"USP7 DESUBIQUITINASE REGULATES EARLY DIFFERENTIATION OF ADULT MUSCLE PROGENITORS."

Thesis delivered to the Pontificia Universidad Católica de Chile in partial compliance with the requirements to opt for the Degree of Doctor of Biological Sciences with mention in Cellular and Molecular Biology.

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November 2018

Thesis Committee Approval Act

A mi abuela Norma y mi patita pequeña,
que hace cada día más feliz que el anterior

Acknowledgements and funding

Firstly, I would like to express my sincere gratitude to my advisor professor Hugo Olguín. You have been a tremendous mentor to me. I would like to thank you for the trust you deposit on me and for encouraging my research and guiding my path to grow as a scientist. Your advice on both my career and personal aspects are invaluable. I feel very fortunate having you as a teacher and mentor; a person of a great quality, thoughtful, and caring with every student

I Also would like to thank my committee members, Professor Enrique Brandan, Professor María Estela Andres, and Professor Patricia Burgos for making my thesis advance an enjoyable moment, and for your brilliant comments and suggestions.

I am infinitely grateful to the funding sources. This work was funded by the CONICYT Scholarships "CONICYT Scholarship for doctoral studies in Chile" and the VRI Scholarship. This thesis was also largely financed by Dr. Hugo Olguín's FONDECYT 1130631 and 1170975 projects.

I thank my laboratory colleagues for their company and collaboration, otherwise, the development of this thesis would not have been possible. I feel especially grateful to Naty for his invaluable collaboration in the development of my experiments. Thank you very much for your advice and constant help, it makes me feel very lucky and pleased that we have also built a relationship of friendship. To Felipe, The Chorizo, for the discussions of results, the gateways to "seminars" and his great friendship. A Fabian, that with its black humor and understandable mumbling makes our day more bearable. To Natasha for bringing us the flavor of Venezuela, and putting in the extra quota of drama necessary, and Jeremy for his infinite knowledge, his great disposition, and to always have at hand the “curious data”. Finally, but not for this less important, I would like to thank Francisco Bustos, we became very close friends into and out of the laboratory; special thanks for his dedication and for provide me with very important tools for the development of my thesis. I know that I am probably forgetting very important people during this period of my life and I want to thank those that I did not name for their great collaboration and friendship.

Thanks to the other laboratories of the Department of Cellular and Molecular Biology of the PUC. I think I have visited almost all the laboratories in search of equipment, reagents, comments or technical help and I thank you all for your willingness to help me. I especially thank the heads and laboratories of Dr. Juan Larraín (Héctor Carrasco), Dr. Enrique Brandan (Víctor Troncoso), and Dr. María Paz Marzolo (Pamela Farfan)

I would also like to thank all the staff who made this process less torturous. I would especially like to thank Maria Teresa Pino for supporting my after-hours ideas and always having a smile on my face to receive the “Pastelitos”. To Gloria Acevedo for efficiently managing practically everything, in addition to her great sense of humor. How could I not also thank Barbarita and Mrs. Sole, without them the building would be a disaster! Thank you very much for your great human quality. With Fer, we love them very much.

I also want to dedicate a a small parragraph to my family. My gradma Norma, my mother Alejandra, and aunt Rocío. They became and a very still pilar for my formation as a professional and human being.

Finally, I want to thank the woman who has been with me in both good and bad times. I am very grateful for her support at all levels, both personal and emotional. I love you very much, little paw. Fercilla, I thank you very much for your great company and love. Paws united in the struggle!

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LIST OF ABBREVIATIONS

ATP:	Adenosin triphosphate
bHLH:	basic Helix-Loop-Helix
CHX:	Cycloheximide
CK2:	Casein Kinase 2
Co-IP:	Co-immunoprecipitation
CSAs:	Cross sectional area
SCs:	Satlite Cells
DAXX:	Death domain associated protein
DM:	Differentiation medium
DNA:	deoxyribonucleic acid
dpi:	Days post-injury
DUBs:	Deubiquitinating
EBNA:	Epstein-Barr nuclear antigen 1
ECM:	Extracellular matrix
eMyHC:	Embrionic myosin heavy chain
FBS:	Fetal bovine Serum
FGF-2	Fibroblast growth factor 2
FOXO4:	Forkhead box protein O4
GM:	Growth medium
GMPS:	Guanine monophosphate synthase
HAUSP:	Herpesvirus-associated ubiquitin-specific protease
HRP:	Horseradish peroxidase

HS:	Horse Serum
IF:	Immunofluorescence
MDM2:	Murine doble minute 2
MEFs:	Mouse embrionary fibroblasts
Mgn:	Myogenin
MJDs:	Machado-Josephine Disease proteases
M-MLV:	Moloney Murine Leukemia Virus
MRFs:	Muscle Regulatory Factors
Myf5:	Myogenic factor 5
Myf6:	Myogenic factor 6
MyoD:	Myogenic differentiation
OTUs:	Ovarian Tumor Proteases
p53:	protein 53
Pax7:	Paired box 7
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
PFA:	Paraformaldehyde
pMBs:	Primary myoblasts
PPM1G:	Protein phosphatase magnesium dependent 1
PTEN:	Phosphatase and tensin homolog
PTM:	Post-translational modifications
PVDF:	Polyvinylidene difluoride
qPCR:	quantitative PCR

REST:	RE-1 silencing transcription factor
RFP:	Ret finger protein
RIPA:	Radioimmunoprecipitation assay buffer
RNA:	Ribonucleotide acid
rRNA:	ribosomal RNA
SCTF:	Factor de transcripción de células troncales
siRNA:	Short interference RNA
TA:	Tibialis anterior
TRAF:	TNF receptor associated factor
Treg:	Regulatory T-Cells
Ubl:	Ubiquitin like
UCHs:	Ubiquitin C-terminal hidrolases
UPS:	Ubiquitin Proteasome System
USP7:	Ubiquitin Specific Protease 7
USPs:	Ubiquitin Specific Proteases
WT:	Wild type

RESUMEN:

Las células satélites (CSs), son progenitores miogénicos quiescentes responsables de la regeneración muscular durante la adultez. Ante un daño muscular, las CSs se activan, proliferan y finalmente se diferencian. Este proceso es sostenido por la expresión secuencial de factores reguladores de músculo (MRFs), y por la disminución de los niveles del factor de transcripción Pax7. Por otro lado, se ha establecido que las células que no se diferencian y retienen la expresión de Pax7, participan en la autorenovación de las CSs. Trabajo previo de nuestro laboratorio, indica que la regulación diferencial de los niveles proteicos de MRFs y Pax7 -vía el sistema ubiquitina-proteosoma (UPS)- son críticos en la determinación del destino de las CSs. Hemos demostrado que Pax7 interactúa con un grupo de proteínas relacionadas a la función de UPS, incluyendo la ligasa de ubiquitina E3 Nedd4-1 (Bustos, et al 2015), la cual regula directamente Pax7 mediante ubiquitinación y la desubiquitinasa USP7. Sin embargo, el rol de USP7 durante la miogénesis adulta no ha sido explorado. De esta forma, los objetivos de este estudio comprenden la caracterización de la expresión de USP7 en precursores musculares y determinar si USP7 es específicamente requerida para la miogénesis.

Mediante el uso de varios modelos celulares, técnicas de biología celular y molecular, inhibición farmacológica, experimentos de pérdida y ganancia de función e inducción de daño *in vivo* en músculo esquelético, investigamos el rol de USP7 en diferenciación temprana de CSs. Los resultados mostrados corresponden al trabajo de esta tesis doctoral, en la cual demostramos que el MRF miogenina, es regulado post-traduccionalmente por la enzima deubiquitinasa USP7, en etapas tempranas de la diferenciación de mioblastos. Nuestros

resultados sugieren que USP7 regula el estado de ubiquitinación de miogenina, con la consiguiente estabilización y aumento de sus niveles de proteína lo cual definiría el destino de las células precursoras de músculo esquelético. Estos resultados establecen por primera vez un reóstato que regula los niveles críticos necesarios de miogenina una vez que las células se encuentran comprometidas (MyoD+) se gatilla el programa de diferenciación terminal permitiendo la expresión de genes músculo específico. Asegurando la irreversibilidad del proceso de diferenciación. La identificación de estos mecanismos tiene una implicancia profunda en el entendimiento de la biología y función en la homeostasis y enfermedad del tejido muscular.

ABSTRACT

Satellite cells (SCs) are quiescent myogenic progenitors responsible for skeletal muscle regeneration during adult life. Upon muscle damage, SCs activate, proliferate and finally differentiate; a process supported by the sequential expression of muscle regulatory factors (MRFs) and the down-regulation of the transcription factor Pax7. On the other hand, it has been established cells that do not differentiate and retain Pax7 expression are thought to participate in SC self-renewal. Work from our laboratory indicates that differential regulation of MRFs and Pax7 protein levels -via Ubiquitin Proteasome System (UPS)- are critical for determining SCs fate. We have shown previously that Pax7 interacts with several proteins related to UPS function, including the E3 ligase Nedd4-1 (Bustos, et al 2015) –which directly regulate Pax7- and the deubiquitinase USP7; however, the underlying mechanism of USP7 during adult myogenesis has not been explored. Thus, the aims of this study are to characterize USP7 expression in muscle precursors and to determine if USP7 is specifically required for myogenesis.

By the use of several cell models, cellular and molecular biology approaches, pharmacological inhibition, gain, and loss of function assays and induced *in vivo* skeletal muscle damage, we investigated the role of USP7 in SCs early differentiation. The results showed are from the work from this Ph.D. thesis, in which we demonstrated that the MRF, Myogenin, is regulated post-translationally by the deubiquitinating enzyme USP7 in early stages of myoblasts differentiation. Our results suggest that USP7 regulates myogenin ubiquitination status by removal of ubiquitin, with the following stabilization and increase of

the myogenin protein levels, which define skeletal muscle precursors fate. These results establish for the first time a rheostat that regulates the required critical levels of myogenin when the cells are compromised (MyoD+) and trigger the terminal differentiation program allowing the expression of specific muscle genes. This assures the irreversibility of the differentiation process. The identification of these mechanisms has profound implications in the understanding of satellite cells biology and function in homeostasis and disease.

GENERAL INTRODUCTION:

I.- Skeletal muscle is a crucial component in the body's homeostasis:

The skeletal muscle is an organ of great complexity in charge of a series of critical physiological functions for the organism constituting, in the human being, about 40% of the total weight (Zurlo et al., 1990). It is a highly conserved organ among species, with muscle tissue being observed in organisms as diverse as mollusks, insects and other higher organisms. Among the major functions of skeletal muscle, we can highlight that it i) participates in the generation of voluntary movements (also involuntary movements such as the reflex arch), ii) contributes to energy metabolism by participating in the absorption of glucose mediated by insulin and in the β-oxidation of acylglycerols, and iii) maintenance of body heat.

The structural and functional unit of the skeletal muscle is made up of multinucleated cells that work like a syncytium, called muscle fibers. As a morphological characteristic, these cells have their nuclei oriented in the periphery, adjacent to the plasma membrane or sarcolemma (Alexander, 1961). The cytoplasm of the muscle fiber is predominantly occupied by the contractile apparatus, the sarcomere. Tethered and repetitive sarcomere sections are the basic unit of myofibrils in striated muscle. Sarcomere backbone is made up by three major filament systems: myosin-based thick filament, an actin-based thin filament (associated to the regulatory proteins tropomyosin and troponins, and the filament system titin. Parallel arrangements of thin actin filament constitute the I-band which span in a thick section with myosin filaments in the A-band, and titin encompasses the whole sarcomere limited by Z-band at each extreme and crossing at the center with the M-band (Figure I-1). Sarcomeres subunits

are arrayed on myofibrils, as well as myofibrils are organized in parallel bundles into the muscle fiber, which explains the functional uniaxial contraction of the striated muscle. On the other hand, this high specialization and functional specification of skeletal muscle have the consequence that this tissue is in a post-mitotic state that prevents its proliferation. In addition to muscle fibers, skeletal muscle tissue is composed of other cell types, acting cooperatively to nourish and support the muscle function and integrity. Of the cell types of greatest physiological importance are motor neurons that will innervate each muscle fiber in a focal point of contact known as the neuromuscular junction (Burden, 1998). There are also vascular endothelial cells that will form the blood vessels that nourish the muscle with oxygen and nutrients (Christov et al., 2007), fibroblasts that participate in the generation of the extracellular matrix (Mathew et al., 2011; Murphy et al., 2011) and components of the immune system (Arnold et al., 2007; Lescaudron et al., 1999; Sonnet et al., 2006). Finally, there are satellite cells, the function of which is discussed in section III.

