Myst1 acetyltransferase regulates Pax7 function

Tabitha Rosembert

Supervisor: Dr. Michael Rudnicki

This thesis is submitted as a partial fulfillment if the M.Sc. program in Cellular and Molecular Medicine

University of Ottawa

74 Laurier Ave.E

Ottawa Ontario

K1N 6N5

April 5th, 2018

Abstract

Pax7 is essential for the function of muscle satellite cells. It was previously determined that Pax7 methylation is essential for its transcriptional activity and function in satellite cells. We investigated whether Pax7 displays other post-translational modifications playing a role in its function. By mass spectrometry using immunoprecipitated FLAG-Pax7, we identified two lysine residues (K105 and K193) within the Pax7 protein that are acetylated. Pax7 transcriptional activity was monitored using a luciferase reporter under the control of Myf5, a Pax7 target gene. Treatment with Trichostatin A, a histone deacetylase inhibitor, increased significantly luciferase activity, but this activity was progressively loss when the created Pax7 mutants (K105R, K193R) were introduced. This suggests that acetylation plays a role in Pax7 transcriptional activity. To identify the acetyltransferase modulating Pax7 activity, we used a candidate approach. Myst1 is expressed in muscle satellite cells. Myst1 is known for its interaction with Wdr5 and MLL1/2, a known Pax7 partner. We detected an interaction between Pax7 and Myst1 by co-immunoprecipitation in fibroblasts and in primary myoblasts. Myst1 knockdown decreases Pax7 acetylation status suggesting Myst1 as Pax7's acetylase. Myst1 siRNA knockdown negatively impacts many Pax7's target genes as well as primary myoblast proliferation. Moreover, primary myoblasts treated with siMyst1 express higher levels of MyoD. In satellite cells Myst1 reduction through siRNA knockdown significantly reduces satellite cells progenitor expansion as well as increases MyoD expression. In all, Myst1 modulating Pax7 activity through acetylation represents a novel mechanism in muscle stem cell biology.

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Abbreviations

Ac	Acetylation
Cas3	Caspase 3 mediated degradation
ChIP-seq	Chromatin Immunoprecipitation sequencing
FGF	Fibroblast growth factor
IF	Immunofluorescence
IgG	Immunoglobulin G
IP	Immunoprecipitation
siRNA	Small interfering RNA
HA	Influenza hemagglutinin
K	Lysine
K/R	Lysine to arginine substitution
KAT8	Lysine acetyltransferase 8
Me	Methylation
MOF	Male One First
Myf5	Myogenic Factor 5
MSL	Male Specific-Lethal complex
MRF	Myogenic Regulatory Factor
MRF4	Myogenic Regulatory Factor 4
MiR	Micro-RNA
NSL	Male non-specific lethal complex
P	Phosphorylation
R	Arginine
RA	Retinoic Acid
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Shh	Sonic hedgehog
SUMO	SUMOlyation small or (small ubiquitin like modifiers)
Ub	Ubiquiation

Acknowledgements

I would like to acknowledge Dr. Michael Rudnicki for his supervision, his mentorship and opportunity to work on this project. I would also like to thank Dr. Marie-Claude Sincennes for all her mentorship, collaboration and support through the course of this project. I would also like to acknowledge Hong Ming for his help with protocols as well as Dr. Caroline Brun for her mentorship with fiber isolation techniques.

Introduction

Skeletal muscle - an overview

Skeletal muscle, one of the largest organs in the body, is a highly organized and heterogeneous population. Myofibers are multinucleated cells part of the basic contractual unit. Remarkably, muscle can regenerate to almost uninjured level following exercise, injury, disease, etc. Muscle regeneration is a multistep process where many cell types are involved in driving and maintaining a proper homeostasis (Seale., 2000). This process starts in all cases with satellite cells, who are in a resting quiescent state to undergo activation. Stem cells, during their commitment phase can proliferate at a rapid pace where they enter the differentiation stage in which these myoblasts become myocytes and are able to fuse to new or existing myofibers (Figure 1) . This process is regulated by many transcription factors crucial for this process as well as regulated through other molecular mechanisms which will be further discussed.

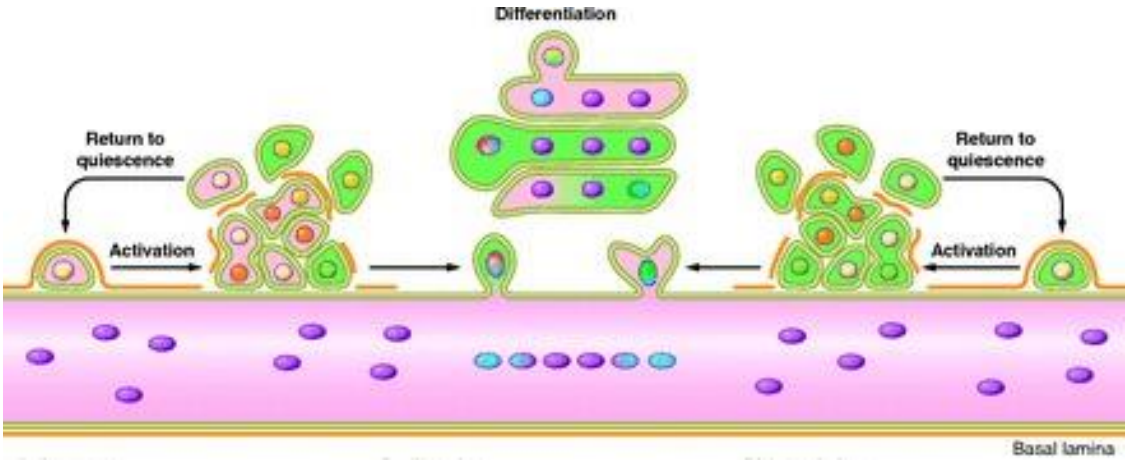

Figure 1: Schematic diagram depicting the skeletal muscle myogenic process. (Adapted from Yin and all, 2013).

Embryonic myogenesis

Adult and embryonic myogenesis are two different processes. Embryonic muscle starts its formation around embryonic day 9 to day 18.5, close to birth. The precursor cells that will become muscle originate from the presomitic paraxial mesoderm (PSM) (Bentzinger et al., 2012). The ventral portion of the PSM gives rise to the dermomyotome which will produce most of the skeletal muscles. This process is driven in major part by the gradient expression of FGF's, Wnts, Shh, and RA proteins (Tajbakhsh et al., 1998). The myogenic precursor cells are marked by the expression of Pax7/Pax3. Limb muscle require Pax3 expression and their migration is facilitated through proteins such as N-Cadherin and Fibronectin. The same pathway that controls the formation of head muscles do not seem to be the same in trunk muscles.

Myogenic regulatory factors

During myogenesis, different transcription factors called myogenic regulatory factors (MRF) are expressed and regulated to drive this process. Upon satellite cell activation and entry into the cell cycle, Myf5 as well as MyoD are rapidly expressed. These proteins belonging to the basic helix-loop-helix family of transcription factors play a crucial role in myogenic specification along with myogenic commitment. MyoD and Myf5 double knockout mice are lethal due to the lack of muscle formation (Rudnicki et al., 1993). Surprisingly, single knockouts do not display severe abnormalities in the skeletal muscle. Early studies have indicated the possibility that Myf5 and MyoD have compensatory roles though newer genetic approaches have led to their distinct function. Characterization of Myf5 and MyoD in satellite cells have defined their roles in commitment and differentiation respectively. MyoD-/- mice display an overall deficit in muscle regeneration due to a higher level of committed satellite cells but a lack in the proliferative progenitor population (Megeney et al., 1996; Sabourin et al., 1999). In the adult muscle, deletion of MyoD results in early upregulation of Myf5 expression as well as decreased MRF4 expression, resulting in an impaired differentiation (Rudnicki et al., 1992, Cornelison et al., 2000). Myf5 -/- mice in development display aberrant rib formation resulting in their death due to respiratory problems (Braun et al., 1992). However, the skeletal muscle develops normally, as the myogenic cells are still able to differentiate and express MyoD as well myogenin. Myogenin and Mrf4 (myf6), other members of the basic helix-loop-helix family are expressed during the proliferative phase to start the terminal differentiation program. Myogenin is integral to the myofiber formation process. Myogenin-deficient

mice died prenatally due to a significant defect in the muscle (Nabeshima et al., 1993). These mice have a lower level of myosin heavy chain and actin expression as well as contain fewer and smaller myofibers. Many Mrf4 depleted mice have been generated wielding various phenotypes such as aberrant rib formation similar to the Myf5 null mice, back muscle defects at birth or mouse lethality. One specific group created a MRF4-null mouse with normal viability and muscle. These mice showed increased expression of myogenin suggesting their compensatory role in terminal differentiation. The same group also generated MFR4/MyoD knockout mice which recreated the same phenotype seen in myogenin-null mice also suggesting that MRF4 and MyoD may have some redundant roles (Hernández-Hernández et al., 2017, Braun 1995, Rawls et al., 1998, Zhang et al., 1995).

Satellite cells

Satellite cells are crucial for proper regeneration of the muscle fiber due to injury, physical activity or disease (Chargé and Rudnicki, 2004; Seale and Rudnicki, 2000; Sincennes et al., 2016) . Compared to other stem cells, satellite have a particular anatomical location being beneath the basal lamina and outside the plasma membrane of the muscle fiber. As most adult stem cells, they remain in a quiescent state and represent 2-10% of the cell population in the muscle (Wang et al., 2014). In response to an external stimulus, they become activated, enter the cell cycle and proliferate to give rise to myogenic precursor cells. The precursor cells then enter terminal differentiation and fuse to new or existing myofibers.

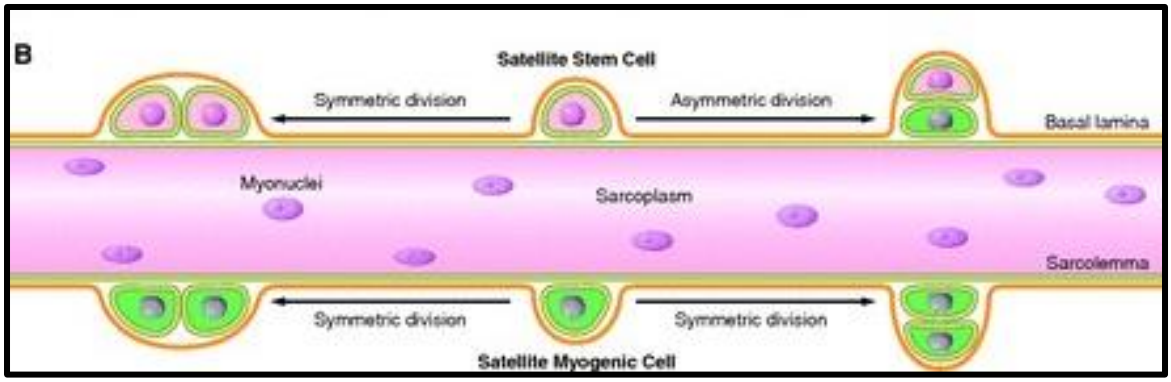

Figure 2: Schematic diagram representing a satellite cell symmetric vs asymmetric division. (Adapted from Yin et al., 2013).

Satellite cells are capable of symmetric and asymmetric divisions (Rudnicki et al., 2008). An asymmetric division is when a satellite cell divides and gives rise to one satellite cell and one cell that is more committed to the myogenic program. Committed cells express the transcription factor Myf5 and give rise to myogenic precursor cells that go along the myogenic lineage to fuse to new or existing myofibers. During symmetric self-renewal, a satellite cell divides and gives rise to two identical daughter satellite cells that don't express Myf5 and retain their stem cell state. During symmetric commitment, the two daughter cells express Myf5 and are more committed to the myogenic lineage (Figure 2).

Satellite cells are distinguished from other muscle or stem cell populations by the expression of different cell surface markers such as α7-integrin, M-cadherin, CD34 and Vcam1(Sincennes et al., 2017;Seale et al., 2004). Their expression is also delineated by the fact that they do not express Sca-1 as well as lineage markers Cd11b, Ter-119, Cd45 and Cd31.

Molecular regulation of satellite cells

Satellite cells are also marked by Pax7 expression, a master regulator of the early stages of myogenesis. The Pax family are Paired box DNA binding proteins and are involved in development and maintenance of specific tissues. Pax proteins are divided into subgroups which Pax7 and Pax3 share due to their very similar roles in organ specification as well as similar binding targets (Kuang et al., 2006; Soleimani et al., 2012). This transcription factor is known for the implication of neurons and well as the esophagus patterning (Chihara et al.,2015, Budry et al., 2012). More importantly, Pax7 is crucial for their specification of the myogenic lineage (von Maltzahn et al., 2013; Seale et al., 2000). Pax7 null mice display several striking characteristics such as a 50% reduction in weight compared to their wildtype counterparts and a reduction in muscle fiber size. Most importantly, Pax7 null mice do not possess satellite cells, leading to their death around the two-week mark due to a lack of muscle regeneration and lack of functioning diaphragm. Pax7 is undoubtedly a master regulator of early myogenesis as its transcription is essential for satellite cell and myoblast cell cycle progression and proliferation.

