated clone #18, was infected with the resulting retrovirus.

Infected C₂C₁₂ cells were then induced with 200 ng/mL doxycycline (dox) for 16 hours. Cells were sorted via FACS (fluorescence-activated cell sorting) four times, with the first sort selecting for cells that responded to addition of dox by expressing GFP from the reporter cassette, the second and third sorts selecting for cells that did not express from the reporter cassette in the absence of dox, and the fourth sort again selecting for cells that responded to the addition of dox. These four rounds of sorting were used in order to isolate cells with a low expression level from the cassette in the absence of dox, but with a high expression level in the presence of dox.

Following these FACS of C₂C₁₂ populations, C₂C₁₂ cells were then sorted as single cells following 16-hour induction with 200 ng/mL of dox. Single cells were placed in 96-well plates. Luciferase assays to determine amount of induction with dox were then performed on the single cell clones according to Promega protocol, with a non-induced sample as a control, and a dox-induced sample for analysis. Induced samples were induced with 500 ng/mL dox for 24 hours. Differentiation capabilities of single cell clones were also tested by seeding cells on gelatin-coated coverslips in C₂C₁₂ differentiation media and observing myotube and sarcomere formation. Differentiation proceeded for 12 days, with induced samples being induced with 500 ng/mL dox. The differentiation media was changed daily. Immunofluorescence staining was carried out according to the protocol in Weidenfeld et al., 2009. Antibodies used were mouse F59 IgG1 directed against myosin head groups (Leinwand lab) and goat anti-mouse Alexa Fluor 568 IgG1 (Invitrogen). Imaging of samples was performed on a Nikon A1R confocal microscope.

After luciferase assays and differentiation studies, clone 18-8 luc/GFP was chosen for further studies due to its high level of induction upon addition of dox and its ability to differentiate into myotubes containing sarcomeres.

C₂C₁₂ luc/Myh3 Cell Line Generation from Reporter Cell Line 18-8 luc/GFP

C₂C₁₂ reporter cell line 18-8 luc/GFP was transfected with pCAGGS-Flpe-IRESpuro and SF2-Myh3 luc/Myh3 (WT, R672C, or R672H) FRT3 according to Lipofectamine 2000 protocol (Invitrogen). Following 6 hours of transfection, a round of antibiotic selection was carried out with 5 µg/mL puromycin added to the normal growth media. Cells were then induced with 500 ng/mL dox for 48 hours and sorted via FACS. Cells chosen for sorting had low fluorescence, indicating the exchange of the luc/GFP cassette for the luc/Myh3 cassette. Two subsequent sorts were carried out using the same conditions to eliminate as many cells containing the luc/GFP cassette as possible.

Cassette exchange was confirmed by isolating genomic DNA using the Qiagen DNeasy Blood-and-Tissue kit, and performing PCR with primers for the luc/GFP cassette and primers for the luc/Myh3 cassette. Control primers for the rtTA transactivator were also included. All primer sequences and PCR cycles can be found in the Supplementary Materials. Primers were ordered from Invitrogen.

Differentiation Studies of C₂C₁₂ luc/Myh3 Cell Lines

Cells were seeded onto gelatin-coated coverslips and allowed to reach 100% confluence in C₂C₁₂ growth media. Growth media was then exchanged for differentiation media (day 0 of differentiation), and cells were allowed to differentiate for 14 days. Differentiation media was

changed daily. Samples were induced with 500 ng/mL dox for varying amounts of time, with one non-induced control per experiment.

Following differentiation, cells were fixed and stained according to the protocol in Weidenfeld et al. 2009. Antibodies used were mouse 9B11 IgG2a directed against the Myc tag of the transgenic embryonic myosin (Cell Signaling Technology), mouse F59 IgG1 supernatant directed against endogenous myosin head groups (Leinwand lab), goat anti-mouse Alexa Fluor 647 IgG2a (Invitrogen), and goat anti-mouse Alexa Fluor 568 IgG1 (Invitrogen). Imaging of immunofluorescence samples was performed on a Nikon A1R confocal microscope.

Results:

1. Generation of the C₂C₁₂ Reporter Cell Line

In order to generate the reporter cell line, C₂C₁₂ cells were transduced using a retrovirus containing the reporter gene cassette, SF2cLM2CG-FRT3. The reporter gene cassette contains the luciferase and GFP genes under the control of the Tet-On promoter. Cells that stably integrate the reporter gene cassette into their genomes will respond to the addition of dox, therefore expressing luciferase and GFP. The expression of these two proteins allows for selection of cells that have integrated the cassette, as well as quantification of the amount of induction upon addition of dox. Selection of cells can be carried out by FACS, which monitors the fluorescence of the cells due to the GFP expressed from the reporter gene cassette. Quantification of induction amount can be calculated from luciferase assay data, which measures the light produced from the reaction of luciferase with its substrate, luciferin.

1.1 – Isolation of single dox-responsive cells using FACS

Four rounds of FACS were performed following viral transduction of C₂C₁₂ cells (Figure 7). For each round, a non-induced control, as well as a non-infected control was included. Data shown are for rtTA clone #18. Induced samples were induced with 200 ng/mL dox 16 hours prior to sorting. The first round was an “on” sort, in which cells that responded to the addition of dox were sorted out. The second and third rounds were “off” sorts, in which cells that did not express from the reporter gene cassette in the absence of dox were sorted out. The fourth sort was an additional “on” sort. These four sorts allowed for the selection of cells that did not express from the cassette in the absence of dox, and did express in high amounts in the presence of dox.

Following the fourth FACS sort, single cells were then sorted into 96-well plates. Luciferase assays were performed for these clones to determine which had the highest level of inducibility (Figure 8 and Table 1). From these assays, it was determined that clone 18-8 had a high level of inducibility. Differentiation tests were then performed to determine whether or not the clones were still capable of fusing to form myotubes with sarcomeres (Figure 9). Testing differentiation is critical because sarcomeres are necessary in order to study the behavior of embryonic myosin as it occurs in developing skeletal muscle. After differentiation tests, it was determined that clone 18-8 still differentiated to form myotubes with sarcomeres.