II - Satellite cells are the stem cells of muscular tissue.

Skeletal muscle is an organ with a remarkable plasticity, highlighting its ability to respond to damage (Collins and Partridge, 2005), repair and requirements during the growth of an individual (Neal et al., 2012), this capacity lies on the stem cells of skeletal muscle known as satellite cells (SCs); initially identified by electronic microphotography by its particular spatial location. These cells are located between the sarcolemma of the muscle fiber and the basal lamina in a proliferative resting state (quiescence) (Christov et al., 2007), comprising a discrete population of about 3-5% of the total nuclei of a myofiber. SCs, as an adult stem cell,

have the characteristic of generating mature tissue through differentiation, as well as the ability to self-renew, to maintain the resident population and respond to further stimuli.

Upon damage, these cells leave the state of proliferative rest, being activated and quickly entering the cell cycle to proliferate. It is believed that once a sufficient number has been reached, mechanisms are triggered that regulate the arrest of the cell cycle in order to begin differentiation until a mature myofiber is formed. This whole process is coordinated by a conserved family of transcription factors, known as Muscle Regulatory Factors (MRFs).

III.- Function of the Muscle regulatory factors (MRFs) in skeletal muscle biology.

Commitment to the muscle lineage and muscle differentiation are controlled by four closely related transcription factors belonging to the class 2 bHLH (basic Helix-Loop-Helix) family, called MRFs. These MRFs are MyoD, Myf5, Myogenin and MRF4 have three conserved domains: Amino and carboxyl transactivations domains and the central region containing the bHLH DNA binding domain (Tapscott, 2005). Their function as transcription factors requires homodimerization and/or heterodimerization with the Class I bHLH proteins, including E47, E12, HEB/HTF4, and E2-2/ITF-2, requirements to proper and stable binding to the E-Box consensus sequence (CANNTG).

In mature muscle, the function of MRFs is restricted to the maintenance of the skeletal muscle tissue, as well as hypertrophy, post-natal growth, and repair. With gene reporter experiments, was established that Myf5 locus is active on resting SCs and is also expressed muscle spindles-stretch-sensitive mechanoreceptors since denervation leads to expression of Myf5 (Zammit et al., 2004). Also, it was demonstrated that Myf5 is poised in SCs and

transcripts are targeted by miR-31 and thereby sequestred and untranslated in mRNP granules, allowing the maintaining of quiescence. Upon SC activation, Myf5 mRNA dissociates from mRNP granules and its rapidly translated (Crist et al., 2012). MyoD, along with Myf5 is the determinant factor of muscle lineage, where ectopic expression of MyoD in cultured fibroblasts conduce transdifferentiation to Myoblasts (Davis et al., 1987; Tapscott et al., 1988). In a healthy adult, muscle is practically undetectable as well as the MyoD transcript. On activated satellite cells, MyoD levels increase to a moderate level of expression in proliferating myoblasts, afterward a shift in the transcriptional mechanism, MyoD levels sharply increse, allowing the down-stream MRF expression, Myogenin, and repressing Myf5 transcription (Myogenin function is section IV). MyoD and Myogenin act synergically and permits the transcription of MRF4 and other late differentiation genes directing the formation of mature myofibers. Conversely, MyoD and myogenin expression are downregulated, whereas MRF continues to be expressed at high levels, being the predominant MRF in healthy adult muscle (Timothy et al., 1991). Moreover, MEF2 (Muscle enhancer factor 2), potentiates the myogenic progression acting in concert with myogenin, mediating the recruiting of chromatin remodeling machinery (as SWI/SNF complex) at muscle-specific genes, to allow the transcriptional activation.

Additionally, MyoD, Myf5, and MRF4 are dispensable for SCs specification. SCs number seems unperturbed in the absence of Myf5 and MyoD, however, double *knock-out* mouse (Myf5:MyoD), have severe defects in formation of muscle with almost complete ablation of myoblasts (Yamamoto et al., 2018), while identity of skeletal muscle in these animals is determined downstream by MRF4, if expression is not perturbed (Golub et al.,

2004). During embryonic development, the transcriptions factors Pax3 and Pax7 are essential for specification of SCs. In the absence of both Pax3 and Pax7, there is a major deficit in skeletal muscle, with an arrest of myogenesis and only early embryonic muscle and myotome forms (Relaix et al., 2005).

IV.- Satellite cell activation.

Satellite cell activation is a multi-step process, determined by the transition from G_0 to G_1. Once they enter the cell cycle, the satellite cells undergo a highly proliferative state, characterized by the upregulation of the transcription factor Pax7. Since this point, the satellite cells progress to the myoblast state, initiating the differentiation process. Pax7 also functions as a counter-regulator of differentiation, inhibiting MyoD activity through protein interaction resulting in ubiquitination and proteasomal degradation of MyoD, a process mediated by E3 ligase RFP. (Joung et al., 2014; Olguin et al., 2007). Once the satellite cells are activated, Pax7 protein levels are rapidly depleted by proteasomal degradation (Bustos et al., 2016), while MyoD is activated by multiple phosphorylations in serine and threonine residues, resulting in a sharp increase in transcriptional activity (Kitzmann et al., 1999; Singh et al., 2015). MyoD forms a heterodimer with protein E type transcription factors. This dimer binds to consensus sequences known as E-Box, inducing the myogenin transcription and other specific genes that regulate terminal differentiation of skeletal muscle lineage. (Ma et al., 1994; Weintraub et al., 1994) (Figure I-2).

V.- The sequential transactivation of MRFs regulates the differentiation of myoblasts.

As mentioned before, commitment to the muscle lineage and muscle differentiation are controlled by the MRFs. MRFs have distinct but overlapping patterns of gene expression during development. (Ustanina et al., 2007). In *Knock-out* models of each one of the MRFs, it has been demonstrated that each has a unique role in muscle differentiation, where Myf5, Myf6, and MyoD are not required for the viability of the organism but do exhibit unique phenotypic characteristics. (Braun and Fludnicki, 2000; Braun et al., 1992; Rudnicki et al., 1993). On the other hand, in the combined absence of Myf5, Myf6, and MyoD, the myogenic specification is lost and skeletal muscle is not developed, resulting in a lethal phenotype. (Braun and Arnold, 1995; Valdez et al., 2000). When only the Myogenin gene is ablated, these mice survive fetal development but dies immediatly after birth and show a severe reduction of mass in all skeletal muscle. Therefore, myogenin is essential for the development of functional skeletal muscle (Hasty et al., 1993; Knapp et al., 2000). Thus, it has been observed that animals with one myogenin allele are viable and exhibit a normal phenotype, whereas null animals for both myogenin alleles die at birth. *Post-mortem* observations have made it clear that these animals present myoblasts, but a reduced number of muscle fibers, suggesting that the function of myogenin is not required for the specification or proliferation of myoblasts, but required during later stages of differentiation such as expression of muscle-specific genes and myofiber fusion. (Meadows et al., 2008).

VI.- Regulation of myogenin during the process of muscular differentiation:

Myogenin is, along with Myf6, one of the MRFs that lead the process of terminal

differentiation in compromised myoblasts. Through the generation of null alleles of myogenin for both alleles targeted by homologous recombination ($mgn^{-/-}$), it has been demonstrated that these animals die in perinatal stages, presumably because of severe deficiencies in the development of the diaphragm and a significant reduction in the total body mass of skeletal muscle. It was also reported that the $mgn^{-/-}$ animals presented an abnormal curvature of the spine and anomalous development of the rib cage. Subsequent analyses performed on a hypomorphic animal model of myogenin levels (25% reduction in myogenin transcription compared to *wild-type*), proportional effects were observed to the animal $mgn^{-/-}$, suggesting that multiple thresholds of myogenin levels are required for proper myogenic development and correct conformation of the rib cage (Vivian et al., 1999); since the observed muscle hypoplasia correlates with the absolute levels of myogenin present. Conversely, myogenin expression does not appear to be essential for *in vitro* differentiation. This is evidenced by continuously cultivating myoblasts obtained from $mgn^{-/-}$ animals which are still able to differentiate. Also when MyoD is ectopically expressed in $mgn^{-/-}$ mouse embryonal fibroblasts (MEFs), myotube formation occurs.

During post-natal myogenesis, myogenin and Myf6 are the latest transcription factors being expressed, resulting in differentiation and fusion of muscle precursors (Mastroyiannopoulos et al., 2012; Naidu et al., 1995), operating downstream of MyoD and Myf5. Myogenin mainly binds to regulatory sequences of genes related to terminal differentiation. It has been observed that although MyoD is necessary for myogenin expression, both proteins share a population of transcriptional targets, which are not mutually exclusive, sometimes even overlapping certain functions. Thus, transcription of late

differentiation genes requires the expression of both MyoD and myogenin. Part of the role of myogenin is to enhance the expression of a subset of genes previously initiated by MyoD. (Cao et al., 2006). Additionally, unique transcriptional targets for myogenin have been described, suggesting that although with MyoD they share the same DNA binding sequence (E-box), there are other regulatory mechanisms independent of the sequence. (Knapp et al., 2000).

Due to the nature of the mechanisms mentioned above, we propose that these mechanisms are finely regulated at the level of post-translational modifications, by balancing the protein levels of these factors, which would be determinant in cellular decisions during development and regeneration.

VII.- Ubiquitin-proteasome system (UPS) regulates protein degradation:

The ubiquitin-proteasome system (UPS) regulates a wide variety of cellular processes and functions during homeostasis, proliferation, differentiation and cellular senescence. Unbalances in the UPS, have been associated with a series of deleterious and/or pathological consequences at the cellular and physiological level. (Milan et al., 2015; Ross et al., 2015).

Protein ubiquitination engages the hierarchical activity of three families of enzymes, constituting an elaborately regulated protein destination system. The ubiquitin cascade initiates with the ubiquitin activating enzyme (E1), which starts the cascade by activating ubiquitin, a highly conserved protein of 76 amino acids, through an ATP-dependent mechanism. The E1 enzyme forms a covalent bond between the C-terminal end of ubiquitin and a cysteine residue on its active site. The thioesterified ubiquitin is then transferred from the active E1 site to the

following enzyme: E2, ubiquitin conjugator. Finally, the enzyme E3 (Ubiquitin ligase) interacts with the enzyme E2 covalently bound to ubiquitin and the protein substrate, catalyzing or facilitating (depending on the type of E3), the transfer of ubiquitin to the target protein. It is worth mentioning that mammals only need two E1 enzymes to catalyze the conjugation of ubiquitin to one of about 40 E2, which in turn is able to interact with some of the approximately 600 E3 ubiquitin ligases that determine substrate specificity. (Deshaies and Joazeiro, 2009) (Figure I-3).

The ubiquitin molecule is covalently transferred to the substrate protein via an isopeptide bond between a glycine at the C-terminal end of the ubiquitin and the primary amine of a lysine residue of the target protein. By this way, it is possible to generate modifications by monoubiquitination as well as polyubiquitination (homotypic and heterotypic) with several types of union between lysine, which constitutes differential signals for the regulation of the tagged protein. (Xu et al., 2009). In a ubiquitin chain, ubiquitin moieties can be conjugated through one of their lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal methionine residue (M1), offering countless possibilities to assemble a specific polymer. Such regulations comprise ubiquitin chains in residues of lysine K48 and K11 of the target protein (a canonical mark of proteasomal degradation). K63-formed chains have been associated with the generation of scaffolds which mediate the recruitment of regulatory proteins, and K27 linkage as a regulator of the DNA damage response. It is important to highlight that almost all proteins can be modified by ubiquitination, by regulating processes ranging from cell cycle control, recruitment of transcription factors, histone code dynamics, and subcellular protein destination, among others (Oh et al., 2018).

VIII.- Deubiquitinating enzymes (DUBS)

Ubiquitination is a dynamic process that can be reversed by the activity of a group of protease enzymes known as deubiquitinases (DUBs), which specifically cleave the molecules bound to ubiquitin after the G76 residue on ubiquitin bound to the target protein. (Komander et al., 2009). This capability allows DUB enzymes to participate in almost all processes that are also catalyzed by E3 ligases. Thereby, DUBs can participate in i) correction/edition of polyubiquitin chains, ii) removal of polyubiquitin chains, allowing the rescue of proteins subject to degradation by the 26S proteasome and iii) processing of ubiquitin precursors during ubiquitin synthesis.