ChIP-seq experiments have shown that Pax7 has multiple targets for cell growth and proliferation (Soleimani et al., 2012). One important target is the myogenic regulatory factor Myf5. A previous study has shown that that Pax7 regulates Myf5 expression by recruiting the Trithorax complex to the Myf5 promoter (McKinnell et al., 2008). The Trithorax complex can induce a trimethylation mark on histone H3 lysine 4 (H3K4) on the histones surrounding the Myf5 promoter and this causes the chromatin around the area to be less condensed, allowing the transcription of Myf5 and entry into the myogenic program. Through mass-spectrometry and co-immunoprecipitation experiments it has been

shown that the members of this complex that are able to interact and are recruited by Pax7 are MLL1/2, Wdr5, Ash2L and Rbbp5.

Mechanisms of Pax7 regulation

Pax7 is known to be regulated through many processes which can occur at the post-transcription level and post-translational level. Pax7 is known to be regulated by multiple micro-RNAs *in vitro* as well as *in vivo* (Dey et al., 2011, Wu et al., 2015, Chen et al., 2010). MiR-206, MiR-1 and MiR-486 have been shown through multiple papers to directly regulates Pax7 expression through repression of mRNA levels driving myoblast differentiation.

Posttranslational modifications play an essential role in modulating histone as well non-histone proteins. In non-histone proteins, they play a role in modulating protein activity, protein-protein interaction, protein recruitment to the chromatin region, protein degradation or stability (Schönichen et al,.2013). These modifications such as methylation, ubiquitination, acetylation and SUMOylation are provided by specific proteins possessing enzymatic domains allowing this action to occur.

In the literature, there has been instances in which Pax7 posttranslational modification has been shown to affect either Pax7 levels or transcriptional activity These modifications are found on various residues throughout Pax7's domains. (Figure 3)

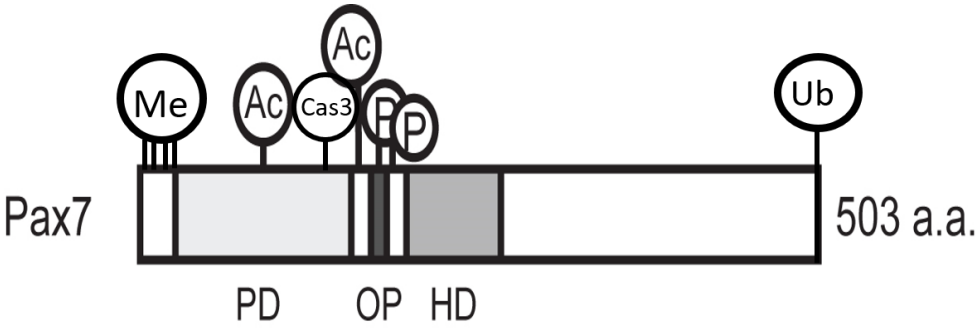

Figure 3: Schematic diagram displaying Pax7 protein and known or discovered posttranslational modifications in mouse.

Pax7 protein levels have been shown to be affected through caspase-mediated degradation (Dick et al., 2015). Pax7 is cleaved by Caspase 3 on aspartic acid 187 residue resulting in 20kDa and 60kDa fragments. Pax7 degradation via Caspase 3 is implicated in satellite cell and myoblast differentiation. Interestingly, CK2-mediated phosphorylation of Pax7 at serine 201 and 205 inhibits Pax7 cleavage promoting satellite cell renewal. CK2 and Caspase 3 are important players in modulating satellite self-renewal to differentiation transition.

Another group has shown that Pax7 is also ubiquitinated at its C-terminal region by NEDD4, an E3 ubiquitin ligase (Bustos et al., 2015). Ubiquitination of Pax7 by NEDD4 results in Pax7 protein degradation by the Ubiquitin Proteasome-System (UPS). Pax7 downregulation results in an exit from the cell cycle and shift towards myoblast differentiation (.

Pax7 has also been shown to be SUMOylated in chick embryos as well as in C2C12 cells (Luan et al., 2013). Ubc9 mediated SUMOlyation of Pax7 at lysine 85 plays a vital role in neural crest development through the expression of later neural crest genes such as Snail2, Fox9 and FoxD3.

Pax7 has been shown to display a post-translational modification that is necessary for its transcriptional activity. Pax7 is methylated by Carm1/PRMT4 on 4 arginine residues located in the N-terminal region (Kawabe et al., 2012). Methylation of Pax7 by Carm1 allows binding to MLL1/MLL2 of the Trithorax complex. Pax7's binding to the HMT complex allows H4K3 tri-methylation to occur on the Myf5 locus and transcription of Myf5 transcripts. This process is critical during an asymmetric division for the expression of the committed satellite cell, deletion of Carm1 or inhibition increases the number of asymmetric divisions as well as the number of committed satellite cells

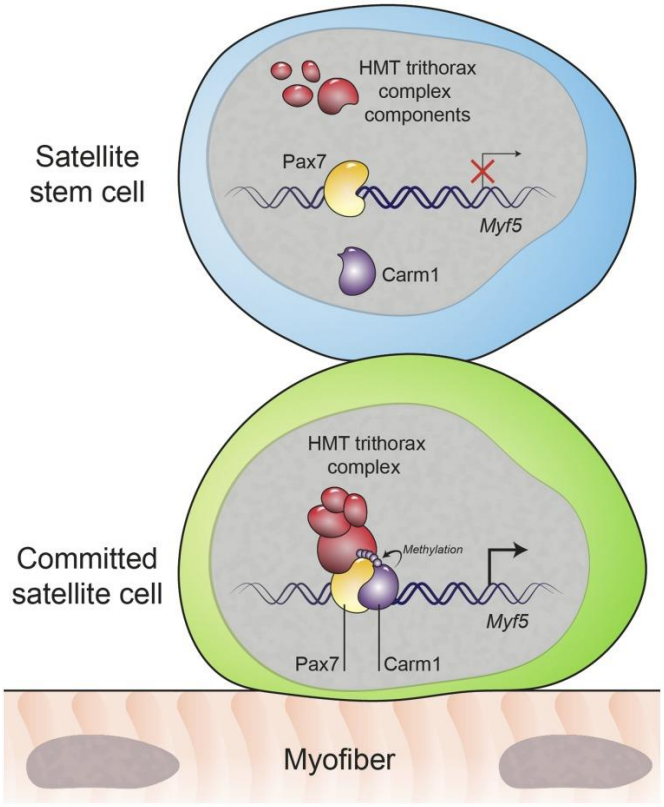

Figure 4: Schema representing Carm1 role in establishing asymmetric cell division. Sincennes and Brun, 2016.

We are interested in determining if Pax7 displays other posttranslational modifications, in addition to methylation. Possible Pax7 acetylation sites have been identified through mass spectrometry on two lysine residues, lysine 105 and lysine 193 (Unpublished Data). Both lysine 105 and lysine 193 are conserved throughout all species. Lysine 105 is conserved on all Pax proteins and lysine 193 is only found on Pax7 proteins. Through site-directed mutagenesis, single and double mutants carrying lysine to arginine substitutes were generated. These mutants as well as wildtype Pax7 were challenged to a luciferase assay using the reporter fused to Myf5 -111kb enhancer, a known Pax7 target.

Cells were also treated with Trichostatin A (TSA) a histone deacetylase inhibitor. Combined TSA treatment and Pax7 expression induced a robust luciferase reporter activity compared to those not treated with TSA. In addition, single or double mutants were introduced in place of the wildtype construct and surprisingly reporter activity was progressively loss, more so with the 193R mutant. This data suggested that acetylation plays a role in modulating Pax7's transcriptional activity.

Acetylation is a process defined by the action of an acetyl group being added to specific lysine residues (Choudhary et al., 2014). The lysine residue found on these proteins are positively charged once they are protonated or the physiological pH of the environment changes. The acetyl group added by these enzymes are negatively charged which neutralizes the lysine residues. Consequently, this can result in a change in mediating protein-protein interactions, protein activity, DNA binding, protein localization, chromatin recruitment and more. This process is mediated by enzymes known as histone acetyltransferases (HATs) or lysine acetyltransferases (KATs) that utilize acetyl-CoA to perform this reaction

Overview of Myst1 acetyltransferase

There are 4 main lysine acetyltransferase families: the GNC5/PCAF family, p300/CBP family, the SRC family, as well as the MYST family (Wang et al., 2009). In order to identify the acetyltransferase responsible for Pax7 acetylation, we chose nine KAT candidates based on their expression in satellite cells, and their known interaction with Pax7 partners (Carm1 or members of the Trithorax complex) or known Pax family acetylase (Asher et al., 2008; Chen et al., 1999; Dias et al., 2014; Guelman et al., 2009; Jakkaraju et al., 2005; Lu et al., 2011; Roth et al., 2003; Teyssier et al., 2002). Through individual overexpression of these nine chosen KAT candidates (Ncoa1, Ncoa2, Ncoa3, Myst1, Tip60, p300, Clock, Crsp2bp and Mettl8) in combination with overexpressed Pax7-FLAG in HEK293T cells and co-immunoprecipitation of these proteins, we were able to see an interaction between Myst1 and Pax7 (see Results section). This interaction was also seen with endogenous proteins through co-immunoprecipitation in primary myoblasts. This has led us to identify Myst11 as a promising potential candidate for Pax7 acetylation.

The MYST proteins are named after the founding protein members MOZ Ybf2/Sas3, Sas2 and Tip60 and this family is composed of many members such as Myst1, Myst2, Myst3 and Myst4 (Thomas and Voss, 2007). Myst1, also known as Kat8 or MOF, was one of the first established members and was discovered in *Drosophila melanogaster* as part of a complex named MSL (Male-specific lethal) essential for X chromosome-linked dosage compensation (Gelbart and Kuroda, 2009; Li and Dou, 2010). The MSL complex is composed of Myst1, MLS1, MLS2, MLS3, JILI, MLE and two roX (RNA on the X) non-coding RNAs. This complex that is specific to the male flies is believed to be implicated in X chromosome dose compensation through H4K16 acetylation of

surrounding target genes on the X chromosome. In female flies this complex cannot be formed due to the degradation of MLS2 RNA transcripts (Kelley et al., 1995). In mice, this complex is also present and seems to play a role in X chromosome inactivation in the female mice (Gupta et al., 2008). In addition, a second complex in mammals called the NSL complex (Non-specific lethal) is composed of Myst1, NSL1-3, WRD5, PHF20 and MCRS1/2 and seems to be mostly implicated with global H4K16 acetylation (Lam et al., 2012). Moreover, the NSL complex has been associated with gene transcription as well as DNA repair and nuclear architecture (Horikoshi et al., 2013; Li et al., 2010). Myst1 has also shown to be physically interacting with MLL1/2 and this interaction plays a role in the crosstalk between H3H4 methylation and H4K16 acetylation.(Taylor et al., 2013, Zhao et al., 2013)

Myst1 also acts outside of its complex and is known to acetylate a few non-histone proteins such as p53 (Sykes et al., 2006) as well as ATM (Gupta et al., 2014). Emerging papers are linking the role Myst1 in the regulation autophagy-related genes through H4K16 acetylation or acetylation of these proteins (Füllgrabe et al., 2013).

Myst1 is known to be negatively regulated by Sirt1, a Class III histone deacetylase through lysine deacetylation (Lu et al., 2011; Peng et al.). This lysine deacetylation occurs on residues K274 on the Myst1 protein and seems to cause Myst1 inactivation as well as an increase in Myst1 proteasome degradation . Though, Myst1 acetylation on lysine 274 seems to be only necessary for non-histone protein activity and may not be necessary for acetylation of H4K16 residues. Some other groups have reported that other residues play a role in the function of Myst1 enzymatic activity but K274 is the most studied (Yang et al., 2012; Yuan et al., 2012).

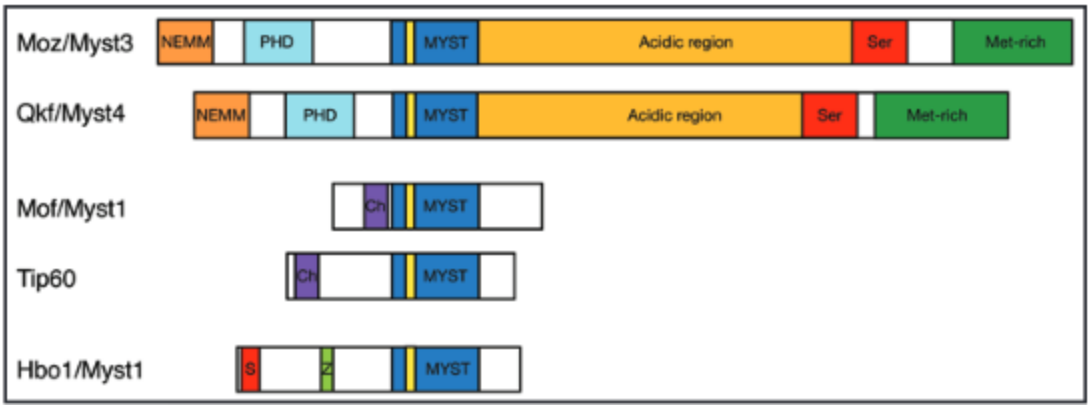

Figure 5: MYST1 family domains (modified from Thomas T and Voss AK, 2007). The chromodomain is in purple, the Zinc finger domain is represented in yellow and the MYST domain is represented in blue.