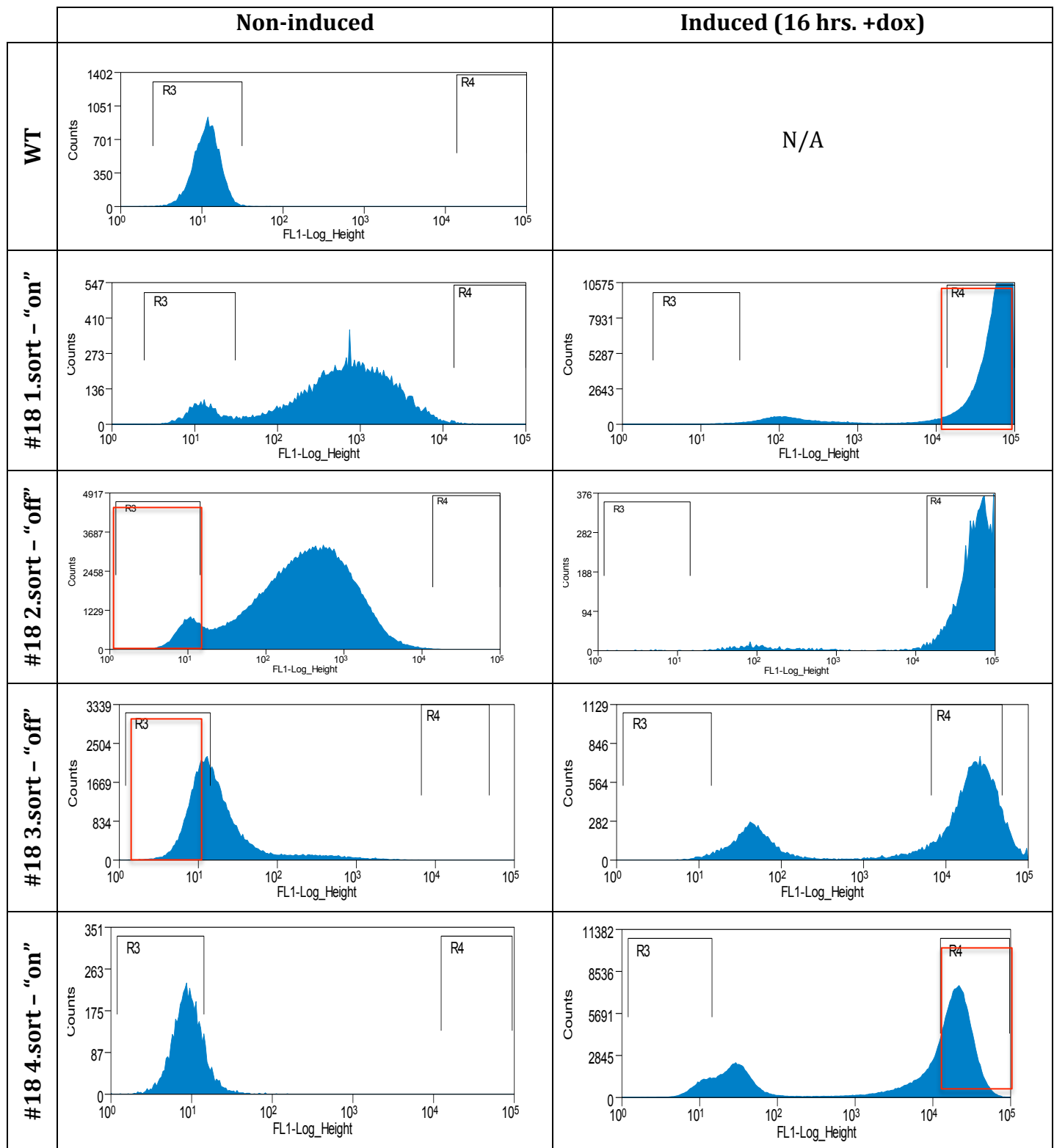


Figure 7: Four rounds of FACS were performed on rtTA clone #18. Non-induced and induced samples were included for all rounds, as well as a non-transduced control (WT). Induced samples were induced for 16 hours with 200 ng/mL dox. Cells that were sorted for each round are boxed in red. Data provided courtesy of Ina Weidenfeld.

1.2 - Quantification of expression in single C_2C_{12} clones using luciferase assays

Luciferase Assays of #18 luc/GFP Clones

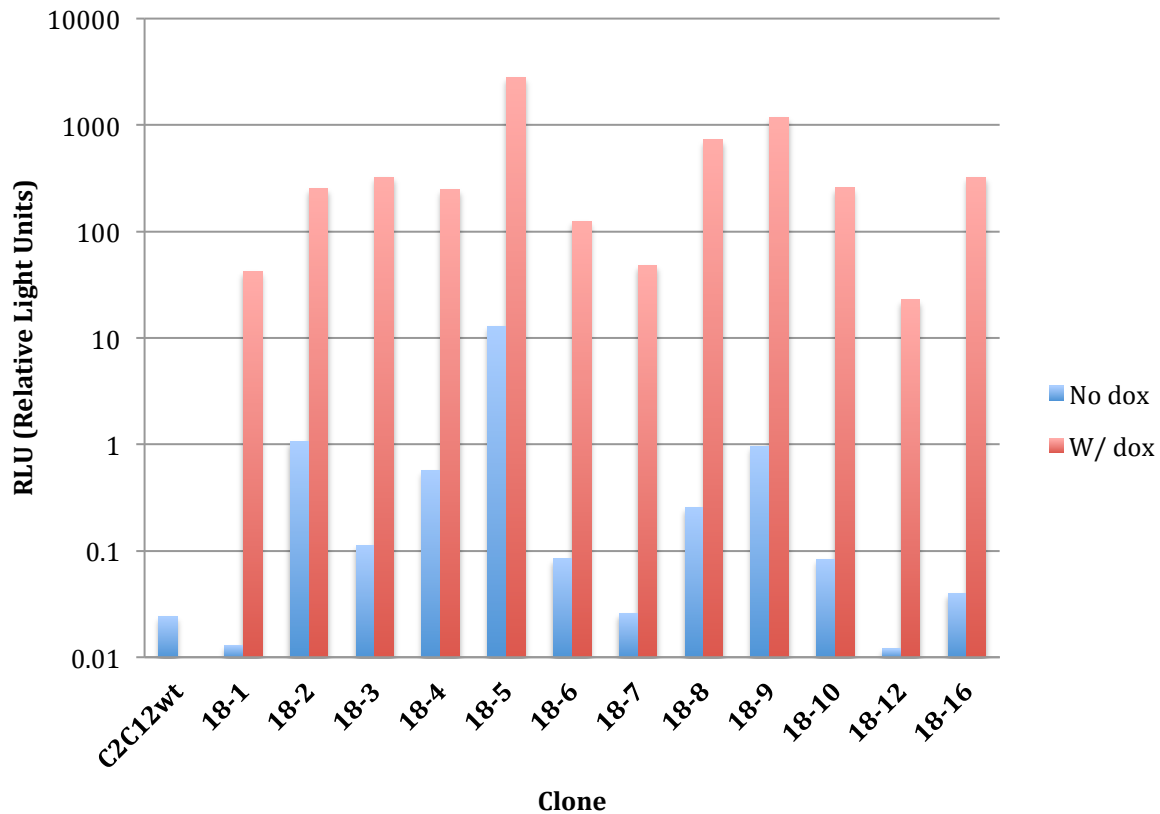


Figure 8: C_2C_{12} luc/GFP clones of #18 tested via luciferase assay. Blue indicates a non-induced sample, and red indicates an induced sample. Samples were induced with 500 ng/mL dox 24 hours prior to collection for the luciferase assay. Data provided courtesy of Ina Weidenfeld.