More than 90 deubiquitinases enzymes have been described in the human genome. These proteins have been classified into two major classes referred to as DUB enzymes with cysteine protease activity and another class of DUBs with metalloprotease activity. Being the first the most abundant, composed of four superfamilies of DUBs, while only one family has been described in the superfamily of metalloproteases.

VIII.I.- DUB enzymes with cysteine protease activity:

Within the DUBs with cysteine-protease activity are included four groups of DUBs called: Ovarian Tumor proteases (OTUs), Ubiquitin C-terminal hydrolases (UCHs), Machado-Josephine Disease proteases (MJDs) and Ubiquitin Specific Proteases (USPs). All these DUBs share a similar architecture and ubiquitin removal mechanism. This group of enzymes possesses a cysteine residue at their catalytic sites. The Thiol group of cysteine residue performs a nucleophilic attack on the carbonyl of the isopeptide bond between ubiquitin and

the lysine of the target protein. This nucleophilic attack results in the removal of the ubiquitin molecule which is released from the DUB enzyme by hydrolysis.

VIII.II.- The Superfamily of Ubiquitin-Specific Proteases (USPs):

USPs constitutes the largest group of DUBs, with approximately 74 different members. (Hutchins et al., 2018). These enzymes possess about 350 amino acids in their catalytic core. (Ye et al., 2009). USPs enzymes have a highly conserved region called the Cys box, composed of 19 amino acids and a His box (60-90 amino acids). These regions are fundamental in the ubiquitin protease catalytic activity of the DUBs belonging to this family. In addition, these proteins possess a great size, due to their long sequences in amino and terminal carboxyl. It has been suggested that these sequences facilitate protein-protein interactions in substrate identification, serve as recognition sequences for regulatory and modulating proteins, and signals that determine their subcellular location (discussed below).

IX) USP7/HAUSP

USP7/HAUSP (Herpesvirus-associated ubiquitin-specific protease) is a member of the USP family of deubiquitinase enzymes. It is highly expressed in several tissues and a wide variety of interacting proteins have been described. In addition, this DUB does not have significant homology in its N-terminal and C-terminal domains with other DUBs of the same family, which suggests unique regulatory mechanisms. USP7 in N-terminal region has a portion without a defined structure rich on glutamine (poly-Q tract), followed immediately by a TRAF (TNF receptor- associated factor) domain, which is crucial for nuclear localization.

(Bouwmeester et al., 2007). The TRAF domain has been described as important for interaction with substrate proteins (Sheng et al., 2006), but it does not affect deubiquitinase activity over a minimal ubiquitin substrate if deleted. (Faesen et al., 2011). By crystallography, it has been established that USP7 interacts with their substrates through a P/AXXS degenerate motif (where X represents any amino acid), which would explain the wide variety of substrates that USP7 can interact with. Within the validated USP7 substrates it is important to highlight p53, PTEN, FOXO4, and EBNA1. (Horst et al., 2006; Jagannathan et al., 2013; Sheng et al., 2006; Song et al., 2008). The C-terminal end of USP7 contains 5 repetitions of ubiquitin-like motifs (Ubl 1-5), connected to the catalytic domain by an alpha helix of 26 residues. This region is believed to allow some flexibility of the Ubl motifs, which is thought to affect its activity. This domain participates in the allosteric activation of USP7 by the metabolic enzyme guanine monophosphate synthetase (GMPS), through direct interaction with Ubl 1,2. Deletion experiments for these motifs comprise a significant reduction in USP7 activity levels on a synthetic diubiquitin substrate. (Faesen et al., 2011).

The catalytic core of USP7 is composed by a catalytic triad, composed by a reactive cysteine (C223), a deprotonating Histidine (H464) and an Aspartate residue (D481), where alterations in their spatial distribution mobilize these residues to their active conformation. *In vitro* experiments with USP7-motifs deletion mutants have shown that the catalytic region is sufficient for USP7 activity, but its C-terminal region is required to positively modulate its activity, as mentioned above.

Among the most studied substrates of USP7, it is relevant to mention its regulation on p53/MDM2 axis, that has turned this DUB into an interesting target for the development of

antitumor therapies. USP7 directly regulates the stability of p53 by de-ubiquitination (Cummins et al., 2002), and the ubiquitin ligase (MDM2) that targets p53 for proteasomal degradation (Li et al., 2004). Under normal conditions, USP7 binds to p53, possibly in association with DAXX as an adapter that would protect MDM2 from being self-ubiquitinated (Tang et al., 2006), enabling the ubiquitination of p53 by MDM2 and its subsequent proteasomal degradation. On the other hand, under conditions of DNA stress, USP7 preferentially binds to p53, being rescued from degradation and thus allowing its proapoptotic signaling. (Meulmeester et al., 2005; Tang et al., 2006). It is interesting to note that both p53 and MDM2 share the same binding site to USP7, suggesting that this complex operates as a switch regulating the stability of p53. Other USP7 substrates described in the literature include FOXO4, PTEN, and proteins of the polycomb group, among others. Some of these substrates will be reviewed in the following section.

X) The UPS role in regeneration and USP7 function in cellular differentiation:

Stem cells are defined by the following two characteristics: i) they are capable of self-renewal, thus maintaining the stem cell reserve, and ii) produce progeny that undergo differentiation, resulting in a more specialized cell type. (Gage, 2000). The balance between stem cell reserve maintenance and differentiation is strictly controlled at various levels. Within these regulations, the post-translational modifications (PTM) of key proteins in the transition from stem cell to a differentiated status are noteworthy. Ubiquitination (referred to in Section

V) is one of the main forms of PTMs in eukaryotic organisms and functions as a fundamental regulator of protein stability and activity.

It has been proposed that the specification of stem cell fate is controlled by an antagonization between the ubiquitination and deubiquitination pathways of stem cell transcription factors (SCTFs). (Huang et al., 2011a). Following this line, each SCTF should be regulated by at least one pair of an E3 ubiquitin ligase and a DUB enzyme. In situations where there is a dominance of ubiquitin E3 ligase activity, the SCTF will be destined to proteasomal degradation, releasing the transcriptional repression of differentiation induction genes. On the other hand, the dominant activity of DUB will lead to the removal of ubiquitins and stabilization of SCTF, resulting in the repression of genes that control differentiation and allowing the maintenance of the stem cell reserve. Thus, the net balance of ubiquitination and deubiquitination will determine the fate of the stem cell (Chandrasekaran et al., 2016).

In neuronal progenitors, repressor element 1-silencing transcription factor (REST) undergoes proteasomal degradation through $SCF^{\beta\text{-}TrCP}$ during neuronal differentiation. (Westbrook et al., 2009). On the other hand, USP7 stabilizes REST through direct deubiquitination, promoting the maintenance of neuronal progenitors and antagonizing the activity of $SCF^{\beta\text{-}TrCP}$. (Huang et al., 2011b). As part of the differentiation of functional T lymphocytes, it is known that the stable expression of Foxp3 is necessary for the development of regulatory T lymphocytes (Treg). In this context, it was demonstrated that USP7 counter-regulates the polyubiquitination of Foxp3, by rescuing it from proteasomal degradation. (Loosdregt et al., 2013). In addition, a recent study proposes that USP7 is required for osteogenic differentiation of stem cells derived from human adipose tissue, although the

underlying mechanisms governing this transition have not yet been elucidated. (Tang et al., 2017).

In our laboratory, we have identified ubiquitin ligase E3 Nedd4-1 as the enzyme responsible for Pax7 monoubiquitination in SCs and its subsequent degradation by the proteasome. This negative regulation of Pax7 leads to de-repression of MyoD activity, allowing the expression of myogenin and subsequent muscle differentiation. (Bustos et al., 2016).

In this work, we propose an additional level of regulation required for the stable expression of myogenin. Once a threshold of myogenin level/activity is reached, this would trigger downstream pathways leading to differentiation of the SCs progeny, allowing the appropriate amplification of muscle progenitors and myofiber formation. As a consequence and based on the antecedents already presented, we formulate the following hypothesis and research objectives:

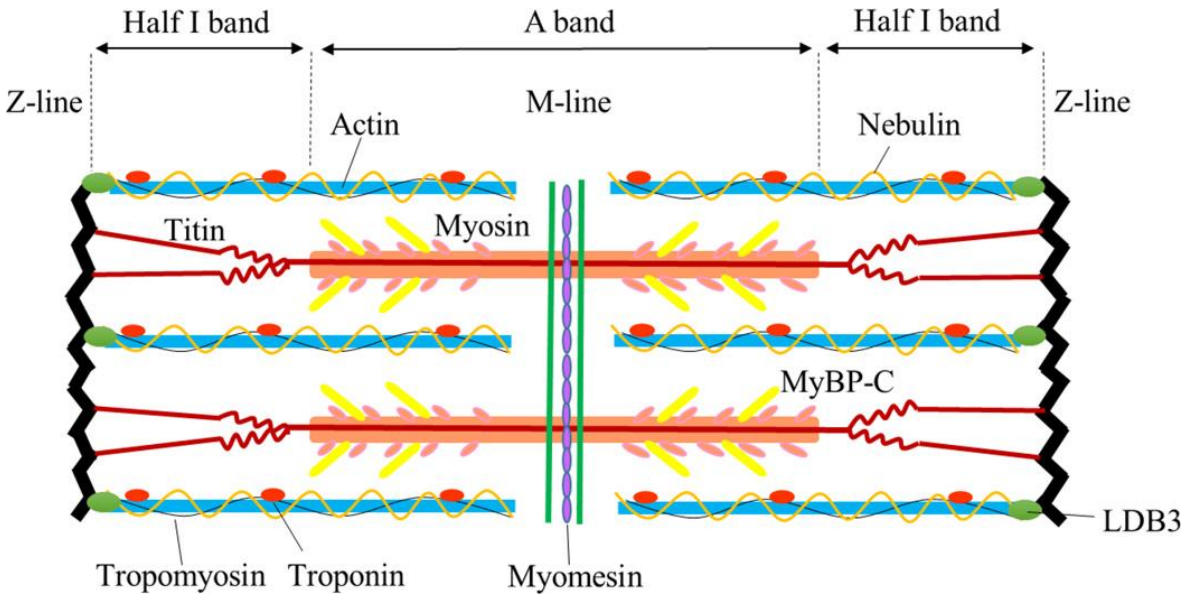

Figure I-1: Sarcomere ultrastructural organization. Schematic representation of a skeletal muscle sarcomere, illustrating the three major filament systems: actin-based thin filaments (light blue), myosin-based thick filaments (light red), and titin (red). The lateral boundaries of the sarcomere are the Z-discs. The I-bands surrounds the Z-disc and is a region where thin filaments are not superimposed by thick filaments. The A-band region contains thin filaments and thick filaments. The M-band falls at the center of the sarcomere, where thick filaments do not interdigitate with thick filaments, represented by myomesin.