The MYST protein family is composed of three known functional domains (Thomas and Voss, 2007). The bromodomain, which is specific to Myst1 and Tip60, is situated in the N-terminal portion of the protein (Figure 5). MYST proteins are able to recognize lysines residues within this domain for acetylation. There is also a zinc domain which is situated very close to the enzymatic domain of the protein and seems to be implicated in many processes such as protein or DNA binding, gene transcription and mRNA trafficking. In MYST proteins its purpose seems to be for nucleosome recognition. The last known domain is the lysine acetyltransferase domain which is situated in the C-terminal portion and carries the enzymatic function of the MYST proteins. The enzymatic domain could be inhibited by the use of anacardic acid, a newly discovered HAT inhibitor that can cause this inhibiting action by binding to the same site as acetyl-CoA (Wapenaar et al., 2015). Myst1 is known to display multiple lysine residues which are acetylated

through Myst1 auto-acetylation. Lysine residue 274 within the KAT domain seems to be the primary residue critical for its enzymatic activity.

Myst1 has not yet been discovered to play a role in myogenesis but it has been known to play a role in mouse embryonic stem cell maintenance as well as embryo development (Chelmicki et al., 2014). Myst1 null mice are embryonic lethal at around E5.5 and are unable to implant. These mice do not develop a regular inner cell mass as well as trophectoderm. It has also been shown to play a role in the expression of developmental genes such as Hoxa9, Sox2, Nanog and Oct4 through H4K16 acetylation of histones (Li et al., 2012; Ravens et al., 2014).

Recently there has been an emergence of publications underlining the role of Myst1 in tumorigenesis in different cancers such as leukemia, renal carcinomas and prostate cancer cells (Jaganathan et al., 2014; Su et al., 2016; Zhang et al., 2013). In these studies, aberrant expression of Myst1 resulted in differential H4K16 acetylation or a change in expression of its target ultimately resulting in an increase in cell proliferation.

In all, Myst1 has been shown to acetylate many non-histone proteins. This protein has been shown to work in complex as well as interact with Trithorax proteins, MLL1 and Wdr5. Furthermore, it's been shown to play important roles in various biological processes such as embryogenesis, autophagy and DNA damage. In all, solidifying Myst1 as a suitable candidate for Pax7 acetylation.

The objective of this project is to characterize the function of Pax7 acetylation and is done through 3 specific aims:

1. By investigating Myst1's capacity of acetylating Pax7 on lysine resides 105 and 193 and its effect on Pax7 target genes;
2. By investigating the consequence of Pax7 acetylation being involved in protein stability, protein-protein interaction, protein recruitment, etc.
3. By investigating the role of Myst1 on satellite cell population.

This project will help gain a further understanding of new molecular mechanisms which regulates satellite cell function. This through further information on how Pax7 a master regulator is regulated in muscle. Moreover, further insight into molecular mechanisms regulating satellite cells can help us obtain more information on muscle diseases such as sarcopenia, muscular dystrophies, Rhabdomyosarcoma and other prevalent muscle diseases.

Material and Methods

Antibodies and Plasmids

Commercial antibodies raised against Myst1 (Abcam), Pax7 (DSHB), HA (Bethyl), Myc (Bethyl), acetyllysine (cell signaling), GFP (Abcam), Pan Myosin (DSHB), tubulin antibody (Sigma), Flag M2 (Sigma), FLAG (Sigma), rabbit anti-Myc (Sigma), Rabbit control IgG (Santa-Cruz), MyoD (Santa-Cruz), Sirt1 (Millipore), Ki67 (Abcam) were purchased from individual vendors. Myst1 Plasmid was purchased from Open-bio source LLC.

Cell cultures

Primary myoblasts were obtained from whole hindlimb of 2-month BL6 mice using Magnetic Activated Cell Sorting (MACS) protocol (Sincennes, 2016). Primary myoblasts were cultured in HAMs F-10 supplemented with 20% FBS and 1250ng of beta fibroblast growth factor (bFGF) and 1% Penicillin/Streptomycin. All plates used for primary myoblasts culture were pre-coated with 0.001% collagen before use. C2C12 and 293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Bovine Calf serum and 1% Penicillin/Streptomycin.

Immunofluorescence

Cells and myofibers were fixed using 4% PFA diluted in PBS for 10 mins. Cell were permeabilized in solution (0.1% Triton, 0.1M glycine diluted in PBS) for 10mins. Cells were then incubated for 2hrs RT in Blocking solution (5% Horse serum, 2% BSA, 0.1%

Triton in PBS) before adding appropriate primary antibody overnight. The next day, the myofibers were washed three times for 5mins each using 1x PBS and incubated in fluorescence-conjugated secondary antibody (1/1000) for 1hr RT diluted in blocking solution. Samples were then washed three more times for 5mins each using 1x PBS and stained with DAPI for 10mins (Hochest 1/1000 in PBS). Samples were finally mounted in permafluor solution. The myofibers were then imaged using inverted fluorescent microscope (Zeiss) with 200x objective. All myofiber culture experiments used 3 mice per experiment and over 120 fibers were counted per condition Statistical analysis were performed using student's t-test.

Indirect acetylation detection

C2C12 cells were either treated or not with 2 rounds of 50nM of Myst1 Smartpool siRNA (Thermos) before transfection with Pax7-FLAG. Cells were lysed using Triton IP (50mM Tris pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA, 0.5% Triton X-100, 1uM TSA, 1mM NAM, 10mM sodium butyrate and fresh protease inhibitor cocktail). Samples were lysed on ice for 30 mins and cleared for 15 mins in 4^{o}C centrifuge at max speed. Whole cell extracts were subjugated to FLAG immunoprecipitation for two hours using M2 FLAG beads (Sigma). Samples were then centrifuged at 2.0x RT and washed three times using higher Salt Concentration Triton IP solution (50mM Tris pH 7.5, 300mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA, 0.5% Triton X-100, 1µM TSA, 1mM NAM, 10mM sodium butyrate and fresh protease inhibitor cocktail). Samples were then eluted using 30µl 6x laemmeli buffer heated at 65^{o}C for 10minutes. β-mercaptoethanol diluted 1/10 in laemmeli buffer were added to samples then boiled at 100^{o}C for 5min before loading on SDS-PAGE gels for 2hrs. Samples were transferred for 1hr to PVDF membrane. Membranes were subjected

to Western blot starting by 1 hr blocking using 5% BSA in TBST. Acetylation levels were detected using pan-acetyllysine antibody diluted in 5% BSA in TBST by incubating overnight at 4^{o}C. Membranes were then washed three times using 2x TBST for 5 mins each at room temperature. Rabbit HRP light-chain specific secondary antibody (Jackson immunochemistry) were added to the membrane (diluted 1/5000 in 5% BSA with TBST) for 2hrs. Samples were then washed three times using 2x TBST for 10 minutes each before exposing using ECL (GE life technologies) or the Immobilon HRP substrate (Millipore).

Immunoprecipitation

For all immunoprecipitations (IP), cells were lysed using IP buffer (50mM Tris pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA, 0.5% Triton X-100 and fresh protease inhibitor cocktail) on ice for 30 minutes. Lysates were cleared by centrifugation and from those lysates, 500µg of protein extract were used for IP. 25µg (5%) of protein extracts were used as input. Samples were incubated overnight at 4°C with 30µl of M2 FLAG antibody-coupled beads (Sigma), then washed three times with IP buffer. For IP anti-HA, samples were incubated overnight at 4°C with 5µg of anti-HA antibody (Bethyl). For the endogenous immunoprecipitations, samples were incubated with 5µg of anti-Kat8 antibody (Abcam) or the IgG control overnight at 4°C. Protein A beads (Sigma) were added the next day and the samples were incubated for 2hrs at 4°C, then washed three times in IP buffer. All samples were eluted with 30µl of laemmli buffer and incubated at 65°C for 10 minutes. All samples and inputs were loaded with 6x of loading buffer containing 1/10 β-mercaptoethanol and incubated at 100°C for 5 minutes. All samples were loaded on SDS-PAGE and analyzed by Western Blot.

Myofiber culture experiments

EDL (Extensor Digitorum Longus) were isolated from hindlimbs either 6-8-week-olds Myf5-Cre Rosa-YFP mice or C57BL/6J mice. Incubated with collagenase type I (Worthington Biochemical Corp) in low glucose DMEM (Gibco) for 1 hr, fibers were triturated for 10mins then washed 3 times by transferring new into FBS coated plates containing low glucose media. After washes fibers are incubated for an hour in low glucose DMEM for 1hr. Cells are then incubated in fiber media composed of low glucose DMEM (Gibco) , 0.01% Chicken embryo extract (Cedarlane), Fibroblast growth factor, 10% Fetal bovine serum and 1% penicillin and streptomycin.

Table 1: Forward and reverse primers used for qPCR experiments

Primer	Forward sequence	Reverse sequence
Myst1	GTCACACCTAAGCTGGTAGAG	CAGACAGAGTCCACTGTGATAG
Pax7	GACGACGAGGAAGGAGACAA	ACATCTGAGCCCTCATCCAG
Myf5	TGACGGCATGCCTGAATGTA	ATCTGCAGCACATGCATTTGATA
Perp	TGTGGCTTTATCATCCTGTG	CAGCAGGGTTATCGTGAAG
Ebp4	CCCTGTCAATATTGCTGCTC	GCTTCTTCCAGCTCTTCCTT
Stmn2	GCAATGGCCTACAAGGAAAA	TTCACCTCCATGTCGTCGTA
Postn	AACCAAGGACCTGAAACACG	GTGTCAGGACACGGTCAATG
MyoD	TGATGGCATGATGGATTACAG	GTAGTAGGCGGTGTCGTA
Myogenin	CAACCCAGGAGATCATTTG	CATATCCTCCACCGTGAT
MyhI	CTGGAGAAGACGAAGCAGC	CTGCCAGGATCTTGTCAAAGT
Fst	CAGTGACAATGCCACATAC	CCTCTTCCTCCGTTTCTT
Crlf1	GCAGAAGTCACACAAGAC	GGATGGCCTATCCTTAGAG
Pde10a	CCTGCTATACCACCTTGA	CCTTCTCCCACTGATTGA
Dio2	ACTCGGTCATTCTGCTCAAG	GATTCAGGATTGGAGACGTG
Eps8	GAACGGAGCACAACTCTTT	ACTCGGAGCTTCCATTACT

Total RNA isolation

Appropriate cell types were lysed from samples in 6 well plates (VWR) using Trizol solution (Life Technologies). 200ul Chloroform was added to samples and centrifuged for 15mins at 21x g at 4^{o}C. Supernatant was collected, and RNA was precipitated using 850ul of isopropanol and 1.5ul glycoblue solution (Life Technologies). Samples were left to precipitate for 1hr at -20^{o}C. Samples were washed using 200ul of 70% ethanol and spun at 21x for 5mins at 4^{o}C. Samples were left to dry for 2-3 mins before reconstituted in 20ul sterile water. 1ug of RNA was used for RT reaction using Random primer (Invitrogen), OligodT (Invitrogen), 5mM dNTP (Invitrogen), RNase Out (Invitrogen) and SuperScript III Reverse transcriptase (Invitrogen) in a total volume of 20µl per RNA sample. Samples were incubated at 42^{o}C for 1hr and diluted to obtain concentration of 5ng/µl. The samples were then used for qPCR reactions.

Transfection

C2C12 seeded in 150 mm plates at 50% confluency were transfected with 10ug of plasmid using Lipofectamine 2000 (Invitrogen), diluted in Opti-MEM (Life Technologies), according to the manufacturer's protocol. 293T cells were seeded in 100mm plates at 50% confluency and were transfected with 5ug of plasmid using Lipofectamine 2000 (Invitrogen), diluted in Opti-MEM (Life Technologies), according to the manufacturer's protocols. The media for both 293T and C2C12 cells was replaced after 6 hours after transfection. The plates were left for 18-48 hours depending on the experiment, as described in the Results section, then harvested. Primary myoblasts were transfected with

50nM Myst1 siRNA smartpool (Thermos) using Lipofectamine RNAi Max (Invitrogen), diluted in Opti-MEM (Life Technologies), according to the manufacturer's protocols. The media was replaced after 6hrs of transfection and the cells were treated with two rounds of siRNA transfection 18hrs apart. All cells were then harvested for RNA isolation or protein isolation.

Western Blots

The following antibodies were used for Western blots: mouse anti-FLAG (Sigma), rabbit anti-HA (Bethyl), rabbit anti-Myc (Sigma), rabbit monoclonal anti-Kat8 (Abcam), mouse Pax7 (DSHB), mouse tubulin (DSHB), rabbit pan-myosin (sigma). All primary antibodies were diluted to 1/1000 in TBST with 5% milk. The primary antibody was incubated overnight at 4°C. After incubation, the membranes were washed 3 times for 5-10 minutes in TBST. All secondary antibodies were HRP light chain specific (Jackson immunochemistry) antibodies to reduce background signal and were diluted to 1/5000 in TBST 5% milk. The membranes were incubated for 1-2 hours at room temperature. The membranes were washed to remove excess secondary 3 times for 10 minutes in TBST before exposing using ECL (GE life technologies) or the Immobilon HRP substrate (Millipore).

Results

Investigating Myst1 expression pattern in myogenesis

The first objective aimed to investigate whether Myst1 is the acetylase candidate playing a role in Pax7 acetylation. Myst1 has been chosen as a Pax7 acetylase candidate due its known interaction with Trithorax partners Wrd5 and MLL as well as its regulation and interaction with Srt1 deacetylase protein. Although there is no data supporting Myst1 expression in muscle or its pattern of expression throughout different stages in myogenesis, microarray data from activated satellite cells, proliferating myoblasts and differentiating myotubes (data not shown) showed a similar Myst1 detection level in all three of these conditions. To confirm this finding, we wanted to address whether Myst1 is expressed at the protein level as well as measure differential Myst1 expression in these various cell stages. We achieved this by using Western blots to measure protein expression throughout different stages of myogenesis. At the protein level, Myst1 seems to be ubiquitously and strongly expressed in proliferating myoblast as well in differentiating myotubes from day one to day three (Figure 6). In comparison, Pax7 expression was observed only in primary myoblasts and is down-regulated right when differentiation is triggered. Myosin proteins are not expressed in primary myoblasts that are up-regulated right on the first day of differentiation and expression increases during day two and three. We would have expected that Myst1 expression would have a similar expression pattern as Pax7 but seeing ubiquitous expression would suggest Myst1 having another role in muscle. This might be achieved through H4K16 acetylation of genes or the acetylation of other potential proteins which remain to be elucidated.