#18 luc/GFP Clones												
	1	2	3	4	5	6	7	8	9	10	12	16
Fold Induction	3259	240	2882	443	219	1493	1848	2862	1235	3113	1905	8030

Table 1: Fold induction of C_2C_{12} luc/GFP clones of #18. The fold induction was calculated from luciferase assay data shown in Figure 8, and is the fold induction from non-induced to induced. Data provided courtesy of Ina Weidenfeld.

1.3 - Differentiation tests of clone 18-8 luc/GFP

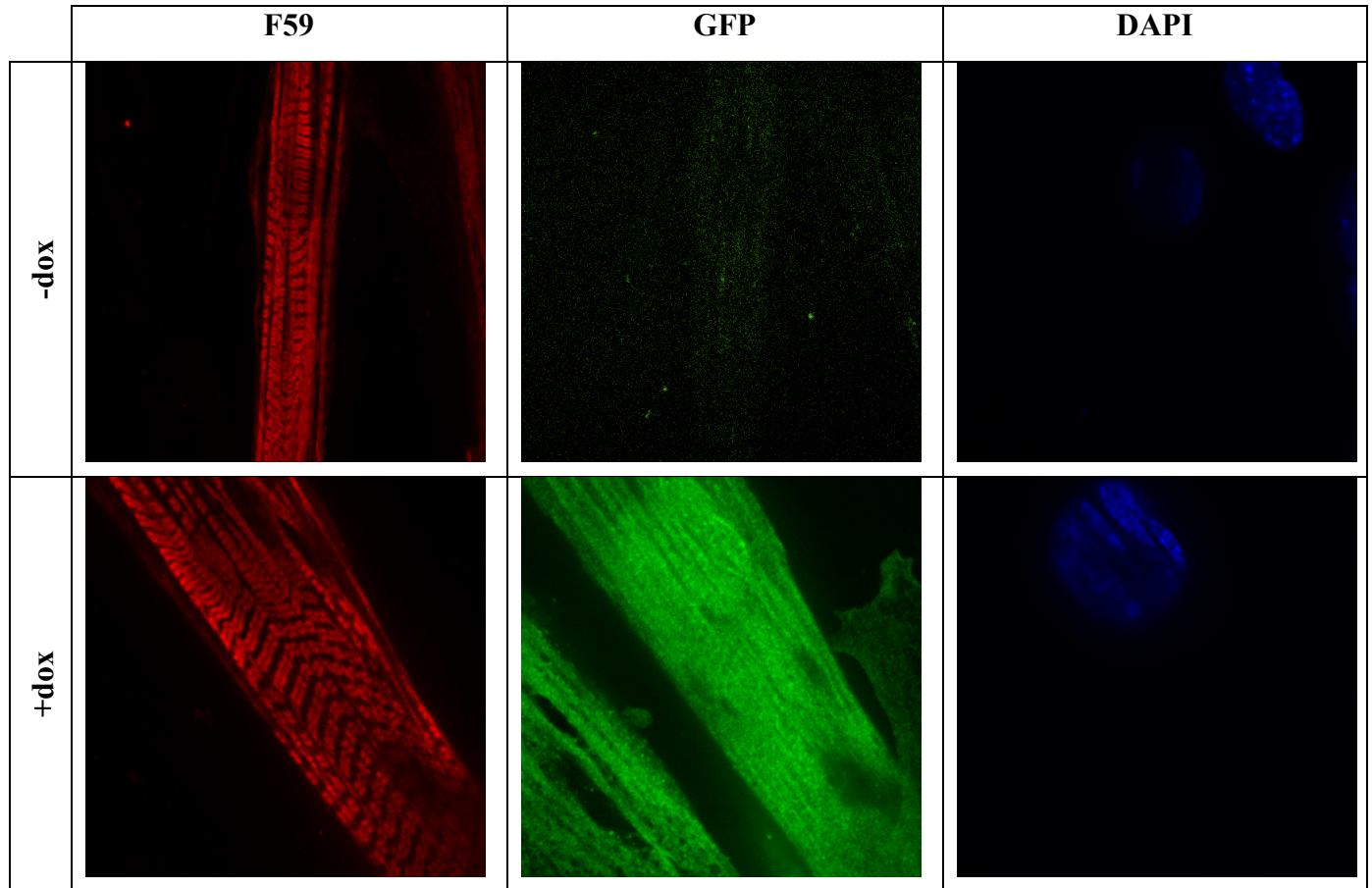


Figure 9: Clone 18-8 luc/GFP after 12 days of myotube differentiation. Immunofluorescence staining was performed for myosin head groups (F59 antibody), and cell nuclei (DAPI antibody). GFP was expressed directly from the reporter gene cassette. The banding pattern of F59 indicates the presence of sarcomeres. Induced samples were induced with 500 ng/mL dox for all 12 days. The +dox sample was imaged at 60X, and –dox at 100X. Data provided courtesy of Ina Weidenfeld.

Following confirmation of the differentiation capabilities of clone 18-8 luc/GFP, recombinase-mediated cassette exchange was performed in order to exchange the luc/GFP reporter cassette for a luc/Myh3 cassette. Cells were isolated via FACS by looking for the loss of fluorescence and sorting out those cells, as this indicates the loss of the luc/GFP reporter gene cassette and the gain of the luc/Myh3 cassette. The luc/Myh3 R672H cell line went through three

rounds of FACS in order to eliminate as many luc/GFP reporter cassette-positive cells as possible. Cassette exchange was verified via PCR on genomic DNA samples (Figure 10).

It should be noted that in the 18-8 luc/Myh3 R672H genomic DNA sample, the luc/GFP reporter cassette is still amplified during PCR. Given that the isolated cell lines were not fluorescent during both FACS and fluorescence microscopy, this indicates the presence of additional cassettes in the genome that are silent and cannot be exchanged via RMCE. These additional cassettes are likely due to the use of too much virus during the initial transduction of the C₂C₁₂ cells to generate the reporter cell line.