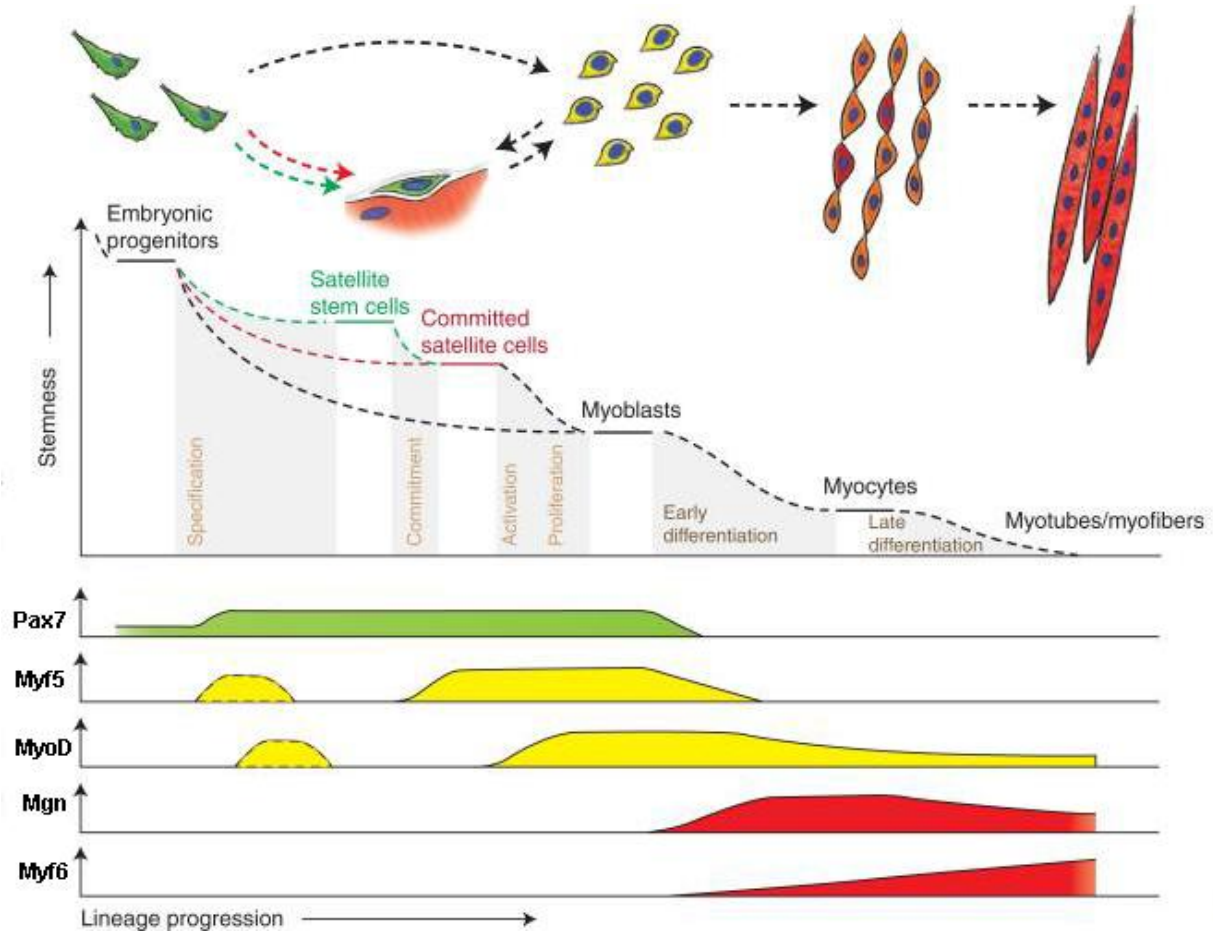

Figure I-2: The hierarchy of myogenic transcription factors in the progression of muscle differentiation. The satellite cell in quiescent state expresses the transcription factor Pax7, that decays rapidly when the satellite cell begins its differentiation process; at the same time the master regulator MyoD is overexpressed and stabilized. Activated and compromised satellite cells can reverse this process by increasing Pax7 levels and reducing MyoD expression. (Olguin et al., 2007). The regulatory network of transcription factors governing muscle differentiation is hierarchically and irreversibly once terminal differentiation MRFs such as Miogenin and Myf6 are expressed. (modified from (Bentzinger et al., 2012)).

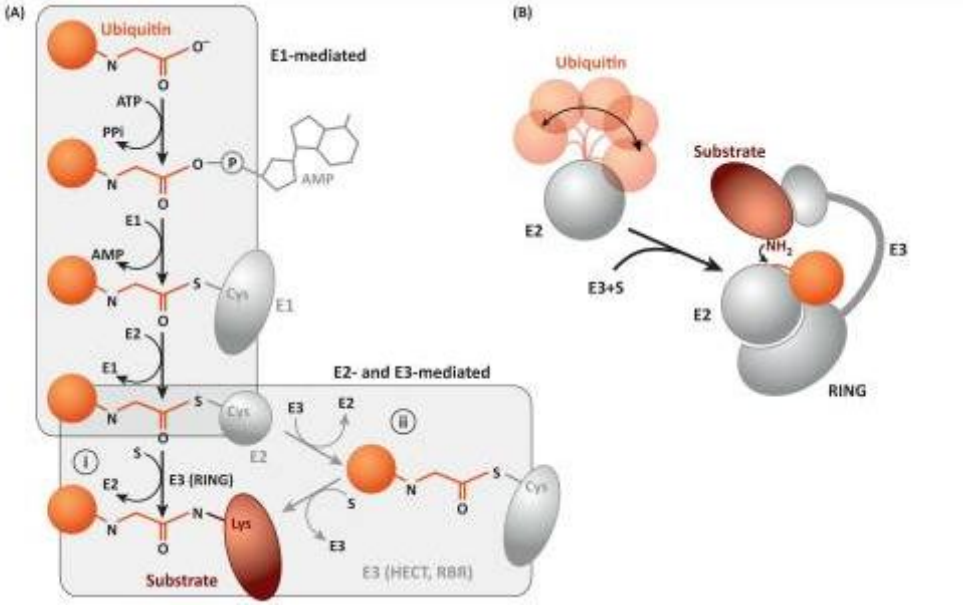

Figure I-3: Ubiquitination Mechanism: The different modifications that occur to the terminal carboxyl of the ubiquitin molecule during ubiquitination are shown. (A) The first step is the activation of the ubiquitin molecule by the activating enzyme E1, that catalyzes the transfer of an AMP molecule through the hydrolysis of ATP. The ubiquitin will subsequently form a covalent bond between the terminal carboxyl of the ubiquitin and a cysteine residue at the catalytic site of E1. The thioesterified ubiquitin is transferred to the next member of the cascade, the ubiquitin-E2 conjugating enzyme. Finally, the ubiquitin E3 ligase binds to both: the ubiquitin E2 complex and the substrate, catalyzing/facilitating (depending on whether an E3 ligase type HECT or RING finger) the transfer of ubiquitin to the substrate protein. (B) The configuration of ubiquitin and E2 is labil, presumably because of its flexible tail. The binding of the E2-ubiquitin complex (bound via thio-ester bond) to E3 allows to secure the ubiquitin in its terminal carboxyl tail in E2, accelerating the transfer rate of ubiquitin to the substrate protein.

HYPOTHESIS:

"The desubiquitinase activity of USP7 is required for the myogenic progression of activated satellite cells during the regenerative process."

OVERALL OBJECTIVE

To determine and characterize the role of USP7 desubiquitinase in the differentiation of adult muscle precursors.

SPECIFIC OBJECTIVES:

1.- To characterize the expression and function of USP7 in the early differentiation of adult muscle progenitors.

2.- To study the effects of differential expression of USP7 in satellite cells in a live model.

RESULTS

The results presented in this section were obtained to achieve most of the objectives of this doctoral dissertation, which have been previously described. Of note, additional results obtained after the submitting of this manuscript, are presented in the section “Manuscript annexed results”. Additionally, in the “Annexed figures” section, ares discussed some extra data.

The manuscript was sent to a mainstream scientific journal in the area of cell biology.

USP7/HAUSP-DEPENDENT REGULATION OF MYOGENIN STABILITY IS REQUIRED FOR TERMINAL DIFFERENTIATION OF SKELETAL MUSCLE PROGENITORS.

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Eduardo de la Vega: conception and design, data collection, analysis and interpretation, manuscript writing.

Felipe Cabezas: data collection, analysis and interpretation.

Natasha Blanco: data collection, analysis and interpretation.

Hugo C. Olguín: conception and design, data analysis and interpretation, manuscript writing.

ACKNOWLEDGMENTS

We thank Dr. Lori Frappier for kindly provide pCAN-*Usp7* WT and pCAN-*Usp7* C223S plasmids. We also thank Dr. Claudio Cabello for FOXO4 antibody. We are in debt with Dr. Enrique Brandan and Dr. María Paz Marzolo for reagents and access to indispensable equipment. Finally, we thank all members from the Olguín' lab for valuable discussions and suggestions.

FUNDING

This work was supported by the National Fund for Scientific and Technological Development (FONDECYT) [grant number 1170975] to HO; Institutional Fellowship for graduate students (VRI-PUC) to EdV and support for EdV and FC from National Doctoral Fellowship, CONICYT.

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Keywords: USP7, HAUSP, myogenin, satellite cells, skeletal muscle regeneration, proteasome, ubiquitin.

ABSTRACT

Satellite cells (SCs) are myogenic progenitors responsible for skeletal muscle regeneration and maintenance. Upon different stimuli, SCs activate, proliferate, and differentiate. This myogenic progression is supported by sequential expression of muscle regulatory factors (MRFs) and down-regulation of Pax7. As a highly regulated process, these key transcription factors are subject to post-translational control affecting their localization, activity and turnover. Moreover, differential regulation of MyoD and Pax7 levels, via the ubiquitin proteasome system (UPS), is critical for determining SCs fate. We showed that Pax7 is targeted by the E3 ligase Nedd4-1 and interacts with several UPS-related proteins, including the deubiquitinase USP7. However, USP7 role during myogenesis has not been explored. Here, we show that USP7 is expressed transiently in adult muscle progenitors, correlating with

the onset of myogeninexpression, while is rapidly down-regulated in newly formed myotubes/myofibers. USP7 knockdown or pharmacological inhibition impairs muscle differentiation *in vitro* and i*n vivo*, by affecting myogenin stability. *In vivo*, acute USP7 inhibition upon muscle injury, results in regeneration delay and persistent expression of early regeneration markers. Together, our results show that USP7 function as a direct regulator of myogenin stability in a novel check-point to control SC fate.

SIGNIFICANCE.

Transcription factor Myogenin is crucial for differentiation of skeletal muscle progenitors. Albeit the in-depth knowledge regarding the transcriptional regulation of muscle differentiation, less is known about the integration of post-translational regulation of Myogenin and the mechanisms controlling muscle stem cell fate during regeneration. Here we show that USP7 is required to allow the up-regulation of Myogenin protein levels, allowing engagement of the terminal differentiation program. In light of the cellular events regulating muscle stem cell function, we propose that USP7 may be part of a "differentiation check-point" that could function to initiate myofiber formation upon proper intra and extracellular conditions.

INTRODUCTION

Skeletal muscle displays a robust regenerative capacity upon physiological cues. This process is supported by a subset of adult stem cells, called satellite cells (SCs), which are key components of skeletal muscle repair and maintenance. These cells are largely considered to reside in a quiescent state between the basal lamina and the myofiber's plasma membrane [1,

2]. Upon local stimuli, SCs become activated (re-entering the cell cycle), proliferate and differentiate to form new fibers. Concomitant to this process, the muscle stem cell pool is also replenished by a sub-population of SCs. Quiescent and activated SCs express the transcription factor Pax7, which regulates the activity of the muscle regulatory factor (MRF) MyoD. At this stage, SCs co-expressing Pax7 and MyoD, are considered proliferating muscle progenitors, with the potential to differentiate. Upon entering the differentiation phase, Pax7 levels are rapidly down-regulated concomitantly with the induction and up-regulation of the MRF myogenin, which drives terminal differentiation [3–5].

Although molecular regulation of muscle differentiation has been studied in detail, the mechanisms that fine tune the levels of transcription factors which promotes stem cell-like vs pro-myogenic fates, are not well understood. These processes are thought to be critical for the control of SC fate, in order to ensure myofiber formation/repair and self-renewal of the quiescent SC pool. In this context, it has been shown that Pax7 [6], MyoD[7–9], Myf5 [10] and myogenin [11, 12] are post-translationally regulated via the ubiquitin-proteasome system (UPS). Through the UPS, the small protein ubiquitin is covalently linked (usually to specific lysine residues) to target proteins [13], acting as a molecular mark for downstream regulatory interactions. Ubiquitination involves the activity of three major proteins: i) E1-ubiquitin activating enzyme, ii) E2 ubiquitin conjugating enzyme and iii) E3 ubiquitin ligase [14, 15]. Activated ubiquitin is transferred to the target protein by the Ubiquitin E3 ligase, which can additionally catalyze the formation of polyubiquitin chains. While E1 and E2 enzymes are highly conserved, each member of the large super-family of E3 ligases targets a defined set of proteins, providing specificity to the ubiquitination reaction. The resultant Ubiquitinated

proteins are generally destined to 26S proteasome-mediated degradation [16], however, ubiquitination can also regulate protein function through diverse and complex intricate pathways [17]. As other mechanisms of post-translational regulation modifications (PTM), protein ubiquitination is a dynamic process and can be remodeled by an specific a diverse group of ubiquitin proteases commonly referred as deubiquitinating enzymes (DUBs), which are involved and ubiquitin maturation [18], removal [19, 20] and polyubiquitin chain editing [21]. At least 90 DUBs have been identified in the human genome [22] and grouped into five families according to the conservation of their catalytic domains. These include the ubiquitin specific proteases family (USP), Ubiquitin C-terminal hydrolases (UCH), ovarian tumor domain containing proteases (OTU) and Machado Joseph disease deubiquitinases (MJD4). USP7/HAUSP (Herpes virus-associated ubiquitin-specific protease) is a member of the USP deubiquitinase family, displaying no significant homology (both at N- and C-terminus domains) with other USP proteins, suggesting distinct regulatory mechanisms [23]. USP7 N-terminal region contains a TRAF domain, which is crucial for nuclear localization [24] and protein-protein interactions through a conserved P/AXXS motif [25, 26]. This broad motif, could explain the promiscuity of USP7 on different interaction screenings [27]. At the C-terminal region, a domain containing five ubiquitin-like repeats, serve as modulator of USP7 activity via interaction with E3 ligases [28], other PTMs [24, 29, 30] and allosteric activation by GMP-synthetase [31]. USP7 is well-known for regulating the p53/Mdm2 axis, functioning as a switch for the control of p53 stability [32]. During neurogenesis, USP7 regulates the stability of the RE-1 silencing transcription factor (REST), promoting the maintenance of neural progenitors cells [33]. Additionally, a recent study shows that USP7 is required for

osteogenic differentiation of human adipose-derived stem cells, although the underlying mechanisms are not well understood [34].