Figure 6

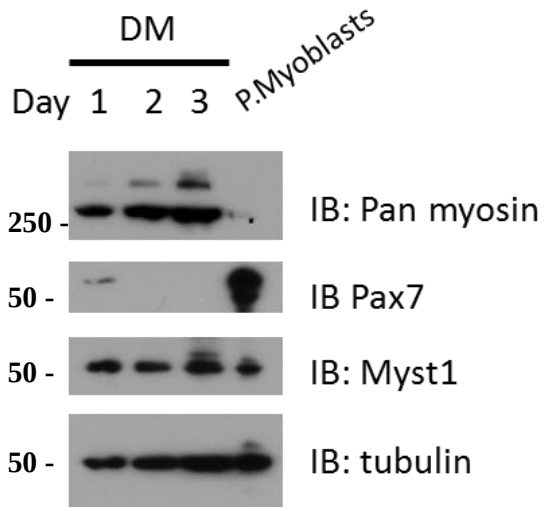

Figure 6: Myst1 pattern expression in proliferating and primary myoblasts. WCE of primary myoblasts in growth media or differentiating media for 24-72hrs subjected to western blot and detected with appropriate antibodies. N=3 biological replicates.

Assessing Myst1 interactions in myogenesis

The addition of any posttranslational mark requires interaction or binding of both substrate and enzyme. To address the question of the first objective we investigated the possibility of Myst1 interaction with Pax7. The interaction was both assessed in overexpressed context as well with endogenous proteins. We achieved this using co-immunoprecipitation experiments. Pax7-FLAG was transiently expressed in the presence or absence of Myst1-HA to test their interaction. Indeed, when Pax7 immunoprecipitated along with Myst1 an interaction was seen and not in the control condition, while Myst1 and Pax7 transfected separately (Figure 7a). The same results occurred when Myst1 was immunoprecipitated through HA tag with transient expression of both Pax7 and Myst1 (Figure 7b). This interaction did not occur in any of the conditions where they were expressed separately. This interaction was also seen in primary myoblasts with endogenous proteins. Myst1 immunoprecipitation showed robust Pax7 interaction compared to isotype control where there was no immunoprecipitation (Figure 8). These co-IP experiments suggest that Pax7 and Myst1 interact robustly in both an overexpressed and endogenously in primary myoblast making Myst1 an excellent candidate for Pax7 acetylation.

Figure 7

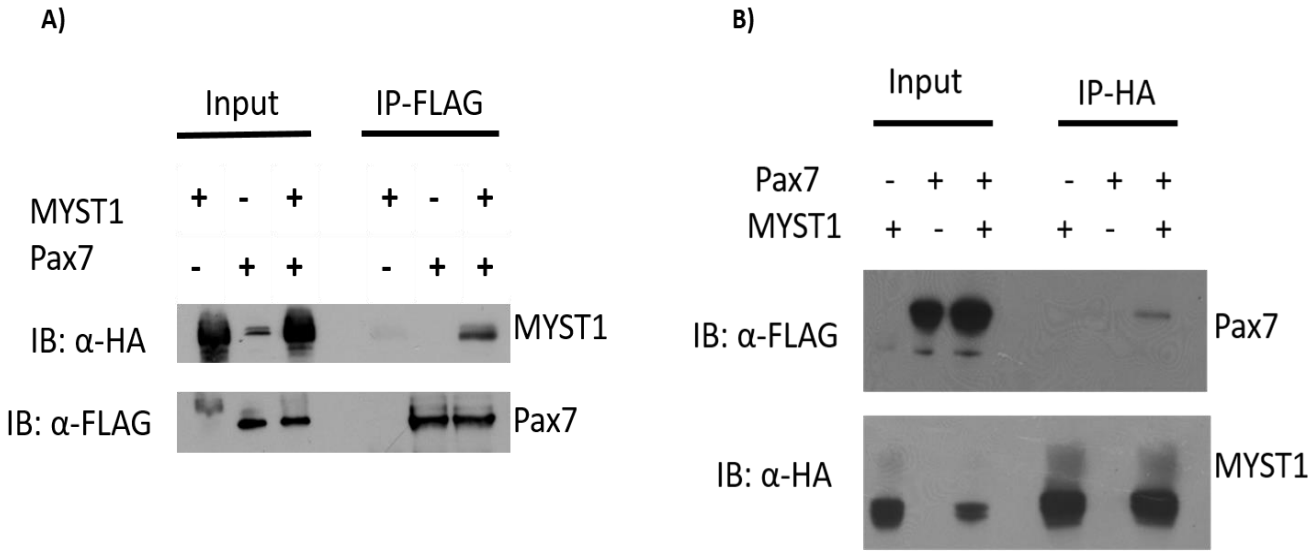

Figure 7: Myst1 interacts with Pax7 in 293T cells. A) Immunoprecipitation of FLAG (Pax7) in WCE containing Pax7-FLAG, Myst-HA or Myst1-HA+Pax7-FLAG overexpression. Immunoblotting, was done with FLAG and HA antibody. B) Immunoprecipitation of HA (Myst1) in WCE containing Pax7-FLAG, Myst-HA or Myst1-HA+Pax7-FLAG overexpression. Immunoblotting, was done with FLAG and HA antibody.

Figure 8

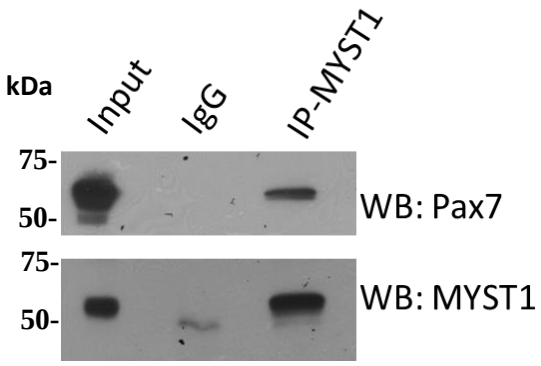

Figure 8: Myst1 interacts with Pax7 in primary myoblasts. Immunoblotting of endogenous Pax7 is detectable through the immunoprecipitation of Myst1 using Myst1 antibody in Whole cell extract. N=3 biological replicates

In the literature, there are instances of the implication of Myst1 interaction with the tri-thorax complex through binding assay, mass spectrometry and co-immunoprecipitation in different systems (Xiao et al. 2013, Dias et al.2014). We were also interested into verifying whether Myst1 interacts or is found to be with other Pax7 interactors namely members of the tri-thorax complex. This was accomplished through co-immunoprecipitation using Myst1 antibody in primary myoblasts. We found that Myst1 did not interact with Wdr5, a common interactor found in other models in primary myoblasts (Figure 9a). This suggested that Myst1-Pax7 interaction occurs in another complex or as a separate event. We did not verify if there were an interaction with MLL1/2 proteins or other MLL1/2 partners which would have to be assessed in future studies. Nevertheless, this data suggests that Myst1 is not found with members with the tri-thorax complex in muscle indicating the possible involvement of another histone-modifying complex such as the MSL or NSL complex.

Figure 9

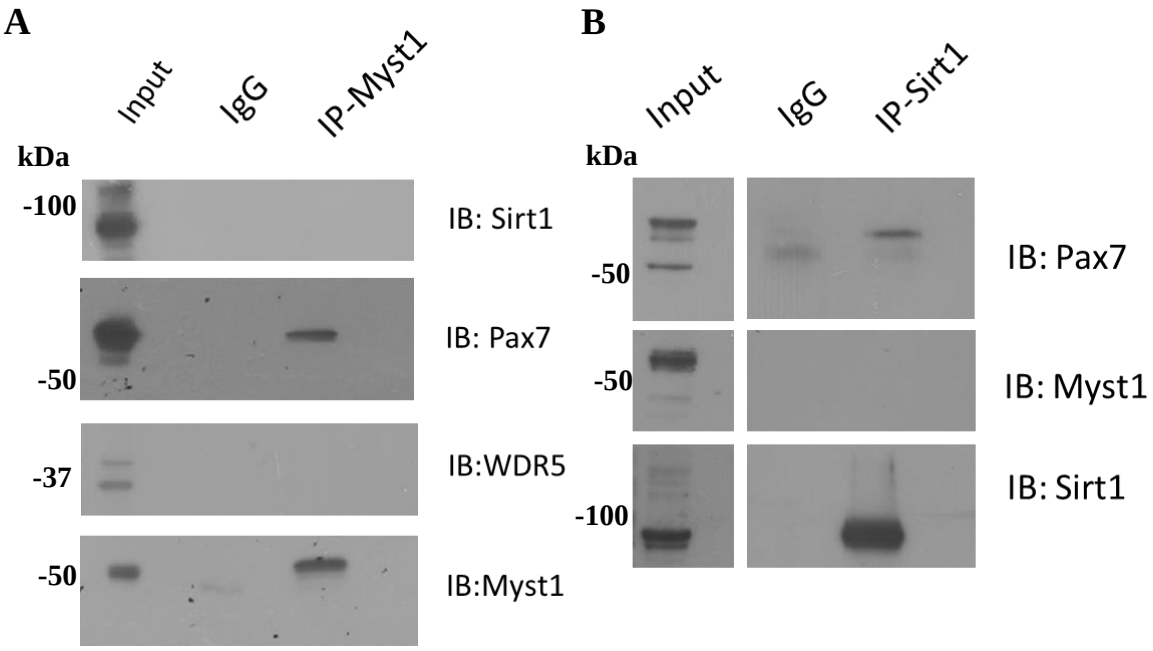

Figure 9: Myst1 and Sirt1 interaction is mutually exclusive or MLL complex member Wdr5. Primary myoblast WCE immunoprecipitated with either **A)** Myst1 antibody or **B)** Sirt1 antibody and were immunoblotted for appropriate antibodies n=3 biological replicate

Myst1 in the literature is known to be negatively regulated by Sirt1 deacetylation at lysine 274 (Fullgrabe et al. 2013, Lu et al. 2011) . Since Sirt1 is part of candidates we've designated for Pax7 deacetylation we sought to answer whether Myst1 and Sirt1 interact in primary myoblast through co-IP. Surprisingly, co-IP experiments have demonstrated that Myst1 and Sirt1 do not have an interaction in the context of primary myoblast either if Sirt1 or Myst1 is immunoprecipitated (Figure 9b). This opens the possibility that Myst1 is regulated differently in muscle and possibly through other acetylation sites or other posttranslational modifications. More importantly, this data shows that Sirt1 and Myst1 are mutually exclusive in their interaction with Pax7, suggesting that they work in different complexes. It would be interesting in future experiments to determine Myst1's interacting complex in myoblasts

Pax7 is target for Myst1 acetylation.

We wanted to address one of the key hypothesis about Myst1's role in modulating Pax7 acetylation since we have shown that Myst1 is expressed in various stages of myogenesis and displays a robust interaction with Pax7. This being the possibility that Pax7 is an substrate of Myst1 acetylation. To answer this question, we decided to assess Pax7's acetylation levels through immunoprecipitation. (Figure 5). We decided to perform this experiment in 293T cell with transiently transfected FLAG-Pax7 and HA-Myst1 plasmids. The reason 293T cells were used instead of C2C12 was because Myst1 overexpression in C2C12 consistently causes cell death. The cell extracts were

immunoprecipitated using FLAG beads and blotted for acetylation status using a pan-acetyllysine antibody. Equal loading was monitored by Pax7 immunoprecipitation levels. Pax7 on its own is consistently seen with high levels of acetylation (Figure 10b). In the condition where Myst1 is also expressed we see a slight increase in acetylation levels, suggesting that Myst1 plays a role in Pax7 acetylation. The minimal change in Pax7 acetylation levels are probably seen due to the Pax7 that is very heavily acetylated in 293T thus overexpressing Myst1 doesn't induce a higher level in Pax7 acetylation status. To further confirm the involvement of Myst1 in Pax7 acetylation we decided to deplete Myst1 expression using siRNA knockdown and assess Pax7 acetylation levels through co-IP and Western Blot (Figure 10a). Under this condition, there is a significant decrease in Pax7 acetylation levels compared to the non-treated control solidifying the capability of Myst1 acetylating Pax7. This critical piece of data supports our hypothesis that Myst1 plays an important role modulating Pax7 acetylation status. In addition, these findings open the possibility of a novel role of an acetyltransferase in muscle as well as a novel target for Pax7 activity.