2. - Verification of cassette exchange of luc/GFP for luc/Myh3 in reporter cell line 18-8

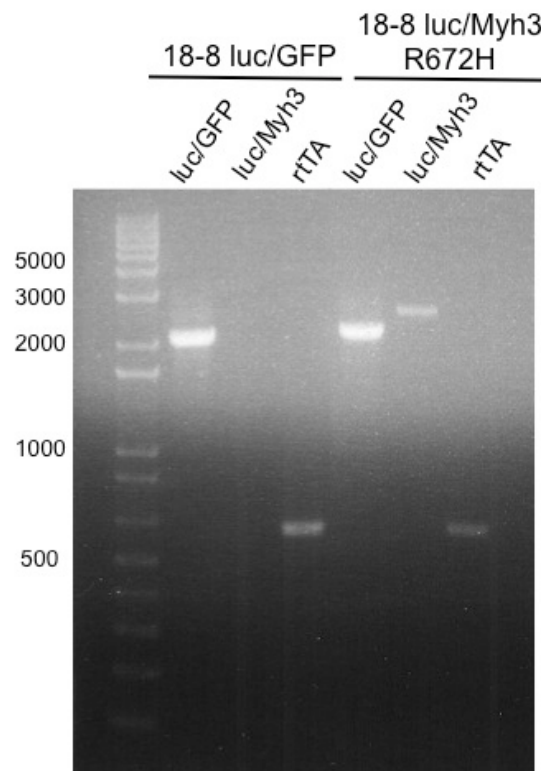


Figure 10: PCR verification of cassette exchange in clone 18-8. Primers amplified either the luc/GFP cassette, the luc/Myh3 cassette, or amplified the rtTA transactivator as a control. Genomic DNA samples tested were reporter cell line 18-8 luc/GFP and 18-8 luc/Myh3 R672H. A 1 kb+ ladder was used as a size marker for the gel.

3. - Localization of Mutant R672H Embryonic Myosin in C₂C₁₂ Sarcomeres

Once cassette exchange was confirmed, differentiation studies began to determine how the mutant R672H embryonic myosin behaves in the sarcomeres of C₂C₁₂ myotubes. Data currently have only been generated concerning the localization of the mutant R672H protein in the sarcomeres. In order to assess where the mutant R672H protein localizes in the sarcomere, cells were differentiated into myotubes for 14 days. Immunofluorescence staining was then done to detect both endogenous myosins as well as the Myc-tagged mutant embryonic myosin in the sarcomeres of the differentiated cells (Figure 11). By staining for both of these, comparisons could be made between the normal localization of myosin versus the localization of the mutant embryonic myosin in sarcomeres.

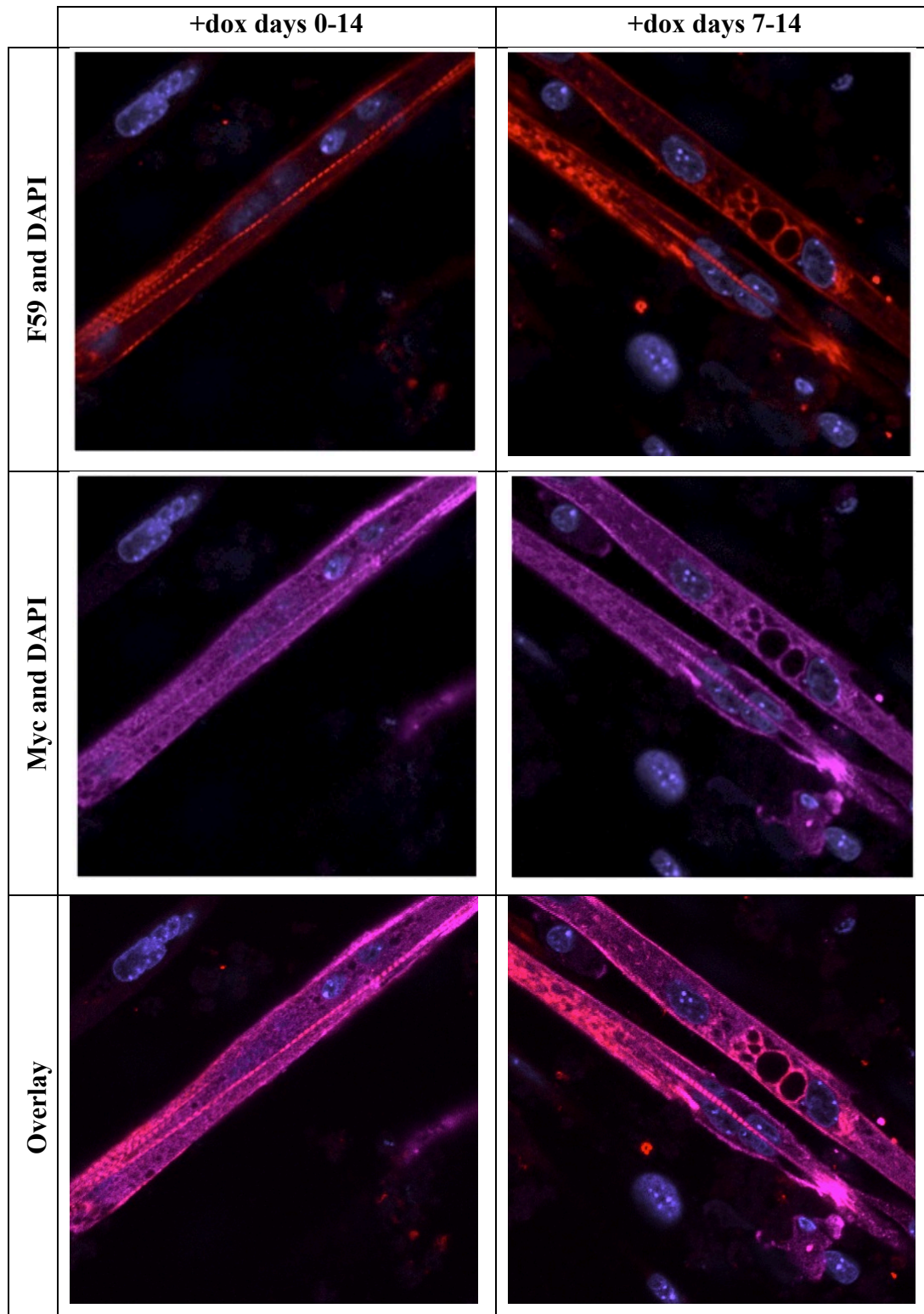


Figure 11: Immunofluorescence of cell line 18-8 luc/Myh3 R672H after 14 days of myotube differentiation. F59 antibody stains for endogenous myosin head groups, while Myc stains for the mutant embryonic myosin expressed from the integrated cassette. Sarcomeres can be seen, which appear as striated bands running through the cells. Samples for these images were induced for either all 14 days of differentiation, or for days 7-14 of differentiation with 500 ng/mL dox. Samples imaged at 100X.