Here we show that USP7 regulates the myogenic progression of muscle progenitors, cell fate by preventing myogenin protein degradation during the transition to terminal differentiation. Accordingly, acute inhibition of USP7 activity *in vivo*, impairs muscle regeneration. Finally, we propose a model that highlights the requirement of sequential check-points to regulate the transition to terminal differentiation, and their relevance for maintaining the balance between SC differentiation and self-renewal.

MATERIALS AND METHODS.

Cell culture.

Adult primary myoblasts and isolated myofibers were obtained as described [35] and maintained in growth medium (GM), F-12C (Life technologies, USA) supplemented with 15% Horse Serum (HS) (Biological Industries, USA) and 500 pM of FGF-2 at 37°C, 6% O_2 and 5% CO_2. When required, the cells were induced to differentiate by replacing GM with differentiation medium (DM), F-12C, supplemented with 15% HS.

C2C12 myoblasts were maintained in GM, DMEM (Life technologies, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Biological Industries, USA) at 37°C and 5% CO_2. For differentiation experiments, GM was replaced by DM, DMEM, supplemented with 5% HS. For USP7 knock-down C2C12 cells were transfected with USP7 siRNA (sc41521, Santa Cruz

Biotechnology, USA) using the Transit X2 reagent for 48 hours (Mirus Bio, USA). After transfection, cells were maintained in differentiation conditions for 48 h. For USP7 inhibition, C2C12 cells were were maintained in differentiation conditions and treated with HBX 41.108 (Tocris, USA) at 0.5 μM for long-term experiments or 3μM for short-term experiments and 12.5 μM of MG-132 (Cell signaling, USA) when required.

Immunofluorescence staining

Initially, C2C12 cells were plated and maintained as described above. Primary myoblasts were seeded onto gelatin coated glass-slides, and maintained as described previously. When specified, after transfection or incubation with inhibitors, cells were fixed with 4% PFA for 10 minutes, then permeabilized with PBS 0.2% T-X100 for 5 minutes and blocked with 5% fish gelatin (Sigma, USA) during 30 minutes and subjected to standard immunofluorescence staining [6]. Primary antibodies and dilutions were as following: Mouse monoclonal anti-Pax7 1:5 and anti-Myogenin (F5D) 1:5 (Hybridoma conditioned medium, Developmental Studies Hybridoma Bank, USA). Rabbit polyclonal anti-USP7 1:100 (Cell Signaling, USA), anti-MyoD 1:500 (Santa Cruz, USA). Secondary antibodies and dilutions were: donkey anti-mouse Alexa 555, 1:500; donkey anti-rabbit Alexa 488, 1:500; donkey anti-mouse Alexa 488 (Life technologies, USA); 1μg/mL of Hoescht was added to nuclei counterstaining. Fluoromont (Sigma Aldrich, USA) was used for mounting.

Muscle tissue staining.

Tibialis anterior (TA) muscles from 2-3 months old male C57BL/6J mice were dissected and snap-frozen on nitrogen chilled isopentane, cryosectioned (7μm) and subjected to indirect

immunofluorescence. Primary antibodies were: chicken anti-laminin (sigma Aldrich, USA) 1:2000, anti-goat Myogenin N-20 (Santa Cruz, USA) 1:250, anti-rat-MyoD (Millipore, USA) 1:500 and anti-rabbit USP7 (Cell signaling) 1:100. Secondary antibodies Secondary antibodies and dilutions were: 1:500; donkey anti-rabbit Alexa 488, 1:500; donkey anti-chicken AMCA, 1:500; donkey anti-goat alexa 555 and 1:500; donkey anti-rat alexa 555 (Life technologies, USA); 1μg/mL of Hoescht was added to nuclei counterstaining. Fluoromont (Sigma Aldrich, USA) was used for mounting. For Hemathoxilin/Eosin stainig, sections were fixed with 10% formalin, rinsed with distilled water and stained with hemathoxilin for 5 minutes. Then washed with tap water and stained with eosin for 1 minute. Sections were dehydrated on an ascending ethanol concentration battery and mounted with Entellan (Sigma, USA). Collagen content was detected by staining with 1% Sirius red in picric acid [36] and quantification was performed using ImageJ 1.48v software as percentage of total area. Images were acquired with Motic microscope BA410 Elite trinocular coupled to a refrigerated Moticam pro 252B camera and acquired with Motic Images Plus 3.0 software. Images were analyzed with ImageJ 1.48v processing software (https://imagej.nih.gov/ij/). Background subtractor plugin was applied on immunofluorescence images with a rolling ball radius of 30.0 px parameter.

***In vivo* muscle injury and USP7 inhibition.**

Muscle injury was performed as described previously [35]. Briefly, TA muscles from 2 months old male C57BL/6J mice were injected with 50μL of 1.2% of barium chloride ($BaCl_2$) diluted in saline (0.9%NaCl); contralateral TAs were injected with 0.9% NaCl (50μL) as control. 48 h after injury, mice were treated with HBX 41.108 at 0.8 mg/kg (250μL diluted on saline), via intra-peritoneal injection; one dose per day, for three days. TA muscles were extracted at 4, 9,

and 30 days post-injury and processed for immunostaining and/or lysis.

Myogenin stability assays.

C2C12 cells were seeded at 10,000 cells/cm^2 and differentiated, as previously described, during 24 h with 0.5μM of HBX 41,108. Prior to lysis, cells were pretreated for 15 minutes with 175 μM CHX, followed by co-treatment with 175μM CHX and 3μM HBX for 0, 5, 10, 15, 30 and 60 min and immediately lysed (on ice). Myogenin protein levels were assessed by western blot from whole cell lysates.

Western Blotting and co-immunoprecipitation.

Cells were lysed in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% IGEPAL, cocktail protease inhibitor (Merck, USA) and phosphatase inhibitors (2mM sodium orthovanadate, 50mM sodium fluoride) and 10-30μg of total protein was loaded into 10% SDS-PAGE gels and transferred to PVDF membranes for Western blotting with the following primary antibodies and dilutions: rabbit anti-USP7, 1:5000; mouse monoclonal anti-Pax7, 1:5; mouse monoclonal (F5D) anti-myogenin (Hybridoma conditioned medium, Developmental Studies Hybridoma Bank, USA), 1:10; mouse monoclonal anti-actin, 1:10000; (Sigma-Aldrich,USA) mouse monoclonal anti-Gapdh (EMD-Millipore, USA), 1:10000; 1:5000 and mouse monoclonal P4D1 anti-ubiquitin (Santa Cruz Biotechnology, USA). As secondary antibodies HRP conjugated anti-mouse IgG and anti-rabbit IgG (Cell Signaling, USA) were used at 1:5000. HRP activity was detected using the SuperSignal West Dura Extended Duration Substrate (Thermo-Fisher Scientific, USA). For immunoprecipitation, total protein was equalized (～500 μl at 0.5 mg/ml), precleared with 20 μl of agarose-protein G (50%

slurry; Pierce Chemical Co.), and incubated with 25 µl of anti myc-sepharose conjugated (Myc tag clone 9B11-sepharose, Cell signaling, USA) at 4°C overnight. Immunocomplexes were washed five times for 5 min each in buffer RIPA, and eluted by resuspending beads in 60 µl 3X SDS-PAGE loading buffer and boiled for 5 min.

Real-time PCR.

Primary myoblasts were lysed in Trizol reagent according to the manufacturer instructions (Invitrogen, USA) and total RNA was quantified using nanodrop module of TECAN infinite 200 pro plate reader; 500ng of total RNA were retro-transcribed with M-MLV-RT (Promega, USA) enzyme according to the manufacturer instructions. qPCR reactions were performed with SYBR Green master mix (Fermentas, USA), following the manufacturer intructions on 7500 Real-Time PCR System (Applied Biosystems, USA) with the following oligonucleotides: USP7 Fwd: 5'-CAGGCAGATGCTCCCCAT-3', USP7 Rev: 5'-GATGAATCATCGCCCTCTGT-3'; Myogenin Fwd:5'-ATTGTCTGTCAGGCTGGGTG-3', Myogenin RV 5'-TAAATTCCCTCGCTGGGCTG-3'. and as housekeeping gene 18S Fwd: 5'-GAGCGAAAGCATTTGCCAAG-3' 18S REV: 5'-GGCATCGTTTATGGTCGGAA-3' .

RT-PCR:

C2C12 cells were seeded on 6 well plates and treated with an increasing range of HBX 41.108 (0,1, 3, 10 and 20 µM) for 24 hours. mRNA was extracted with Trizol reagent according to manufacturer instructions and cDNA was synthesized with M-MLV RT enzyme (Promega, USA) and random hexamers (Promega, USA) as adapters, following the manufacturer indications. End point PCR was performed to analyze the expression of MyoD, p21 (a down-

stream target of p53) and S18 rRNA as loading control.

Myogenin P/AXXS mutants.

pEMS-*rMgn* plasmid was used as template to produce myogenin point mutants using Quick Change multi-site directed mutagenesis XL kit (Agilent Technologies). To disrupt USP7 interaction motif on myogenin (P/AXXS), serine was changed to alanine with the following primers pairs: 5'-TCCTGGTTGAGGGAGGCGAGCAAGGCCTGTAG-3' and 5'-CTACAGGCCTTGCTCGCCTCCCTCAACCAGGA-3' to rMgn S137A mutant; 5'-GCCCCACTCCGGAGCGCAGGAGGCGCTG-3' and 5'-CAGCGCCTCCTGCGCTCCGGAGTGGGGC-3' to rMgn S170A; and S137A was used as a template with the S170A noted primers to generate the double mutant S137/170A. For Co-IP assays, myogenin mutants were co-transfected with WT-*Usp7* on C2C12 cells during 48 h with Transit-X2 (Mirus, USA). Lysates and Co-IP against myc epitope were performed as described above. For ubiquitination experiment, C2C12 cells were co-transfected with pCDNA3-*Ub-Myc* plasmid and each of the P/AXXS myogenin mutants. C2C12 cells were lysed on ice with 100 μL of ubiquitination lysis buffer (2% SDS, 150 mMNaCl, 10 mM Tris-HCl, pH 8.0) with 2mM sodium orthovanadate, 50 mM sodium fluoride, and protease cocktail inhibitors (Merck, USA). The tubes were placed immediately onto 100°C plate cells and sheared by sonication. 900 μL of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mMNaCl, 2 mM EDTA, 1% Triton) was added and incubated for 60 minutes with agitation at 4°C. Samples were centrifugated at 14.000 rpm for 30 minutes and cleared supernatants were transferred into a new tube. Denaturing IP was performed as described above. Samples were washed 6 times with ubiquitination washing buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1

mM EDTA, 1% NP-40) and eluted by resuspending beads in 60 µl 3× SDS-PAGE loading buffer and boiled for 5 min.

RESULTS.

USP7 is expressed in skeletal muscle during regeneration.