Figure 10

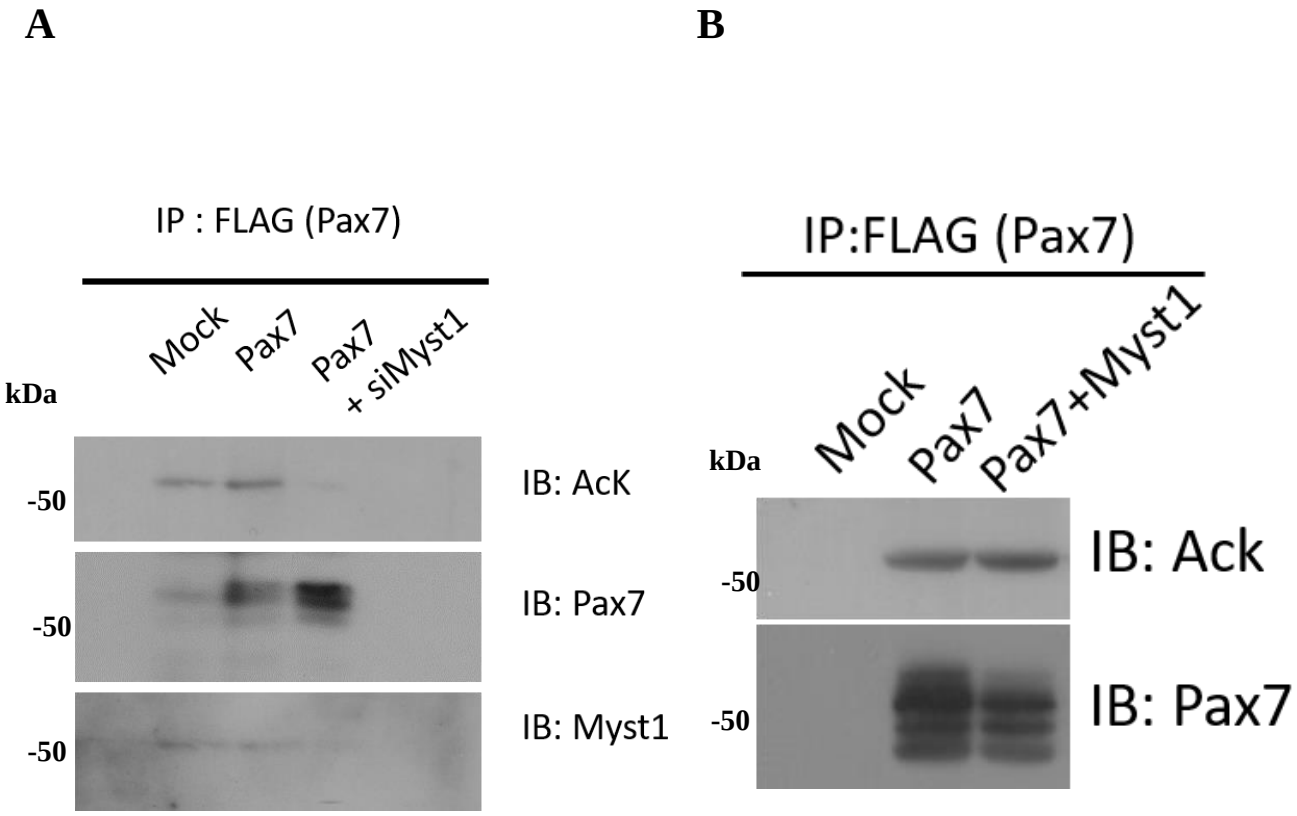

Figure 10: Myst1 plays a role in Pax7 acetylation. **A)** C2C12 cells were transfected with Pax7-FLAG plasmid or left untransfected (mock control) and treated with 2 rounds of 50nM of Myst1 siRNA or untreated as a control. WCE were immunoprecipitated and appropriate antibodies were used for western blot., n=2 biological replicate **B)** 293T cells were transfected with ether Pax7-FLAG or both Pax7-FLAG and HA-Myst1 construct. WCE were immunoprecipitated and appropriate antibodies were used for western blot. Mock control represents untransfected cells n=2 biological replicate.

Myst1 influences Pax7 target genes transcripts levels.

Pax7 acetylation is hypothesized to play a role in its transcriptional activity. Since it has been shown that Pax7 plays an essential role in cell proliferation, adhesion and signaling it would be likely that Myst1 acetylation of Pax7 would play a role in the regulation of these events (Soleimani et al. 2013). To investigate this possibility, we performed siRNA silencing of Myst1 to eliminate its expression and assess its effect on Pax7 target gene expression as well as myoblast differentiation. To achieve this, we treated primary myoblast with two rounds of Myst1 siRNA 12hrs apart and harvested whole RNA for RT-qPCR. For the differentiation experiment, the cells were cultured 48hrs in low serum to induce differentiation.

We assessed Pax7 target gene transcripts (taken from, Soleimani, et al 2013) treated with siMyst1 siRNA or siScramble control and found that many of those transcripts were downregulated compared to scrambled control. Myst1 levels were also assessed and were around 80% knocked down. No change in Pax7 gene expression was observed, which is expected and further confirms that Myst1 is affecting Pax7 on a post-translational level and not on a transcription level. Myf5, one of Pax7's most characterized target was found to be 50% reduced at the RNA level during Myst1 siRNA as well as other Pax7 genes, Gas7 (Figure 11). Other genes were around 60-70% down-regulated such as Perp and Eps8 and surprisingly, some genes transcripts were also upregulated in response de siMyst1 treatment (Fst, Pde10a, Dio2) suggesting that Pax7 could play a role as a negative regulator of these genes. This data in all suggest that Myst1 is required for the expression of Pax7's targets. Of all 13 genes tested, there were only 4 in which had no response suggesting that Myst1, and subsequently Pax7 acetylation may only influence a subset of Pax7 targets.

Figure 11

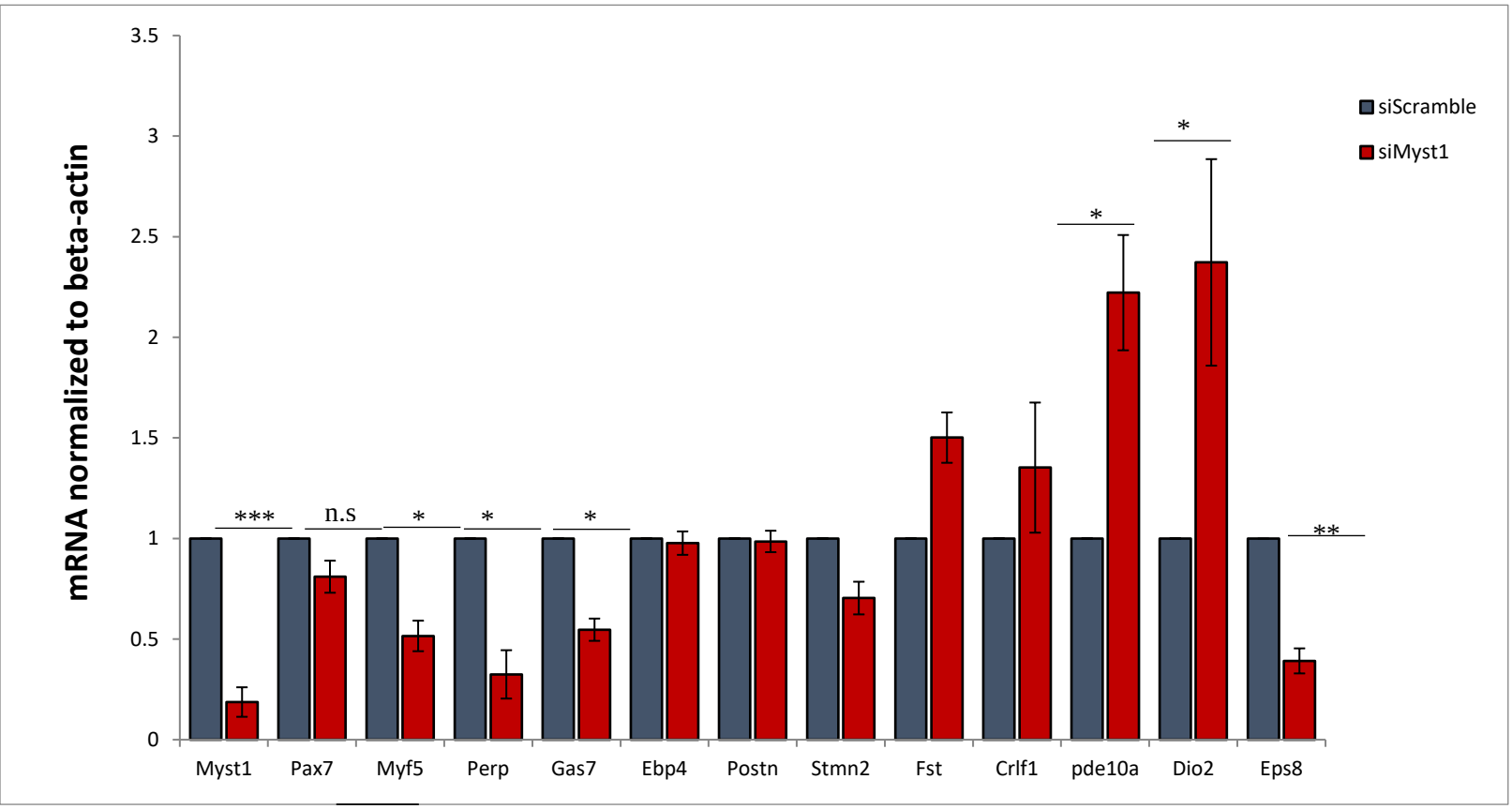

Figure 11: Myst1 depletion effect Pax7's targets. RT-qPCR depicting genes affected by Myst1 depletion. Primary myoblast was transfected with two rounds of siMyst1 24hrs apart and harvested for total RNA. (n=3 biological replicate, error bars represent S.E.M, and *p<0.05, **p<0.01 and ***p<0.001)

In all, this data along with Myst1 depletion causing Pax7 disruption of target genes supports our hypothesis that Myst1 is playing a role to mediate Pax7's acetylation due to its capacity to interact with Pax7, its disruptive effect on Pax7's targets once depleted, as well as its effect its acetylation status.

Myst1 depletion effects myoblasts differentiation

In culture, siMyst1 treated cells tend to not proliferate as quickly and adopt a slightly elongated shape similar to myocytes. This observation led us to investigate whether myoblasts treated with siMyst1 would display a higher differentiation potential by measuring differentiation transcripts such as MyoD, Myogenin and Myosin expression (Figure 12). Surprisingly, these cells do not display higher levels of myogenin or either myosin heavy chain but there was a 50% increase in MyoD expression. Suggesting either Myst1 plays a role in MyoD repression or that reduction in Pax7 acetylation results in a shift in the proportion of differentiating cells.

Figure 12

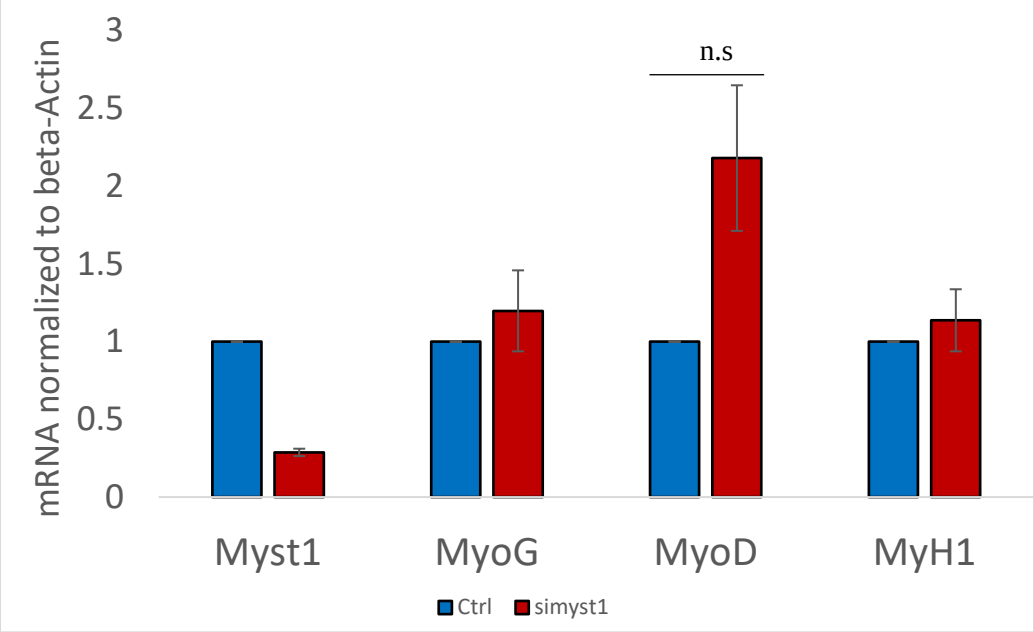

Figure 12: Myst1 depletion does not play a prominent role in myoblast differentiation. RT-qPCR depicting genes affected by Myst1 depletion. Primary myoblast was transfected with two rounds of siMyst1 24hrs apart, put in differentiation media for 42hrs and harvested for total RNA. (n=3, error bars represent S.E.M)

Pax7 acetylation does not play a role in modulating Protein-protein interaction

The second objective was to investigate whether Pax7 acetylation plays a role in protein stability, activity, protein localization or chromatin recruitment. Earlier luciferase experiments have determined that Pax7K105R, K193R double mutant generated through site-directed mutagenesis have a decreased luciferase activation in the presence of TSA (Supplemental 1). Since the mutant was sufficient to impair Pax7 transcriptional activity we sought out to challenge the mutant to various experiments exploring the effect on either protein stability, chromatin, recruitment or protein interaction. We first wanted to address the possibility of acetylation playing a role in Pax7 interaction with Ash2L, Wdr5, Carm1 (known partners). To investigate this possibility, we transfected 293T cells with WT Pax7-FLAG or Pax7105R,193R-FLAG in combination with either Myc-Ash2L, Myc-Wdr5 or Myc-Carm1 (Figure 13). In all the conditions, Pax7 double mutant does not display a differential interaction pattern compared to WT Pax7 suggesting that acetylation does not play a role in mediating the interaction of either of these partners. Acetylation could play a role into mediating other interactions which could be investigated through other experiments such as mass spectrometry.