Discussion:

The C₂C₁₂ inducible cell system provides many advantages to investigating the pathogenesis of Freeman-Sheldon Syndrome. The wild-type or mutant embryonic myosin heavy chain gene can be directly integrated into the genome of C₂C₁₂ cells, producing stable cell lines with a known integration site. The system then allows for fine-tuned control over expression of the Myc-tagged embryonic myosin protein during myotube and sarcomere formation, which is critical for both localization studies and future studies of protein turnover in the sarcomeres. In addition, this system eliminates uncertainties in data due to different cell populations, as all cell lines generated from the reporter cell line are known to have identical genomic integration sites of the cassette. Due to this, the luc/Myh3 cell lines that are generated can be directly compared to one another.

However, the system does currently pose some concerns. Foremost among these is the question of whether or not the system will eventually silence the integrated transgene. In data not provided here, it was seen that after several rounds of RMCE the system was no longer responding to dox. Due to this effect, the methods used for generating the luc/Myh3 cell lines were modified so that RMCE was carried out directly on the reporter cell line 18-8 luc/GFP. Currently this silencing effect does not pose a problem, but it must be considered if the cell lines are to be kept in culture for long periods of time. A second concern is the multiple integration sites of reporter cell line 18-8 luc/GFP. Although data have shown that the additional loci are silent and cannot be recombined, it is possible that they still interfere slightly with the functioning of the cell system. The only way to remediate this issue would be to regenerate the original reporter cell line by performing viral transduction with less virus.

At this stage, data suggest that mutant R672H embryonic myosin enters and localizes in the sarcomere normally (Figure 11). This finding fits with the patient profile – their muscles still develop, indicating that embryonic myosin is able to integrate into the sarcomeres of the developing skeletal muscle, but the function of the embryonic myosin is somehow compromised, leading to contractures.

Moving forward, there are many experiments yet to do in order to elucidate the mechanisms of FSS. The R672C Myh3 mutant and WT Myh3 cell lines must first be created. The localization of embryonic myosin will then need to be investigated in both the WT Myh3 and R672C Myh3 cell lines to complement the data presented here for the R672H Myh3 mutant cell line. In addition to examining the localization of the mutant embryonic myosin, protein turnover in the sarcomeres will be studied to determine if turnover occurs differently in WT versus mutant Myh3 cell lines. These studies will be done by inducing the Myh3 cell lines for several days at the beginning of differentiation and then removing the dox from the differentiation media. Samples will be collected daily for immunofluorescence staining following removal of dox. The amount of Myc-tagged embryonic myosin can then be quantized using confocal microscopy to see at what rate embryonic myosin is being degraded in the sarcomeres. Third, investigations of force production in the Myh3 mutant cell lines versus the WT Myh3 cell line will be performed. These studies will be performed according to the protocol in Khodabukus and Baar, 2009 using 3D tissue engineered constructs created from the Myh3 cell lines. This will give insight into how the muscles of FSS patients function, and may additionally provide insight into the mechanism leading to the muscle contractures in FSS patients.

Along with working with the current version of the luc/Myh3 cell lines, cloning is underway in order to make the isolation of the Myh3 cell lines more efficient. The luciferase

gene in the cassette will be replaced with the mCherry gene linked to a puromycin selection marker. This replacement will allow for isolation of Myh3 cell lines both by antibiotic selection with puromycin and by FACS by looking for the switch from green fluorescence to red fluorescence. Currently, antibiotic selection is not possible following RMCE of the luc/GFP reporter gene cassette for the luc/Myh3 cassette because both cassettes contain antibiotic-resistance markers for gancyclovir. Following successful cloning of this new mCherry-puro/Myh3 construct, new Myh3 cell lines will be generated by using both antibiotic selection and FACS.

In addition to future studies conducted in the cell system, experimentation will be performed in the transgenic mouse lines to look at the spatial and temporal expression of the mutant embryonic myosin. Phenotyping of mice will be performed in order to understand differences between the function of skeletal muscles in transgenic and wild-type animals. Force generation could be investigated by using an *ex vivo* approach on muscles isolated from transgenic or wild-type animals. These studies would complement the work done in the cell system.

Conclusions:

While the C₂C₁₂ inducible cell system does not provide a perfect method of investigation for fully determining the role of embryonic myosin in the pathogenesis of Freeman-Sheldon Syndrome, it does allow for determination of embryonic myosin's location and role in the sarcomeres of differentiating skeletal muscle cells. It has been shown here that the R672H mutant embryonic myosin enters and localizes in the sarcomere normally, but it remains to be seen how it is functioning once within the sarcomere. To this end, further experimentation will

be performed in order to elucidate how force generation and rate of myosin turnover in sarcomeres differs between wild-type and mutant-carrying Myh3 lines of the C₂C₁₂ system. Ultimately, this work will lead to a better understanding of embryonic myosin's role in human disease. More specifically, there may be future applications for potential therapies for FSS, as understanding the pathogenesis of the disease is crucial for the design of therapeutics.

Acknowledgements:

I would like to give thanks to Leslie Leinwand for allowing me to work in her lab as an undergraduate. She has been a great help throughout the entire development of both this project and my future career goals. Secondly, I would like to thank my day-to-day mentor Ina Weidenfeld. Without her, none of this would have been possible. I couldn't have asked for a better mentor, and miss her greatly. I would also like to thank Steve Langer, who has helped me out both with experimental questions and with editing this thesis. Finally, I would like to thank the rest of the Leinwand lab as well as my friends and family for their support and help during my time in the lab.

This project was funded in part by the Undergraduate Research Opportunities Program and the Biological Sciences Initiative's BURST program.

References:

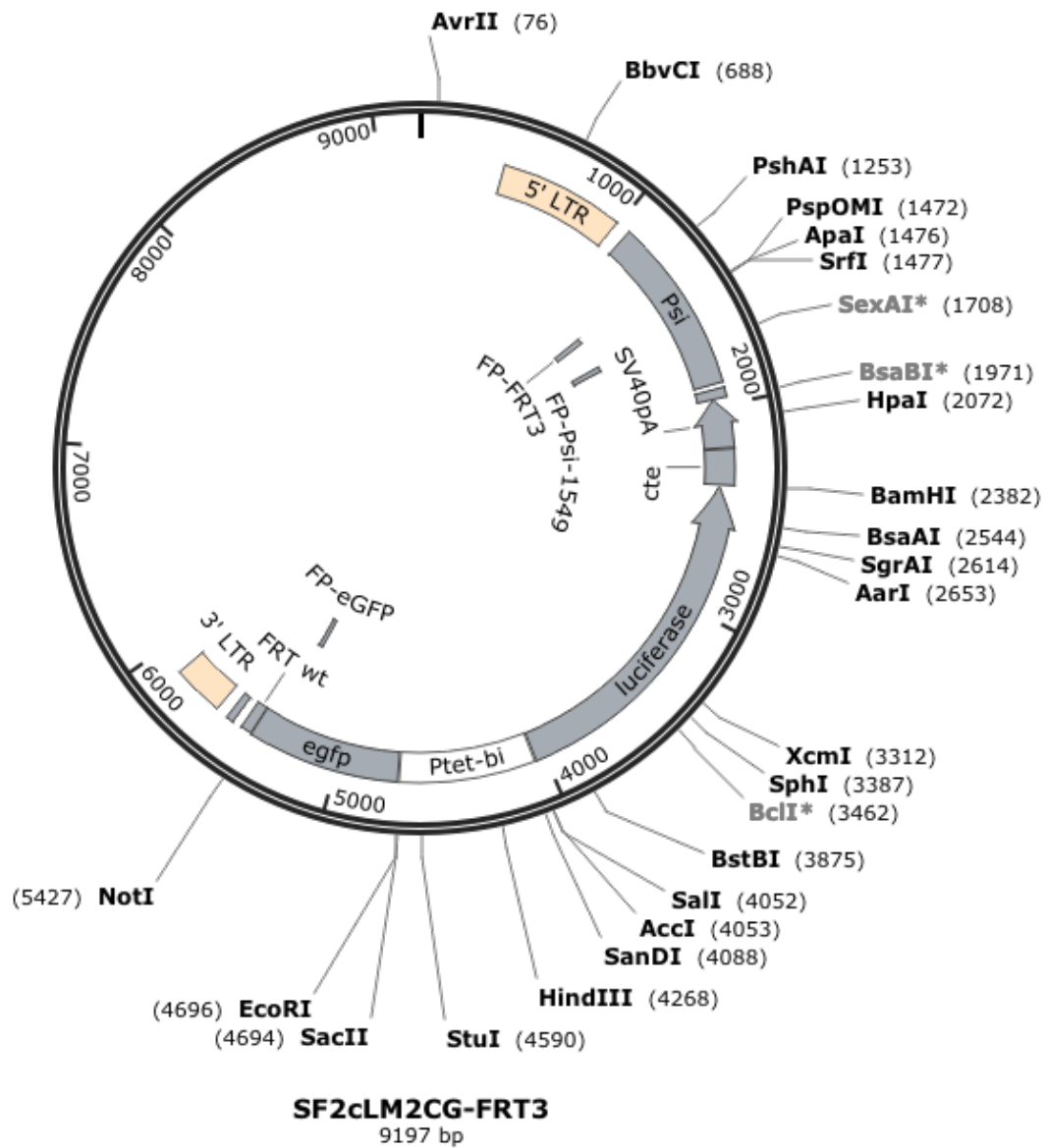
- Alves, A. F., & Azevedo, E. S. (1977). Recessive form of Freeman-Sheldon's syndrome or 'whistling face'. *Journal of Medical Genetics*, 14(2), 139-141.
- Bamshad, M., Van Heest, A. E., & Pleasure, D. (2009). Arthrogryposis: a review and update. *The Journal of Bone & Joint Surgery*, 91(Supplement_4), 40-46.
- Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences*, 89(12), 5547-5551.
- Khodabukus, A., & Baar, K. (2009). Regulating fibrinolysis to engineer skeletal muscle from the C2C12 cell line. *Tissue Engineering Part C: Methods*, 15(3), 501-511.
- Laishley, R. S., & Roy, W. L. (1986). Freeman-Sheldon syndrome: report of three cases and the anaesthetic implications. *Canadian Journal of Anesthesia/Journal canadien d'anesthésie*, 33(3), 388-393.
- Loew, R., Vigna, E., Lindemann, D., Naldini, L., & Bujard, H. (2006). Retroviral vectors containing Tet-controlled bidirectional transcription units for simultaneous regulation of two gene activities. *Journal of Molecular and Genetic Medicine: an International Journal of Biomedical Research*, 2(1), 107.
- Lu, B. D., Allen, D. L., Leinwand, L. A., & Lyons, G. E. (1999). Spatial and temporal changes in myosin heavy chain gene expression in skeletal muscle development. *Developmental Biology*, 216(1), 312-326.
- Rutland, C. S., Polo-Parada, L., Ehler, E., Alibhai, A., Thorpe, A., Suren, S., ... & Loughna, S. (2011). Knockdown of embryonic myosin heavy chain reveals an essential role in the morphology and function of the developing heart. *Development*, 138(18), 3955-3966.
- Sánchez, J. M., Kaminker, C. P., Opitz, J. M., & Reynolds, J. F. (1986). New evidence for genetic heterogeneity of the Freeman-Sheldon syndrome. *American Journal of Medical Genetics*, 25(3), 507-511.
- Schlake, T., & Bode, J. (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry*, 33(43), 12746-12751.
- Stevenson, D. A., Carey, J. C., Palumbos, J., Rutherford, A., Dolcourt, J., & Bamshad, M. J. (2006). Clinical characteristics and natural history of Freeman-Sheldon syndrome. *Pediatrics*, 117(3), 754-762.

- Toydemir, R. M., Rutherford, A., Whitby, F. G., Jorde, L. B., Carey, J. C., & Bamshad, M. J. (2006). Mutations in embryonic myosin heavy chain (MYH3) cause Freeman-Sheldon syndrome and Sheldon-Hall syndrome. *Nature Genetics*, 38(5), 561-565.
- Wang, T. R., Lin, S. J., Opitz, J. M., & Reynolds, J. F. (1987). Further evidence for genetic heterogeneity of whistling face or Freeman-Sheldon syndrome in a Chinese family. *American Journal of Medical Genetics*, 28(2), 471-475.
- Weidenfeld, I., Gossen, M., Löw, R., Kentner, D., Berger, S., Görlich, D., & Schöning, K. (2009). Inducible expression of coding and inhibitory RNAs from retargetable genomic loci. *Nucleic Acids Research*, 37(7), e50-e50.