Previously, we described several Pax7-interacting proteins, identified by tandem affinity purification followed by mass spectrometry [6]. Among other proteins, we identified several components of the UPS, including proteasome subunit alpha type-3 (Psma3), ubiquitin, the E3 ubiquitin ligase Nedd4, and the deubiquitinase USP7. Although our group showed that Nedd4 controlled Pax7 protein levels, the role of USP7 in skeletal muscle progenitors has not been determined. Therefore, we first evaluated USP7 expression throughout differentiation in the myoblast cell line C2C12, by immunofluorescence (IF) and Western blotting. We observed an enrichment of USP7(+) cells in differentiated myotubes, although total protein levels appear to decline at late time points (96-120 h) (S1A, B). Next, we analyzed USP7 expression in primary myoblasts (pMbs), isolated from adult mouse hind-limb muscles. In contrast to the expression pattern observed in C2C12 myoblasts, USP7 expression was not detected in freshly isolated primary cells, measured by qPCR (Fig 1A). Accordingly, USP7 protein was also not detected by IF in proliferating pMbls (Fig 1B, upper panel). Upon differentiation, USP7 mRNA expression was induced (Fig. 1A) while the protein was consistently detected in Myogenin (+) cells (Fig. 1B, middle panel). Interestingly, although expression of USP7 mRNA remained high at 6 days in differentiating conditions (Fig. 1A), USP7 protein was

undetectable in myotubes by IF, despite the expression of Myogenin (Fig. 1B, lower panel). A similar expression pattern was observed in activated SCs associated to isolated myofibers in culture, were USP7 was not detected at 24 and 48h after isolation in ~100% of MyoD (+) cells (Fig. 1C). 6 days post isolation, USP7 appeared differentially expressed in Pax7 (-)/Myogenin (+) cells, whereas Pax7 (+)/Myogenin (-) cells appear uniformly USP7 (-) (Fig 1C, lower panel). Additionally, we evaluated USP7 expression during muscle regeneration, after barium chloride ($BaCl_2$)-induced damaged in the tibialis anterior (TA) muscle. 2 days post injury (dpi), USP7 was expressed in ~60% of myogenic precursors, identified by MyoD expression (Fig. 1D, upper panel). Of note, USP7 was also detected in non-myogenic, interstitial cells, which is expected since USP7 expression has been reported in a broad range of cell types [37, 38]. At 5 dpi, ~98% of Myogenin (+) cells co-expressed USP7 (Fig. 1D bottom panel), consistent with USP7 expression observed in pMbs. Correlating to USP7 expression in pMb (Fig1. B), USP7 was not detected *in vivo* at 1dpi or 15 dpi TAs (Fig. 1E). Together, these results indicate that USP7 expression is dynamic in muscle tissue, where is transiently expressed in differentiating muscle progenitors.

USP7 regulates Myogenin protein stability during early differentiation.

USP7 expression pattern, restricted to early stages of differentiation and the up-regulation of Myogenin expression, suggests a functional requirement during this process. Initially, we sought to analyze the effect of USP7 down-regulation in differentiating C2C12 myoblasts. For this, C2C12 cells were maintained in proliferation conditions and transfected with an USP7-specific siRNA (siU7) or a scramble control siRNA (siCO), and then maintained for 48h in differentiating conditions. Western blot analysis showed a 3.3-fold reduction in USP7

expression (Fig. 2B). Interestingly, Myogenin levels were reduced by 6.8-fold in siU7 transfected cells (Fig. 2B). In agreement with previous work, we also observed a small, but consistent, up-regulation of Pax7 expression. As expected, siU7-induced Myogenin down-regulation, correlated with a significant decrease in the numbers of Myogenin (+) cells and the formation of nascent myotubes, compared to siCO transfected cells (Fig. 2A). To distinguish between the effects of low USP7 expression and reduced deubiquitinase activity on Myogenin expression, C2C12 myoblasts were treated with the USP7 inhibitor HBX 41,108 (HBX) [39] or vehicle, both in proliferation and differentiation conditions. HBX treatment induced a ~3-fold reduction in Myogenin protein levels in differentiating cells, compared to vehicle treated cells (Fig. 2C). Although we detected a basal expression of Myogenin in cells maintained in proliferating conditions, this was not affected by the presence of HBX (Fig. 2C), further supporting a functional role for USP7 at early stages of muscle differentiation. Since USP7 inhibition has been linked to increased p53 stability, we asked if HBX 41.108 activated the p53 signaling in our experimental conditions. Therefore, we determined the expression of the canonical p53 transcriptional target, p21 [37]. RT-PCR analyses showed that p21 expression was not induced by HBX concentrations used in this study (S2A). In addition, as an indirect measure of USP7 inhibition, we monitored nuclear localization of FOXO4 (which is inhibited by USP7 activity) [40] (S2B). These results indicate that the effect of USP7 inhibition on Myogenin expression is likely specific and not a downstream effect triggered by p53 signaling, which is known to halt/inhibit differentiation [41].

Since USP7 has been well characterized as a transcriptional co-regulator [42], we asked if inhibition of USP7 activity affected Myogenin expression at the transcriptional level. As

determined by semi-quantitative PCR, Myogenin mRNA levels remained unchanged in the presence or the absence of 1μM HBX 41.108 (Fig.3A). This result suggested that USP7 inhibition affected Myogenin levels via a post-translational mechanism. We hypothesized that down-regulating USP7 deubiquitinase activity would lead to UPS-mediated myogenin degradation. To test this idea, differentiating C2C12 cells were treated with HBX 41.108 or vehicle, plus or minus the proteasome inhibitor MG132. As expected, Myogenin protein levels were significantly higher upon HBX and MG132 co-treatment, when compared to vehicle treated cells (Fig. 3A). Moreover, we aimed to measure if Myogenin protein half-life decreased upon USP7 inhibition. Thus, differentiating C2C12 myoblasts were treated with cycloheximide (CHX) in order to inhibit general translation, in the presence or the absence of HBX 41.108. Cells were harvested at different time points (0-60 minutes) and Myogenin levels analyzed by Western blot. As shown in Figure 3B, we determined that Myogenin had a half-life of ~23 minutes in vehicle treated cells. This value was reduced by ~50% upon USP7 inhibition (Fig. 3B). Accordingly, over-expression of a myc-tagged USP7 protein (USP7 WT) results in ~3-fold increase of Myogenin levels, compared to cells transfected with an empty vector. Additionally, over-expression of a catalytic inactive myc-tagged-USP7 mutant (USP7-C223S), had no significant effect on Myogenin expression (Fig 3C). Together, these results suggest that USP7 regulates Myogenin stability, presumably, by controlling its ubiquitination status.

Myogenin is a substrate for USP7 deubiquitination in myogenic cells.

In order to identify the mechanism(s) underlying the regulation of Myogenin levels by USP7, we evaluated if USP7 and Myogenin could physically interact in muscle precursors. First, we

performed co-immunoprecipitation (co-IP) assays from C2C12 myoblasts maintained in proliferation and differentiation conditions. Myogenin was IP from whole cell extracts and the presence of USP7 in the eluted fraction was determined by Western blot. As expected, USP7 co-IP was not detected in proliferating myoblasts (Fig.4A, D0). After 24h in differentiated conditions, we observed the induction of Myogenin expression, which gradually increased over time (Fig. 4A, D1-D6). Under these conditions, USP7 was specifically enriched in the co-IP fraction, as indicated by the absence of USP7 when co-IP was performed with a control IgG (Fig. 4A). Of note, USP7 expression in C2C12 myoblasts was detected throughout all stages, highlighting the specificity of the protein interaction in differentiating cells (Fig. 4A, lower panel).

It has been stablished that USP7 TRAF domain binds to degenerate P/AXXS motifs present in various USP7 protein targets [43, 44]. *In silico* examination of the mouse Myogenin primary sequence, revealed two conserved P/AXXS motifs, which could mediate the interaction with USP7 (Fig. 4B, upper panel). Therefore, we generated S > A point mutants in the P/AXXS Myogenin motifs (S137A, S170A and S137/170A double mutant, respectively; Fig. 4B, lower panel), which has been shown to be sufficient to disrupt USP7 protein interactions [45]. Next, Myogenin mutants were expressed in the C2C12 myoblasts, in order to evaluate the interaction with endogenous USP7 via co-IP. Unexpectedly, we observed similar interaction between S137A and S137/170A Myogenin mutants and USP7, compared to WT-Myogenin (Fig 4C). Intriguingly however, Myogenin S170A, was consistently expressed at significantly lower levels. Thus, we hypothesized that S170A was subjected to enhanced turnover. To test this model, P/AXXS Myogenin mutants were co-expressed with myc-Ubiquitin, and we

determined the relative ubiquitinated Myogenin levels via IP followed by Western blotting. Accordingly, despite the significant lower expression, we found and enrichment of ubiquitinated S170A mutant, when compared to S137A, S137/170A and WT-Myogenin proteins (Fig 4D). Together, these results suggest that USP7 can interact with myogenin, regulating its ubiquitinated status.

Muscle regeneration is disturbed upon acute inhibition of USP7 activity.

Based on the results presented above, it is possible to speculate that USP7 regulates SC function *in vivo*. Therefore, we evaluated the effect of inhibiting USP7 activity in a murine model of induced muscle damage and regeneration. *Tibialis anterior* (TA) muscles from adult mice, were injured by intramuscular injection of barium chloride ($BaCl_2$), as described previously [35]. 24 h post injury (1 dpi), mice were separated into two groups, receiving daily doses of HBX 41.108 (8mg/kg/injection) or vehicle, respectively, for three days (Fig. 5A). TA muscles were collected at 4, 9 and 30 dpi and evaluated for regeneration status by i) histological analyses and ii) expression of muscle specific proteins by Western blot. At 4 dpi, H&E staining revealed no major differences in overall muscle histology between mice treated with HBX and vehicle (Fig. 5B). At 9 dpi, however, regenerating TAs from HBX treated mice exhibited alterations in tissue architecture (Fig. 5B). Closer inspection revealed a significant asymmetry in fiber diameter, represented by the frequency and distribution of fiber cross-sectional areas (CSA; Fig. 5B, bottom-left). While in the control group, ≥50% of quantified myofibers were distributed in a CSA range of 1-3 $x10^3$ μm^2; ≥50% of myofibers displayed CSAs between <0.5-1.5$x10^3$ μm^2. Unexpectedly, similar CSAs differences between treatments were maintained at 30 dpi (Fig. 5B, bottom-right). We reasoned that these observations could

be explain at least by three mechanisms: a) increased deposition of extracellular matrix (ECM) proteins, limiting the growth of regenerating myofibers; b) a delay in the myofiber differentiation/maturation process, and/or c) increased cell death of myogenic precursors at earlier regeneration stages. Considering USP7 expression during muscle differentiation, we expected the last possibility to be less likely. Thus, we first evaluated the ECM deposition by Sirius red staining and found no significant differences both at 9 and 30 dpi between treatments (Fig. 5C). Next, we analyzed the expression of differentiation markers in whole muscle extracts by Western blot. Interestingly, at 9 dpi the levels of Pax7 (i.e. activated and/or quiescent SCs) and the embryonic form of myosin heavy chain (eMyHC) were significantly increased in regenerating TA muscles treated with the USP7 inhibitor, while the levels of Myogenin were not significantly different between treatments (Fig. 5D). Moreover, while compensatory hypertrophy was clearly observed at 30 dpi in control TAs, it was absent in HBX treated animals (Supplementary figure 2). Together, these results are consistent with the idea that acute USP7 inhibition disrupts muscle regeneration by impairing and/or delaying the differentiation of muscle progenitors.