Figure 13

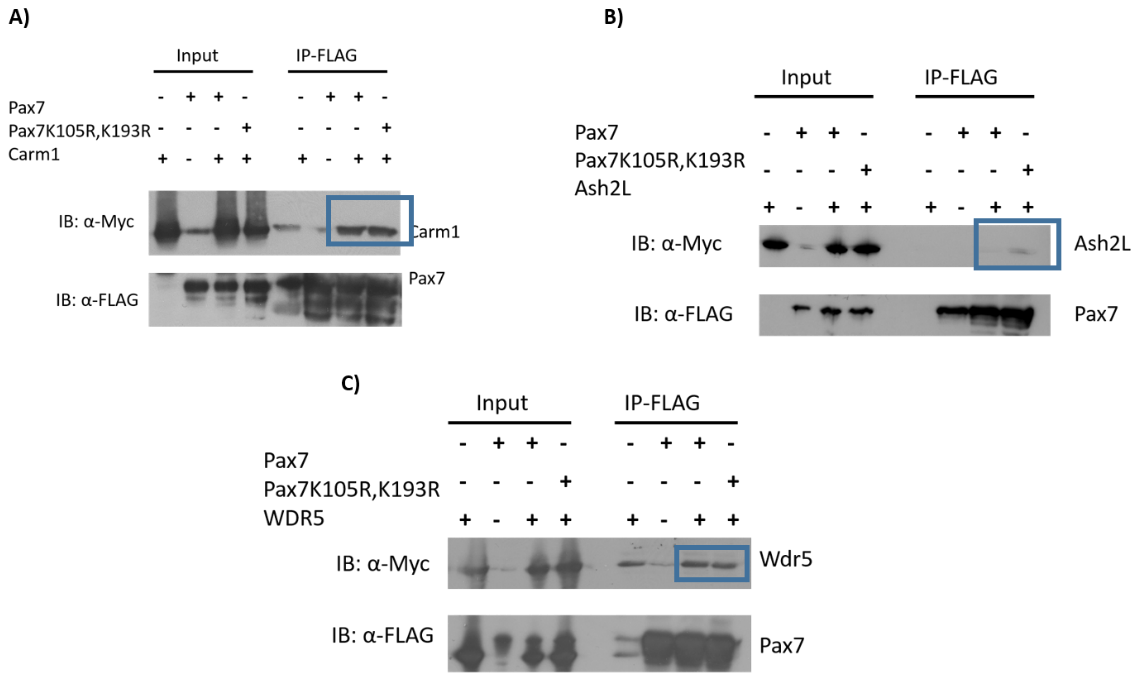

Figure 13: Pax7 acetylation does not play a role in interaction with Ash2L, Wdr5 or Carm1. Western blot depicting immunoprecipitation of WT Pax7 or Mutant Pax7 (FLAG) with different interacting partners. **A)** Carm1-Myc interaction with Pax7-FLAG WT versus Pax7-FLAG K105R, K193R double mutant. 293T cells were overexpressed with plasmids Pax7 –FLAG WT or double mutant and Carm1-Myc. **B)** WDR5-Myc interaction with Pax7-FLAG WT versus Pax7-FLAG K105R, K193R double mutant. 293T cells were overexpressed with plasmids Pax7 –FLAG WT or double mutant and WDR5-Myc. **C)** Ash2L-Myc interaction with Pax7-FLAG WT versus Pax7-FLAG K105R, K193R double mutant. 293T cells were overexpressed with plasmids Pax7 –FLAG WT or double mutant and Ash2L-Myc. N=3 biological replicate.

Myst1 plays a role in satellite cells proliferation.

Through multiple experiments we have established a role for Myst1 in muscle progenitor cells. An avenue that still needs to be explored is determining Myst1's role in satellite cells. Information obtained in primary myoblasts may not necessarily reflect what is seen in the committed or stem cell population. This brought us to the third objective of this study, which aimed to determine the effect of Myst1 protein function in satellite cell through Myst1 siRNA treatment. This set of experiments was performed through immunofluorescence staining of satellite cells found on freshly isolated myofibers in both normal B6L57 mice as well as using the Myf5Cre-R26-YFP model. Myofiber isolation and culture is an excellent method to visualize the state of satellite cells during their first rounds of division (supplemental 4). In a normal, healthy context, at time zero meaning before being put in myofiber culture media all of the satellite cells are quiescent and are marked by Pax7 expression (Kuang et al, 2007) . Within 24hrs those satellite cells are now activated and can be usually marked by MyoD expression. The first division is caught around 36hr mark in which their orientation will determine whether the division will be a symmetric or an asymmetric division. A planar division is most likely to an symmetric division whether it be of the committed cells or of the non-committed cells that will revert back into their quiescent state. At 42h-48hrs a proportion of the cells have divided and will be seen in doublets and will proliferate at a more rapid rate. By 72hrs, the cells will have gone through a couple of division and forms clumps of cells in which they will start differentiating seen by the expression of Myogenin.

Since Pax7 acetylation is mediated through Myst1, we would hypothesize that Myst1 depletion would affect Pax7's function. Causing a disruption in satellite cell

proliferation, or differentiation partially mimicking a Pax7 depletion phenotype. To assess the effect of Myst1 on satellite cell population, the myofibers were fixed at both 42hrs and 72hrs after being put in culture and treated with Myst1 siRNA twice during an 18hrs period. All fibers were then stained with Pax7, Myst1to mark proliferation status of the cells as well as MyoD to mark satellite cell commitment.

Myofibers treated with siMyst1 and fixed at 42hrs to assess the first divisions have a significantly lower proportion of their cells expressing Ki67 proliferation marker compared to scrambled control (Figure 14a). The number of cell themselves seem to remain constant seeing as there are no significant changes between both conditions (Figure 14b). The number of symmetric division seems to be also remain constant. This data suggests that the satellite cells are already losing their proliferative capacity at 42hrs with Myst1 loss of expression. We also assessed the satellite cells proliferative capacity at 72 hrs where there is striking change in the cell numbers in the sMyst1 treated myofibers. 70% decrease of the proportion activated satellite cells marked by Pax7/MyoD/Ki67+ triple positive staining (Figure 15). Moreover, there is an increase of overall Pax7 and MyoD double positive expressing cells in the siMyst1 treated condition suggesting that there is a higher shift to commitment and a loss of progenitor expansion (Figure 16). In all, these data support the notion that absence or depletion of Pax7 acetylation through Myst1 expression mimics in part what is already seen in a Pax7 depleted phenotype.

Figure 14

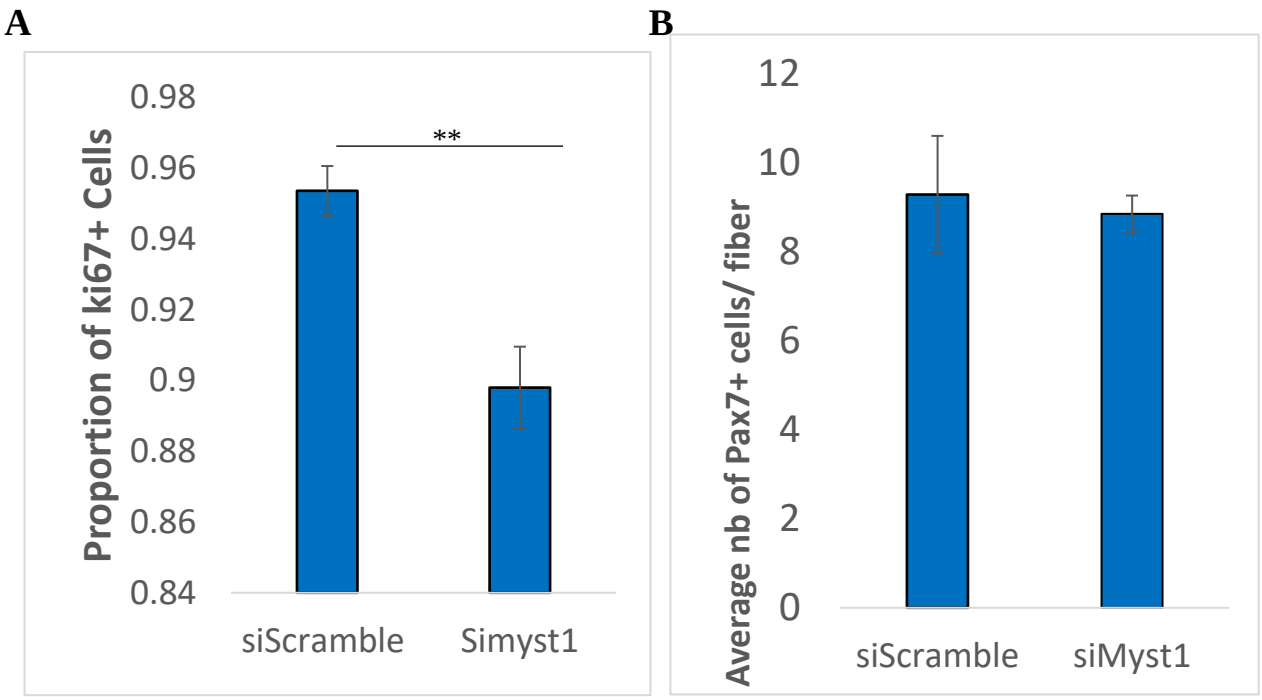

Figure 14: Myst1 depletion effects satellite cell proliferation 42hrs in culture. Freshly isolated myofibers transfected with two rounds of siMyst1 12hrs apart and was let to culture for an additional 24hr then fixed. Pax7 positive and Ki67 positives cells were counted. **A)** Represents the proportion of proliferating cells marked by Ki67 expression. **B)** Represents the average number of Pax7 positive cells per fiber. ~150 fibers per condition. (n=3 mice error bars represent S.E.M, and *p<0.05)

Figure 15

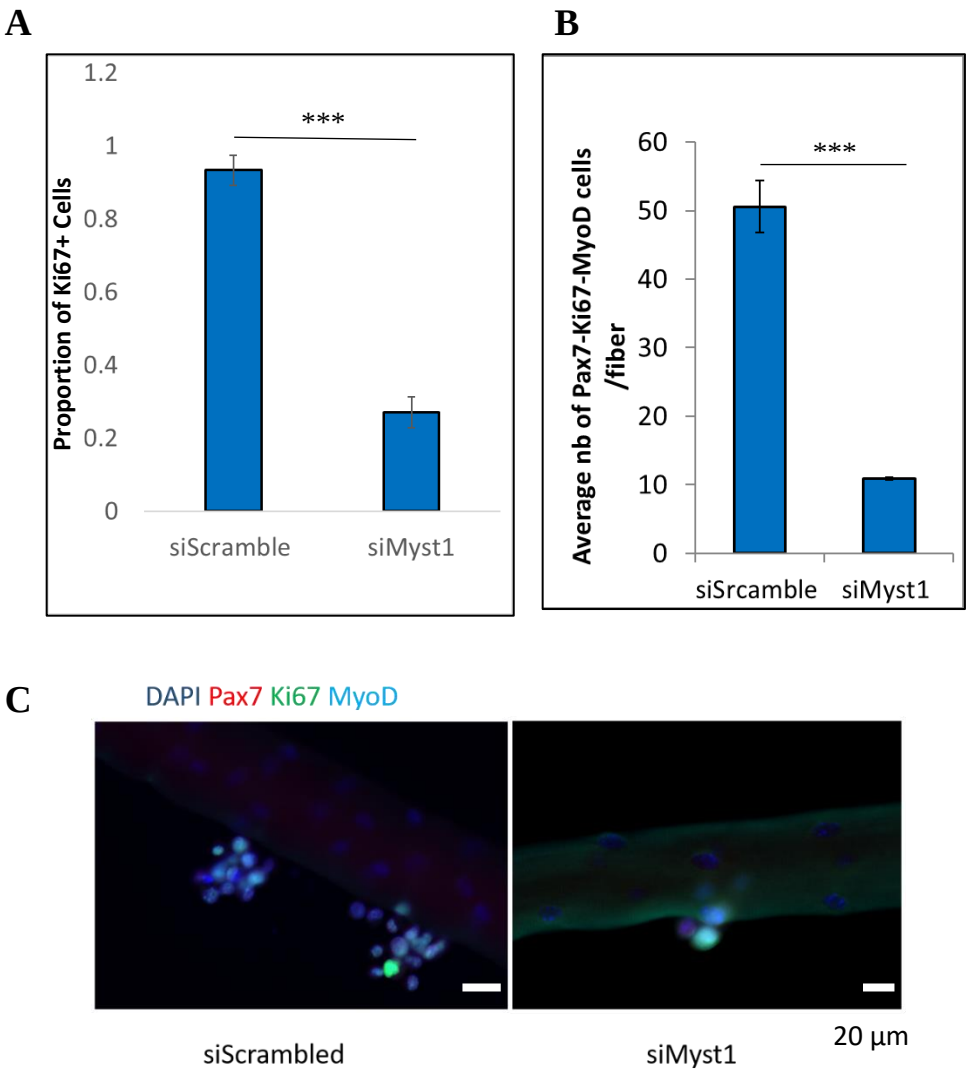

Figure 15: Myst1 depletion in satellite cells at 72hrs decreases the proportion of progenitors. Freshly isolated myofibers transfected with two rounds of siMyst1 12hrs apart and was let to culture for an additional 49hr then fixed. Pax7, Ki67, MyoD positives cells were counted. **A)** proportion of triple positive shown **B)** Average number of cells per fiber **C)** Immunostaining of fibers at 72hr of siScramble vs siMyst1. ~150-200 fibers per condition. (n=3 mice error bars represent s.e.m, and **p<0.01, ***p<0.001).