Supplementary Materials:*I. Plasmid Maps*

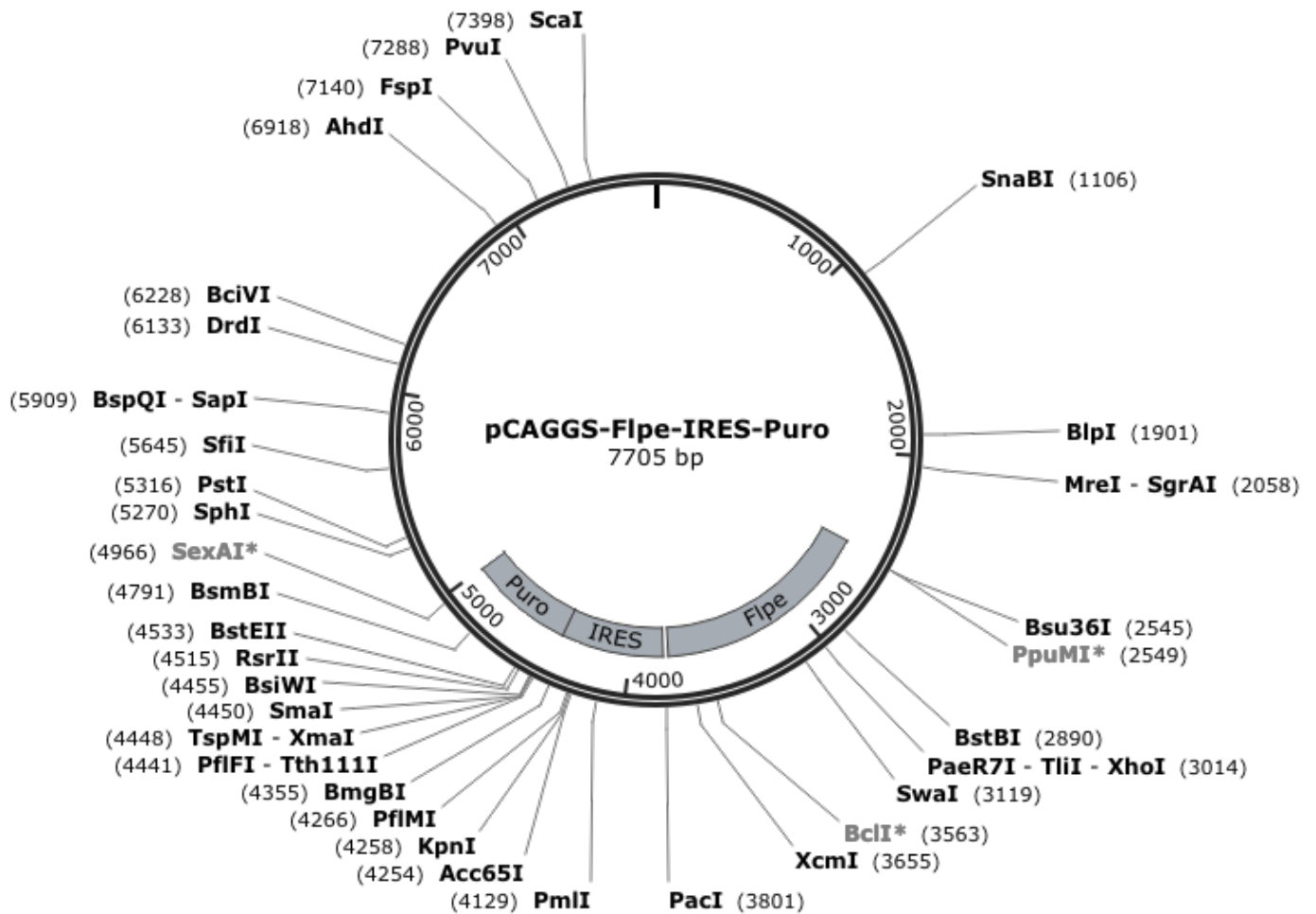
SF2cLM2CG-FRT3 (reporter gene luc/GFP cassette)

Created with SnapGene®



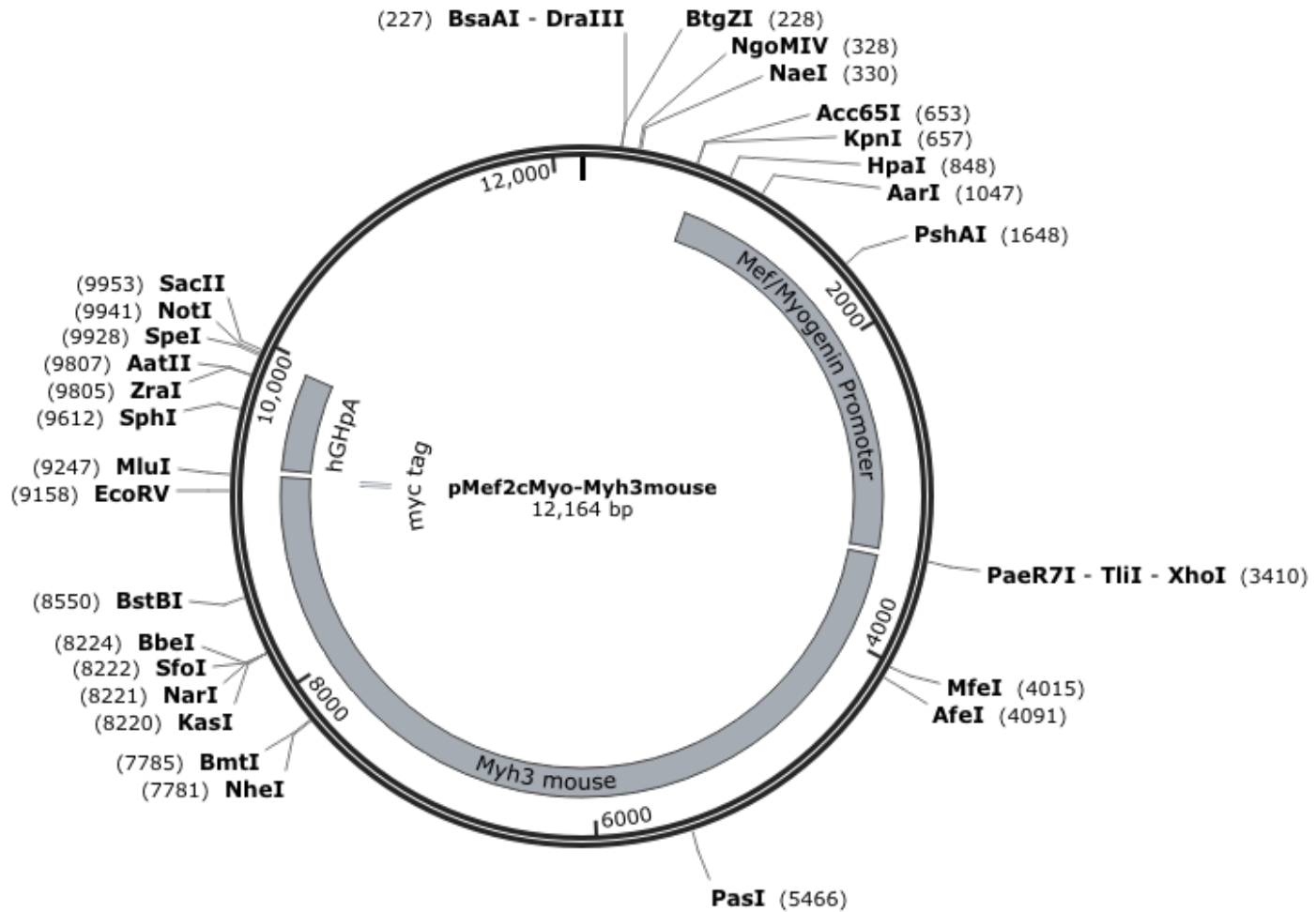
pCAGGS-Flpe-IRES-Puro (Flp recombinase)