DISCUSSION

Transition to early muscle differentiation is driven by cross-regulation between the transcription factors Pax7, MyoD and Myogenin. It has been proposed that such mechanism could fine-tune myogenic progression, in part, by preventing precocious differentiation under sub-optimal cues. In this context, PTMs are thought to play an important role controlling

master regulators of muscle stem cell fate. Indeed, we previously demonstrated that Pax7 and Myogenin expression rapidly became mutually exclusive in differentiating myoblasts, through UPS-dependent Pax7 down-regulation [6, 46]. Nonetheless, it is not fully understood how Myogenin levels sharply increase upon commitment to terminal differentiation. This is particularly intriguing considering that MyoD, functionally upstream of myogenin, is also tightly controlled by PTMs, driven in part by Pax7. The present study highlights the implications of the reversible nature of protein ubiquitination, allowing precise control over myogenic transcriptional regulators. Our results indicate that the ubiquitin-specific protease USP7 is a positive regulator of Myogenin stability at early stages of muscle differentiation. Accordingly, Myogenin protein expression was decreased under USP7 knockdown and conversely, increased upon USP7 over-expression; while expression of a catalytically inactive form of USP7 did not affect Myogenin accumulation. USP7 expression itself appears to be under precise regulation in muscle precursors, both *ex vivo* and *in vivo*, suggesting a specific requirement for USP7 activity during myogenic progression. At the molecular level, inspection of Myogenin protein sequence revealed two potential USP7 binding sites [47]. Site-directed mutagenesis disrupting one of this sites (S170A) results in significantly decreased protein levels upon expression in C2C12 myoblasts, which correlated with increased poly-ubiquitination of the remaining S170A-Myogenin mutant protein. Further supporting its biological relevance, USP7 activity is required for proper muscle regeneration upon induced damage. Remarkably, acute inhibition of USP7 activity upon SC activation (1-4 dpi), resulted in decreased myofiber diameter, persistent expression of early differentiation markers (Myogenin and eMyHC), and the muscle progenitor marker, Pax7. In light of these data, we propose a model whereby Myogenin protein levels need to reach a critical threshold in order

to engage the terminal differentiation program. However, given the reversible nature of this step, Myogenin function is restrained, at least in part, by negative regulation via UPS. In this context, USP7 function counteracts the UPS control, enhancing Myogenin protein stability and, thus, regulating the timing of myogenic progression and terminal differentiation.

Using proteomic analysis to uncover new Pax7-interacting proteins, we identified USP7 as a potential candidate [6]. Intriguingly, we have not been able to detect direct interaction between Pax7 and USP7 and, more importantly, we could not detect muscle progenitors co-expressing Pax7 and USP7 (*ex-vivo* or *in-vivo*). These observation raises the idea that USP7 and Pax7 could co-exist, transiently, in a multiprotein complex. Expression studies using mitotically synchronized myoblast cultures, could provide further insight on this issue; a similar strategy was used to determine that Pax7 and Myogenin were transiently co-expressed during early differentiation [46]. On the other hand, strong Myogenin and USP7 co-expression was observed upon differentiation of primary myoblasts and myofiber-associated SCs cultures. Interestingly, USP7 protein levels are down-regulated in myotubes (i.e. after cell fusion), although no significant change was observed in USP7 mRNA levels. We envision at least three mechanisms underlying these observations: i) an undefined post-translational regulation over USP7 mRNA, were a rate limiting step in USP7 translation is induced in fusing muscle progenitors, ii) a rapid change in USP7 protein stability, leading to its degradation or iii) a different PTM affecting USP7 activity and/or protein interactions, leading to increased USP7 turn-over. From this perspective, a recent report demonstrated that USP7 is regulated by phosphorylation on Serine 18, which leads to protein stabilization and activation [48, 49], while PPM1G mediated de-phosphorylation, reduces USP7 protein stability. As shown by

Gonzalez et al [50], CK2 phosphorylates Pax7 increasing its stability (supporting the proliferating status of myoblasts), while CK2 down-regulation, results in precocious Myogenin expression. From this perspective is known that nuclear CK2 activity increases in cells that have re-entered the cell cycle [51, 52]. If USP7 phosphorylation status is regulated by a similar CK2 dependent mechanism, this could explain in part, the rapid fall of USP7 protein levels observed in differentiated myogenic cells (i.e. cells that have exited cell cycle).

Under local stimuli, MyoD (+) cells rapidly up-regulate Myogenin expression, allowing myogenic progression towards terminal differentiation. As shown in the elegant study by Cao et al [53], when ectopic MyoD expression is induced in CH310T/2 cells, the myogenic process is triggered hierarchically, as observed in muscle progenitors. In this model, endogenous Myogenin protein was detected as early as 12 h after MyoD induction. Interestingly, constitutive expression of Myogenin in MyoD-induced CH310T/2 cells, reveals that Myogenin cooperates with MyoD to regulate the expression of a subset of genes at late stages of the CH310T/2-Myogenin conversion induced by MyoD alone. Moreover, precocious expression of Myogenin can shift the expression of such genes to an earlier time point following MyoD induction. In this context, previous studies show that a switch from Notch to Wnt-dependent signaling, drives the transition from proliferating muscle progenitors to differentiation-committed cells, which in turn is critical to assure proper skeletal muscle regeneration [54]. Indeed, ectopic induction of the Wnt signaling during the proliferation phase, impairs muscle regeneration due to precocious differentiation of the muscle progenitor population [54]. Taken together, these observations give further physiological support to our model, whereby USP7 appears to counteract the tight control over Myogenin levels imposed

by the UPS control. Since USP7 expression itself is restricted to early differentiation, this mechanism could allow fine-tuning over the myogenic progression. Since embryonic USP7 deletion has an embryonic lethal phenotype at E6.5-E7.5 [55], we attempted to determine the effect of USP7 loss of function during muscle regeneration, via intraperitoneal injection of the HBX 41.108 inhibitor. In order to minimize systemic/off-target effects, the drug was delivered as an acute treatment. Since the damaged muscle tissue is highly permeable, we hypothesized that inhibitory concentrations of HBX 41.108 would readily reach the regenerating sites, affecting the activated SC population. As expected, early regeneration was impaired upon HBX treatment. Unexpectedly, however, this effect persisted a later time points, since myofiber size and expression of muscle specific genes remained altered. Of note, USP7 expression is down-regulated in regenerating fibers, suggesting that acute USP7 inhibition, specifically in differentiating muscle progenitors, has long term effects on muscle regeneration. In this scenario, the transcription factor FOXO4 (which participates in metabolic responses in muscle tissue, including the induction of muscle atrophy), is a well-known USP7 target [40]. USP7-mediated de-ubiquitination precludes FOXO4 nuclear localization, therefore regulating the transcription of FoxO targets, including the atrogenic genes [56]. Thus, it is plausible that partial activation of the atrogenic cascade could be involved in the muscle phenotypes observed in our study.

Ongoing studies seek to conditionally ablate USP7 in SCs (Cabezas et al, unpublished data), allowing us to uncover specific mechanisms down and upstream USP7 signaling, which are relevant for SC function and proper muscle regeneration.

FIGURE LEGENDS.

GRAPHICAL ABSTRACT

USP7 controls Myogenin stability. Upon activation, satellite cells become proliferating, committed muscle progenitors co-expressing Pax7 and MyoD. Reciprocal control between Pax7 and the MRFs is thought to allow the amplification of the progenitor pool, while preventing precocious differentiation. Downstream MyoD function, Myogenin expression correlates with the irreversible transition to terminal differentiation. Here we show that Myogenin is efficiently ubiquitinated (1) and degraded by the UPS (2), which may aid to maintain the proliferating phase of muscle progenitors (3). This control is counteracted by USP7 (4), which binds and deubiquitinates Myogenin, resulting in the up-regulation of Myogenin levels and the transition to terminal differentiation (5). In light of the cellular events regulating muscle stem cell function, we propose that USP7 may be part of a "differentiation check-point" that could function to initiate myofiber formation upon proper intra and extracellular conditions.

Figure 1: USP7 is expressed in activated SCs.

(A) USP7 mRNA expression (qPCR) during myogenic progression of primary myoblasts (pMbls). FIM: Freshly isolated pMbls; Prol: Proliferating pMbls; ED: Early differentiating pMbls; LD: Late differentiating pMbls; RQ: Relative quantities of USP7 to S16 rRNA. Bars represent standard deviation of three replicates (B) USP7 is expressed in differentiating primary myoblasts (Mgn+ cells) by 3 days in differentiation culture conditions. Note the

almost undetectable USP7 expression in activated/proliferating myoblasts (MyoD+ cells) and fully differentiated myotubes (D6) (C)USP7, Pax7 and Myogenin expression after 24 to 144 h cultured myofibers, by IF. Note USP7/Mgn co-expression in Pax7- cells. Syndecan-4 (Synd4) was used as independent SC lineage marker. (D) Left panel: USP7 expression during muscle regeneration (TA muscles). USP7 was not detected in SCs from uninjured muscles. After 2 dpi, USP7 is expressed in activated SCs cells (MyoD+). By 5 dpi, USP7 is expressed by ~98% of differentiating myogenic cells (Mgn+). Arrow heads indicates SCs Mgn (+) and USP7 (+), arrows show USP7 (+) only cells. Right panel: Quantification of USP7 cells in myogenic cells, identified by MyoD (2 dpi) or Myogenin (5 dpi) expression. (E) USP7 expression on recently injured (1dpi) TA and 15 dpi, including centrally located myonuclei.

Figure 2: Decreased USP7 expression or activity impairs C2C12 differentiation.

(A) USP7 knockdown (siU7) results in decreased Myogenin protein levels as determined by Western blot. siCO, non-specific siRNA. Representative image from n=3 independent experiments. (B) siRNA-mediated USP7 knockdown in C2C12 myoblasts. Phase contrasts images shows decreased formation of elongated cells (yellow arrows, upper panel) and the expression of Myogenin (IF, lower panel) at 48 h in differentiation conditions. (C) Inhibition of USP7 deubiquitinase activity with HBX 41.108 inhibitor (HBX), results in reduction of Myogenin expression (compare to vehicle, DMSO), determined by Western blot. (D) Quantification of Myogenin levels relative to Gapdh (mean ± s.e.m.) from three independent experiments. Student's t-test *$p<0.05$.

Figure 3: USP7 activity enhances myogenin protein stability.

(A) Left panel: USP7 inhibition does not affect the expression of Myogenin mRNA. Right panel: Inhibition of USP7 deubiquitinase activity decreases the accumulation of Myogenin induced by proteasome inhibition with MG-132 (MG). (B) USP7 inhibition in Cycloheximide (CHX) treated C2C12 cells, reduces half-life of Myogenin protein. Bottom panel: quantification of normalized myogenin levels (mean ± s.e.m.) from three independent experiments. (C) Over-expression of Myc-USP7 (WT) on proliferating conditions, increases Myogenin expression in C2C12 cells, in a dose dependent manner (0-1.5 ug DNA vector). Expression of an inactive form of USP7 (C223S, catalytic site point mutation) has no effect over Myogenin levels. Bottom panel: USP7 gain of function correlates with increase Myogenin expression in proliferation determined by IF. Quantification of Myogenin (+) and Myc (+) cells, shows a ~70% co-expression of the active myc-USP7 with Myogenin, while ~20% of the cells co-express Myogenin with the C223S, inactive form of USP7. A 6.6-fold decreased of myogenin is observed on USP7 C223S mutant transfected cells (mean ± s.e.m. from three independent experiments).

Figure 4: USP7 interacts with Myogenin interacts in vitro and in vivo.

(A) C2C12 cells where transfected with Myc-USP7 vector and maintained in differentiation conditions for 0-6 days. Western blot analysis shows that USP7 is specifically detected upon Myogenin IP (left panel), compared to IP with control IgG (right panel) (representative panel of two replicas). (B) Myogenin primary sequence alignment shows conservation of two P/AXXS putative motifs among several species; Alignment performed with MUSCLE online free software (https://www.ebi.ac.uk/Tools/msa/muscle/). Bottom panel: schematic of Myogenin functional domains showing the location of P/AXXS motifs and highlighting the S

> A site directed mutagenesis. (C) Western blot from C2C12 cells in proliferation conditions co-expressing WT, S137A, S170A or S137/170A Myogenin mutants with Myc-USP7. USP7/Myogenin-mutants interactions were determined by Co-IP. Note the correlation between Co-IP efficiency from cell expressing the S170A mutant Myogenin. (D) *In vivo* ubiquitination of P/AXXS Myogenin mutants determined by Western blot from C2C12 cells expressing myc-ubiquitin (Ub-myc) and Myogenin mutants. Note that upon normalization to the level of expression, S170 mutant is highly ubiquitinated (~4 fold compared to control and the other myogenin mutants). As expected, the S170A ubiquitination was detected even though the expression was very low (For C and D, only one experiment was performed).

Figure 5 Acute systemic inhibition of USP7 results in muscle regeneration impairment.