Figure 16

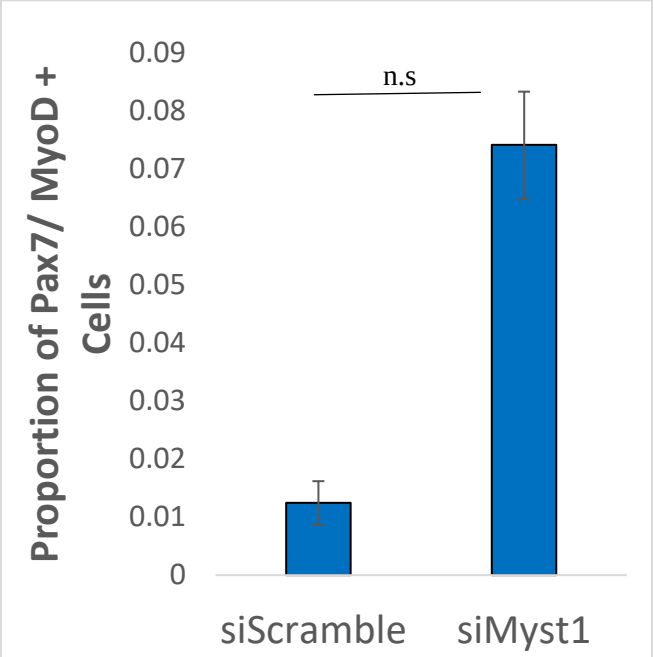

Figure 16: Myst1 depletion in satellite cells at 72hrs cause an increase of Pax7/MyoD Cells. Freshly isolated myofibers transfected with two rounds of siMyst1 12hrs apart and was let to culture for an additional 49hr then fixed. Pax7, MyoD double positives cells were counted. ~120 fibers per condition. (n=3 mice error bars represent S.E.M)

Discussion

Molecular mechanisms in which Pax7 is regulated and its implications in satellite cell function has remained a question. In this study, we identified Myst1, as a Pax7 interactor in overexpressed context as well as in primary myoblasts. We determined that Pax7 is acetylated and that its acetylation status is disrupted in the presence of Myst1 depletion. More importantly, we have shown that Myst1 depletion displays an robust phenotype in muscle progenitors as well as satellite cells such as disruption in Pax7 target gene expression, an increase in MyoD expression and an impairment in satellite cell proliferation. Taken together, this data supports the hypothesis that Myst1 modulates Pax7 acetylation. Pax7 acetylation is required for active satellite cell proliferation and maintenance of the satellite cell's proliferative state. Myst1 depletion experiments through siRNA knockdown on satellite cells and myoblast displays a phenotype resembling Pax7 depletion.

This is particularly interesting since removal of Pax7 methylase Carm1, through knockout or siRNA treatment does not result in proliferation defect, but an impairment in their ability to divide asymmetrically through the lack of Trithorax members recruitment (Kawabe et al. 2012). This information allows us to hypothesize that Pax7 acetylation may be necessary for the maintenance and expansion of the committed satellite cell. Seen by the 70% decrease in the number of satellite cells in treated myofiber culture with siRNA treatment. Consequently, there is a loss in cell clusters seen in the treated cells and they are seen mostly in first or second round of divisions as would be seen 42hrs in culture. There is no change seen in total number of satellite cells at 42hrs, when the first division occur

but there is a significant decrease in the proportion of cycling or proliferating satellite cells. Further, pointing towards the role of Pax7 acetylation being critical of the expanding satellite cells. In all, this data also suggests that Pax7 acetylation is crucial for committed satellite cell expansion.

It would be interesting to investigate through more myofiber culture experiments when during this 42-72hr period does this loss of proliferating satellite cells occur s as well as investigating what the cells become. There are four possibilities in which could be envisioned. The satellite cells may precociously differentiate and fuse to the myofibers, which is the most likely fate due the increase of non-proliferating Pax7/MyoD positive cells seen in the satellite cells. A second possibility is that they may return to quiescence. This hypothesis is unlikely since it seems that the progenitors are the most affected. The third option is that the cells are arrested in cell cycle and go through senescence. The fourth option is that they turn into brown fat. None less the fate of these satellite cells will be explored future directions.

Surprisingly, no interactions between Myst1 and Sirt1 or Wdr5 were observed. This piece of data is very intriguing because, Myst1 is only known so far in the literature to be regulated through Sirt1 deacetylation More importantly, Sirt1 is one of our deacetylase candidates for Pax7 deacetylation, through recent data has pointed towards Sirt2 being the deacetylase candidate playing a role in Pax7 deacetylation. Through Sirt2 has not been found to regulate Myst1 or interact with Myst1, they both do have the same histone target being H4K16 acetylation. Sirt2 is known to be responsible for H4K16 deacetylation during the M phase due to its predominant cytoplasmic localization (Vaquero., 2006) Preliminary IP experiments have shown that Myst1 and Sirt2 do not hold any interaction. However, the

cells were not synchronized nor were there an enrichment of Sirt2 in the nucleus during this experiment thus, we cannot exclude the possibility of an interaction.

Another surprising finding is the lack of Myst1-Wrd5 seen in primary myoblasts. A possible reason which there is no seen interaction between these two proteins in primary myoblast, may since Myst1 is found in another histone-modifying complex such as the NSL complex composed of (NSL1-3, MCRS-2, and MBD-R2). There has been one study that reported that NSL1 is sufficient to allow Myst1 to recognize its substrates (Huang et al., 2012) . Which could explain this lack of interactions in a muscle model.

Through multiple experiments, we have shown that Pax7 acetylation is not implicated in the interaction of Carm1, Wdr5 or Ash2L (members of the tri-thorax complex), in Pax7 protein stability or Pax7 protein localization (supplemental 2). Though we do have evidence that Pax7 acetylation may be required for chromatin recruitment through ChIP-qPCR performed in Pax7 and Pax7K105R, K193R expressing cells (Supplemental 3). Through difference in ChIP enrichment between WT Pax7 and double mutated arginine Pax7 mutant (KR) is minor but statistically significant. Moreover, in future studies these mutants will also be challenged with respect to their ability to homodimerize as well as their ability to bind DNA by means of immunoprecipitation and Electronic mobility shift assay (EMSA) respectively to assess their requirement in Pax7 acetylation. Lastly, we would also like to investigate whether there is differential binding partner between the double mutant and WT Pax7 using Mass-spectrometry.

Luciferase assay experiments have shown that lysine 193 is more potent in its role in regulating Pax7's transcriptional activity. To elucidate the role of Pax7 acetylation *in vivo* lysine to arginine mutant of this residue was generated (Saber, unpublished). These

mice will be used for a panel of experiments ranging from myofiber experiments, to regeneration experiments, verifying differentiation and proliferation capacity and more. We would expect that the experiments performed on these mice will resemble the same phenotype from the siRNA Myst1 treatment experiments cementing the role of Myst1 as Pax7 acetylase.

Myst1 has not yet been demonstrated to play a role in myogenesis. Nevertheless, H4K16 deacetylation modulated by Sirt1 plays a role in maintaining satellite cell quiescence (Ryall et al.,2015). Metabolic loss of NAD+ results in decreased Sirt1 activity in which allows H4K16 acetylation marks to be deposited. They demonstrated that the states between satellite cell quiescence to activation could be regulated in part by this metabolic switch. Myst1's implication in the deposition of most H4K16 acetylation marks, would allow us to speculate that Myst1 may play a role in depositing these marks in satellite cells promoting satellite cell activation. This venue would have to be explored further through ChIP experiments to exclude the possibility of Myst1 modulating H4K16 acetylation in satellite cells having overlap on its role in Pax7 acetylation.

Preliminary data has demonstrated that Pax7 acetylation function may still play a role in the context of Rhabdomyosarcoma (RMS) Pax7-FOXO1 fusion. A subtype of RMS is the translocation of Pax7 binding activity to FOXO1 transcriptional activity resulting in fusion gene with enhanced transcriptional capacity leading to massive proliferation of myogenic progenitors and dysregulation in proliferation leading to cancer phenotype (Sun et al., 2015). Luciferase assay challenging WT Pax7-FOXO1 fusion as well as Pax7-FOXO1 K105R, K193R mutant has shown that Pax7 fusion is also affected by loss of Pax7 acetylation site resulting with 50% reduction in luciferase activity. It would be of interest

to investigate whether Pax7 acetylation levels are higher in rhabdomyosarcoma cells compared to healthy myoblasts. Many groups have shown that Myst1 is dysregulated in different carcinomas and leukemias. It would be an exciting avenue to investigate the role of Myst1 regulating Pax7 acetylation in the context of RMS. We would also investigate the if Myst1 dysregulation plays a role the phenotype of RMS.

In all, we would like to propose a model in which Pax7 acetylation through Myst1 is crucial for the commitment of satellite cells as well as the expansion of committed satellite population. Myst1 acetylation of Pax7 causes an increase of transcriptional activity in which allows an increased activation and repression of its targets including Myf5. This allows proper cellular proliferation and commitment of these satellite cells to replenish the muscle after injury.

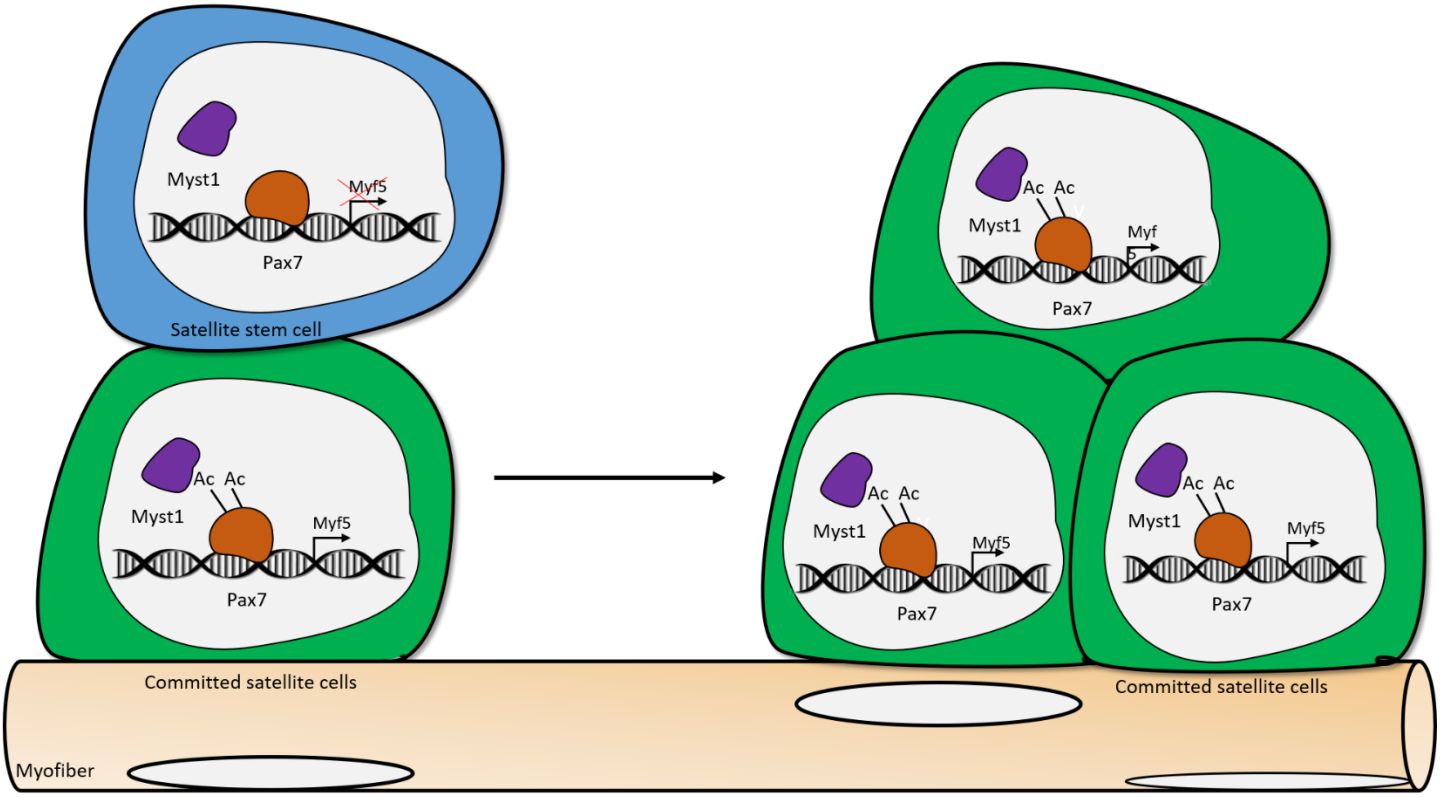

Figure 17: Schema depicting current model regarding the role of Pax7 acetylation.