Created with SnapGene®



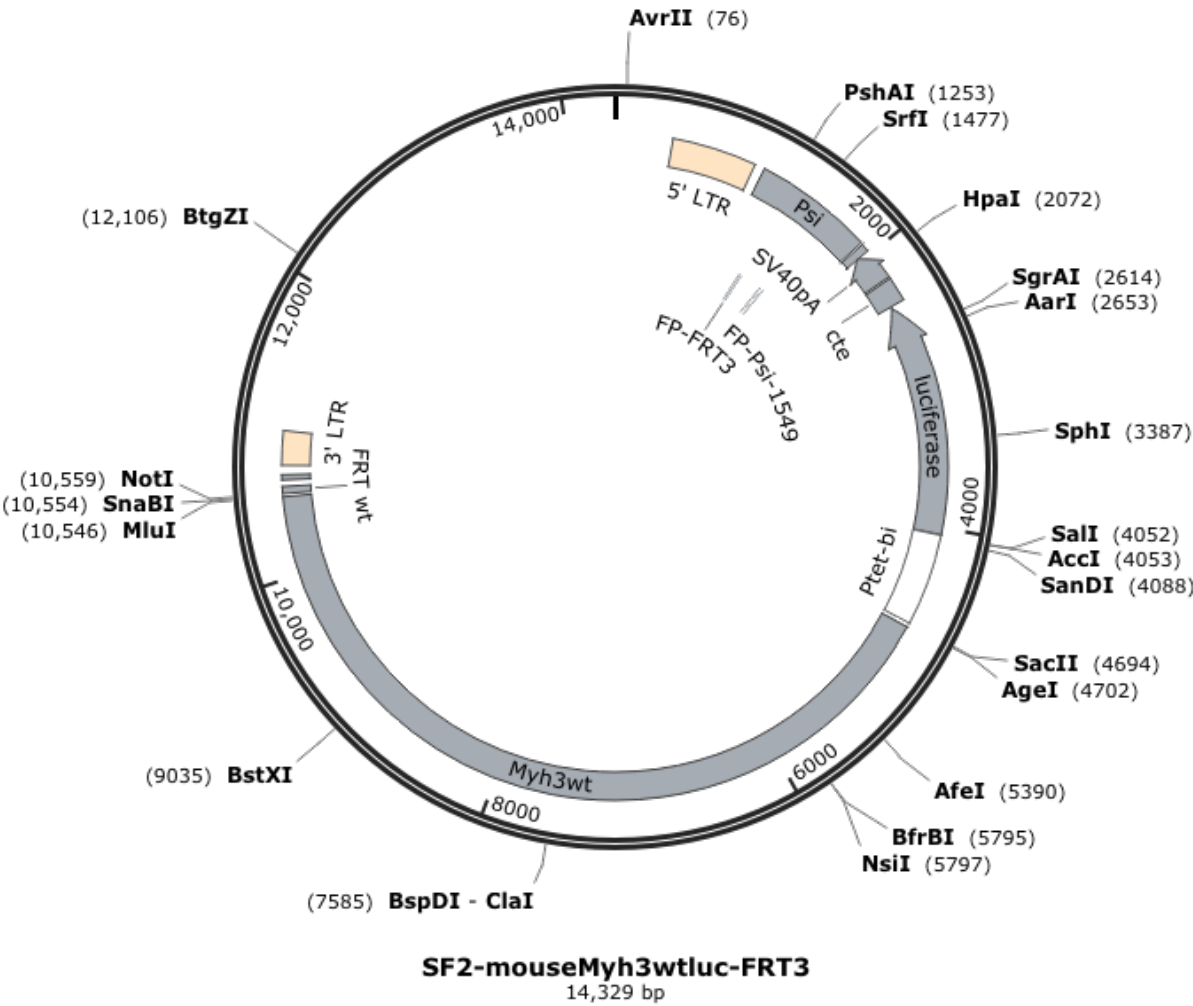
pMef2cMyo-Myh3mouse (wt, RC, or RH) (Myh3 Myc-tagged gene)

Created with SnapGene



SF2-mouseMyh3wtluc-FRT3 (luc/Myh3 cassette)

Created with SnapGene®



*II. Primer Sequences*Primer Sequences for Verification of Cassette Exchange

FP luc/GFP: 5'-CACCTGCGTCGAAGATGTTGGGGTGTGGA-3'

RP luc/GFP: 5'-ACGCTGAACTTGTGGCCGTTTACGTC-3'

FP luc/Myh3: 5'-CACCTGCGTCGAAGATGTTGGGGTGTGGA-3'

RP luc/Myh3: 5'-ACAGTGACACAGAAGAGGCCTGAA-3'

FP rtTA: 5'-CCATGTCTAGACTGGACAAGA-3'

RP rtTA: 5'-CTCCAGGCCACATATGATTAG-3'

Primer Sequences for Sequencing of luc/Myh3 Plasmids

FP 259: 5'-TTAAGCCAGAGGACGTGTATGCCA-3'

RP 421: 5'-ACAGTGACACAGAAGAGGCCTGAA-3'

FP 655: 5'-ACCTTGCCAAGAAGAAGGACTCCA-3'

FP 1312: 5'-ATGCTGTGAACGCTCTCTCCAAGT-3'

FP 1936: 5'-ACGGTGGGAAGAAGAAAGTCGCTA-3'

FP 2582: 5'-TGAGAAGGAGATGGCCACCATGAA-3'

FP 3336: 5'-GAGCAGACCTTGAGCCTCCA-3'

FP 3938: 5'-TGTATCTCAGCTCTCCAGGA-3'

FP 4540: 5'-TGAAACGGGAGAATAAGAAC-3'

FP 5142: 5'-CGGAAGCTGGCAGAGCAGGA-3'

FP 5744: 5'-CCAGCATGAGCTAGAGGAGG-3'

III. Multiple Cloning Site Sequence

5' - AATTCACCGGTTTAATTAAGGCCGGCCACGCGTTACGTAGC – 3'
3' - GTGGCCAAATTAATTCCGGCCGGTGCGCAATGCATCGCCGG – 5'

5' - EcoRI - AgeI – PacI - FseI – MluI – SnaBI- NotI – 3'

IV. PCR Conditions for Genomic DNA PCR

<u>Temp.</u>	<u>Time</u>	
98°	2'	
98°	30"	} 5X
58°	30"	
68°	2' 30"	
98°	30"	} 25X
63°	40"	
68°	2' 30"	
68°	8'	