Future directions

Through different experiments we have demonstrated that Myst1 plays a novel role in regulating Pax7's activity through acetylation. Knowing what has been demonstrated so far there are still some experiments to be performed to complete this story. Firstly, we would like to determine when does the Myst1-Pax7 interaction occurs in satellite cells. We have already determined that Pax7 and Myst1 are interactors in primary myoblasts. We can do this experiment using PLA (proximity ligation assay) to determine when does this interaction occurs in the cells. Through co-IP experiments we have shown that Myst1 does not interact with other members of the tri-thorax members. In the literature, Myst1 is known to work in a separate complex consisting of (MSL1-3, MCRS-2, and MBD-R2). We would like to investigate if Myst1 works in a complex or is found in satellite cells to be interacting with Pax7 on its own. We would also would like to investigate what is the consequence of the satellite cells lacking Myst1 through myofiber culture experiments. More specifically we would like to assess whether removal of Myst1 causes precocious differentiation of the satellite cells evaluating the proportion of Myogenin cells at 42hrs and 72hrs in Control and siMyst1 treated myofibers.

A second overarching question that we would like to investigate is the effect of Myst1 in satellite cells. To properly examine these effect, we would assess the depletion of Myst1 *in vivo* by creating a mouse model in which Myst1 is only removed in satellite cells. This mouse line would be generated by crossing the Myst1 flox/flox mice created by Thomas Voss with our Pax7 CreER mice. Using these mice, we would be able to perform a multitude of experiments as well as validating siMyst1 experiments. We would be able to derive myoblasts from these mice and assess cell proliferation and differentiation as well

as verify Pax7 target genes. EDL myofibers would be isolated from these mice and uncover the proportions of Pax7 positive cells, the proportion of proliferating cells, MyoD positive cells well as evaluate the kinetics of leading them to the first 72hrs. We would also assess satellite cell regeneration using a single injury as well as a triple injury model to measure the efficacy of regeneration of these mice ultimately bridging to the role of Pax7 acetylation *in vivo* by comparing Pax7K193R mutant mice to the Myst1 knockout mice.

We also would like to assess whether H3K4me3 mark are still deposited as efficiently in the context of Myst1 depletion through ChIP experiments by using myoblasts derived from the Myst1 knockout mice. We would expect that since Myst1 depletion reduced Myf5 transcripts by 50% and Pax7 double mutants display lower chromatin recruitment capacity it would be reasonable to hypothesize that the removal of Pax7 acetylase would result in lower H3K4me3 and as a result lower Myf5 expression.

Conclusion

Trough many biochemical, molecular and cellular techniques we have shown that Pax7 contains a novel posttranslational modification playing a role in mediating Pax7 transcriptional activity. Through co-IP we have shown a novel interaction between Pax7 and Myst1 acetyltransferase in muscle. We have also determined that Myst1 plays a role in Pax7 acetylation status through siRNA and co-IP experiments. Using siRNA targeting Myst1 we have determined that Myst1 depletion has a robust impact on Pax7 target genes expression. Myofiber culture experiments using siRNA has also shown that Myst1 is crucial for satellite cell proliferation and maintenance of the committed satellite cells. We have also determined that Myst1 depletion in both differentiating myoblasts and satellite cells causes an increase in MyoD expression in the cells. This study provides evidence of a novel role of an acetylase in which has not yet been shown in muscle as well as a new function for Pax7 proteins. Hopefully this information will help us gain insight into investigating muscle diseases such as muscular dystrophy, sarcopenia and Rhabdomyosarcoma. In all, this novel molecular regulator of Pax7 function well help expand our understanding of muscle stem cell biology.

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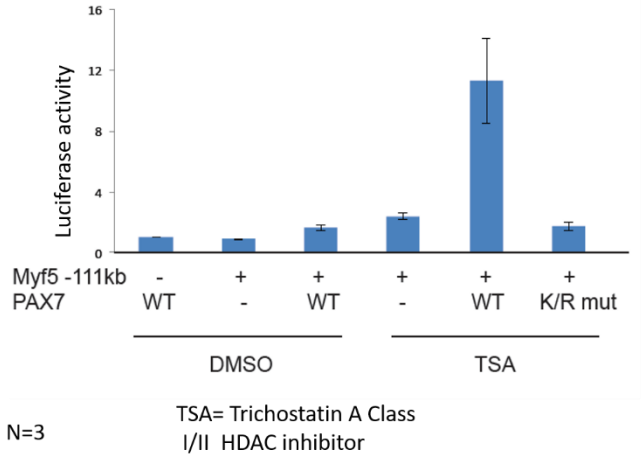
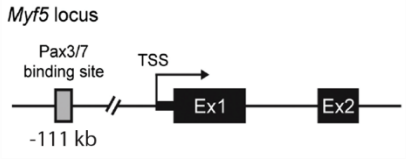
Appendix

Supplemental 1





Luciferase reporter *Luc*-Myf5-111kb

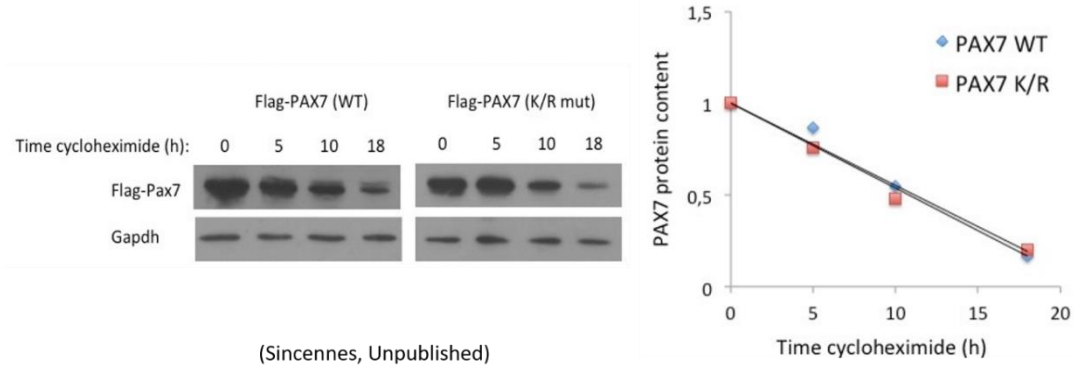
K/R mutants = can no longer be acetylated

(Marie-Claude Sincennes)

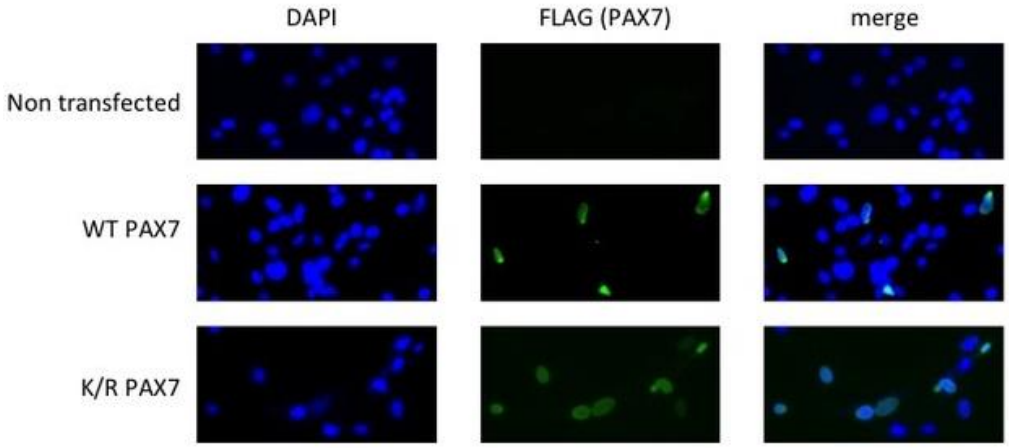
Supplemental 1: Acetylation plays a role in Pax7 transcriptional activity (Sincennes, unpublished). Cos7 cells were transfected with WT Pax7, Myf5-111kb Luc, or Pax7 K/R double mutant. Cells were then treated with TSA for 18hrs and subjected to luciferase assay. Luciferase signal was normalized with Renilla-Luc. (s.e.m represent error bars)

Supplemental 2

A)

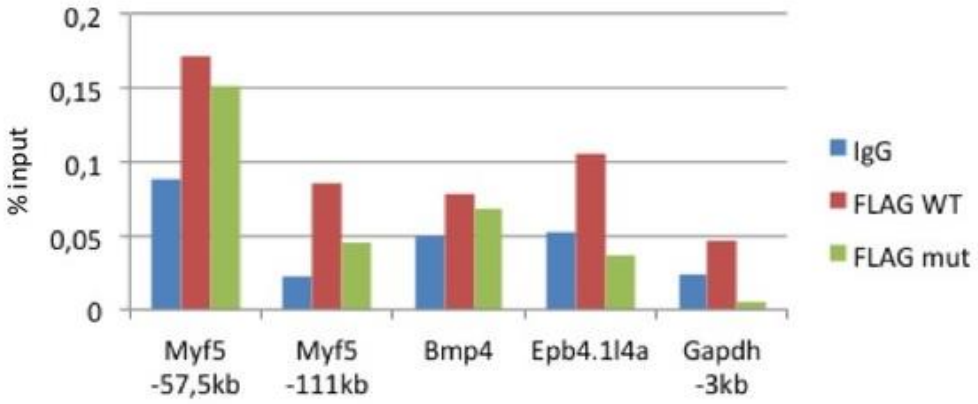


B)



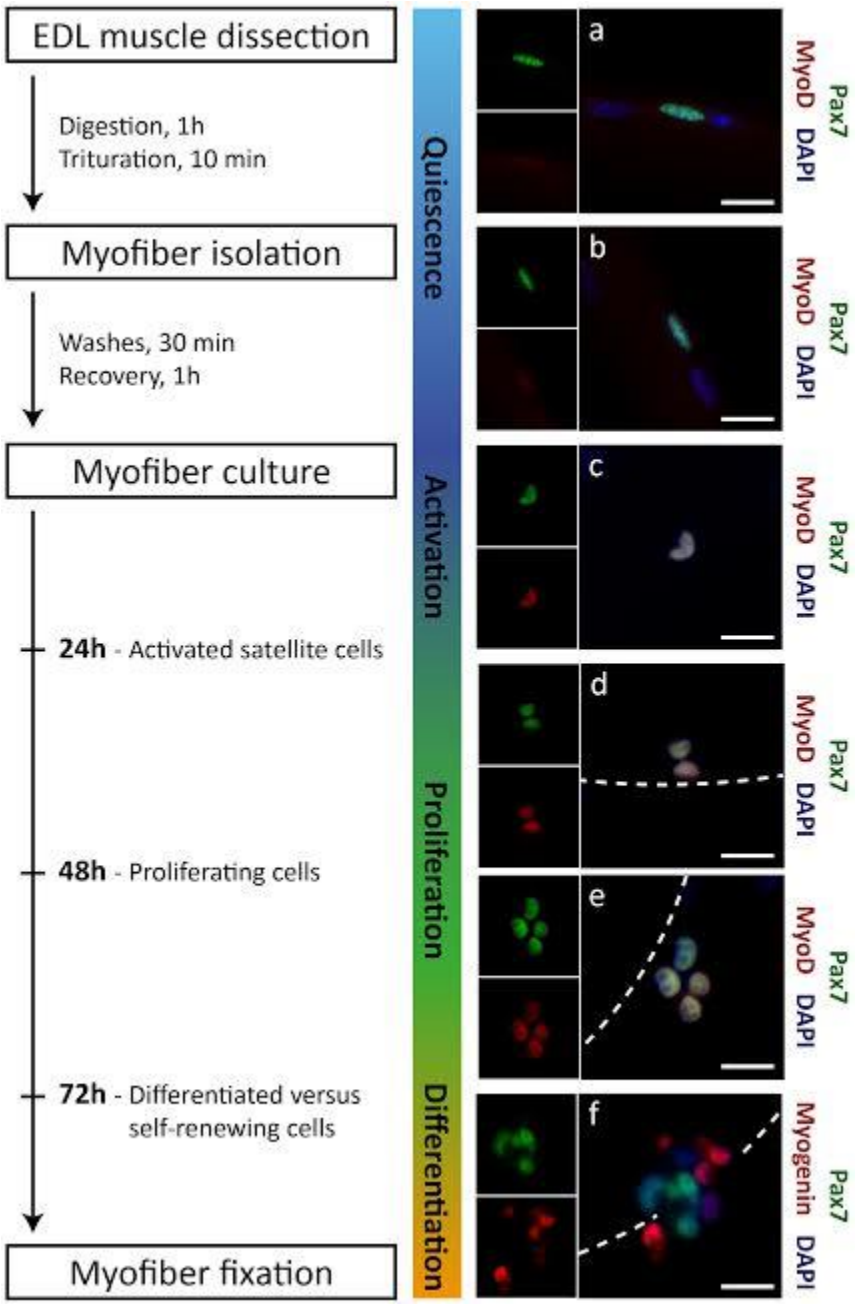
Supplemental 2: Pax7 acetylation does not play a role in either protein stability or protein localization (Sincennes, unpublished). C2C12 myoblasts were transiently transfected with WT Pax7-FLAG or K/R Pax7-FLAG double mutant and subjected to **A)** cycloheximide stability assay or **B)** immunofluorescence.

Supplemental 3



Supplemental 3: Pax7 acetylation plays a role in chromatin recruitment. (Sincennes, unpublished) C2C12 myoblasts were transfected with either WT Pax7-FLAG or K/R Pax7-FLAG double mutant and subjected to FLAG ChIP. DNA fragments were then analyzed through qPCR using primers seen above.

Supplemental 4



Supplemental 4: Schematic representing myofiber isolation protocol. Taken from Brun et al, 2018)