(A) Diagram of the *in vivo* intra-peritoneal HBX 41.108 injection and injury protocol. (B) Top panel: H&E staining of HBX 41.108 treated animals. Tibialis anterior (TA) were dissected after 4, 9 and 30 dpi, snap frozen and cryosectionated. Bottom panels: Quantification of myofiber cross-sectional area (CSA) distribution, in injured TAs at 9 and 30 dpi. Note, the decrease CSA in HBX treated animals, which is preserved after 30 dpi. Only injured muscles are shown. (C) Collagen deposition measured by Sirius red staining, at day 9 and 30 dpi. Total Sirius Red (+) areas were quantified. No significative differences were observed (mean ± S.D. from two independent experiments). (D) Western blot analysis from whole muscle extracts from treated and untreated animals. Early differentiation markers (myogenin and eMyHC), Pax7 and USP7 expression are shown. Note the persistent expression of these markers in HBX treated animals. Day 4, n=3 animals for controls and HBX treated animals; day 9, n=2 for controls and n=1 for HBX treated. Scale bar= 100μm.

Supplementary Figure 1.

USP7 expression in differentiating C2C12 cells A) USP7 expression was analyzed by Immunofluorescence on C2C12 cells were USP7 protein is localized into forming/mature myotubes. B) Western blot analysis of USP7 protein accumulation from proliferating C2C12 myoblasts to 6 days in differentiating conditions.

Supplementary Figure 2.

USP7 inhibitor HBX 41.108 has none off-target effects at working concentrations. A) RT-PCR of C2C12 treated with a increasing concentrations of HBX 41.108. p21 (a downstream target of p53) is not activated at 1 μM B) At this working concentration we observed an effect over nucleo-cytoplasmic shuttle of Foxo, a known target of USP7. Quantification is showed as ± S.D. from two independent experiments.

Supplementary Figure 3.

USP7 inhibition impairs muscle regeneration A) Representative pictures of whole muscle sections from 30 days post damage TA on animals treated with and without acute injection of HBX 41.108 intraperitoneally (Figure 5A: reefer to protocol schematic) B) Quantification of TAs whole area measured on mm^2, represented as mean ± S.D. of two independent experiments

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Graphical abstract

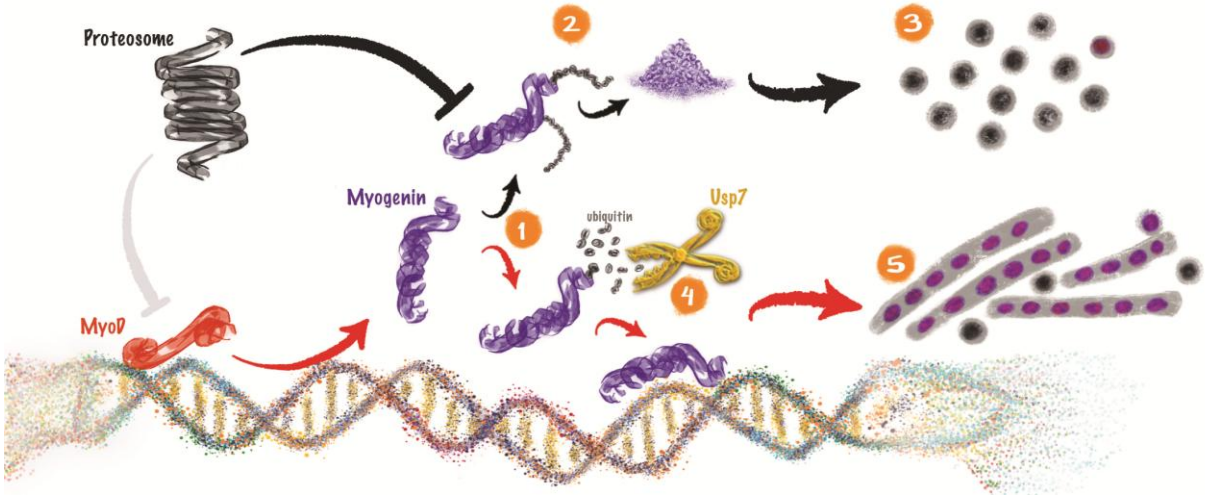


Figure 1:

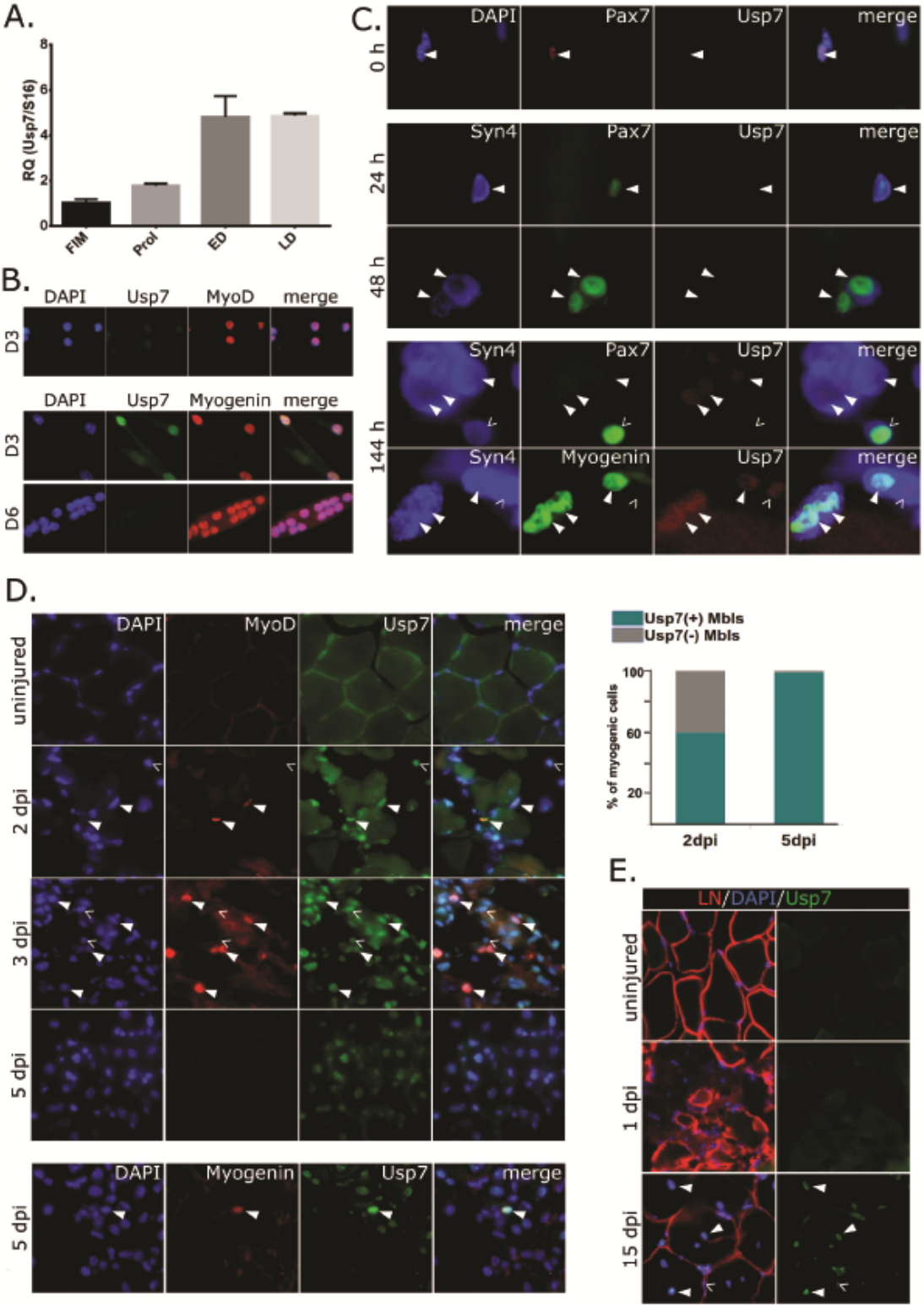

A.
RQ (Usp7/S16)
FIM
Prol
ED
LD
B.
DAPI
Usp7
MyoD
merge
D3
DAPI
Usp7
Myogenin
merge
D3
D6
C.
DAPI
Pax7
Usp7
merge
0 h
Syn4
Pax7
Usp7
merge
24 h
48 h
Syn4
Pax7
Usp7
merge
144 h
Syn4
Myogenin
Usp7
merge
D.
DAPI
MyoD
Usp7
merge
uninjured
2 dpi
3 dpi
5 dpi
DAPI
Myogenin
Usp7
merge
5 dpi
Usp7(+) Mbls
Usp7(-) Mbls
% of myogenic cells
100
60
20
2dpi
5dpi
E.
LN/DAPI/Usp7
uninjured
1 dpi
15 dpi

Figure 2:

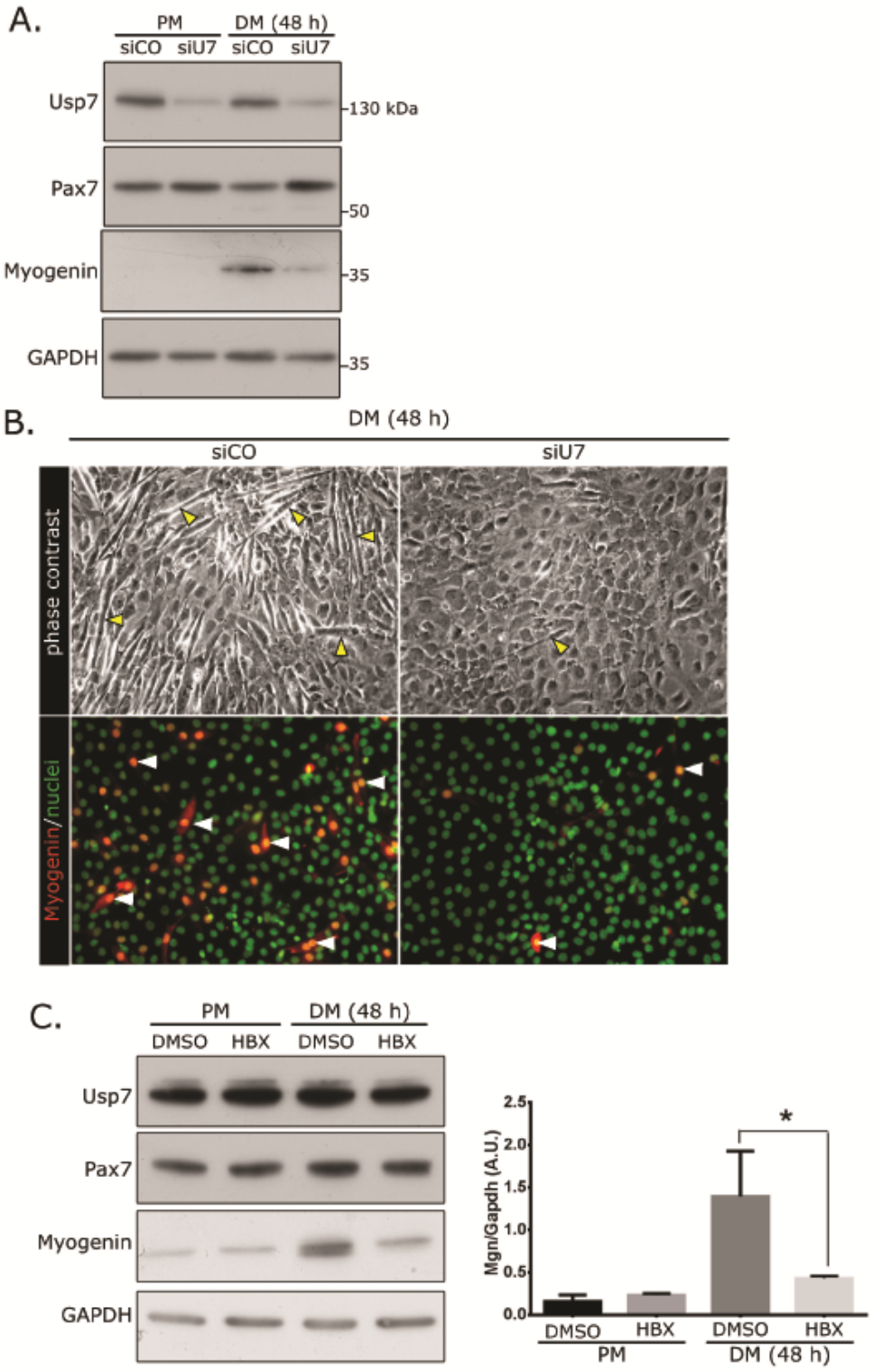

A.
PM
DM (48 h)
siCO
siU7
siCO
siU7
Usp7
130 kDa
Pax7
50
Myogenin
35
GAPDH
35
B.
DM (48 h)
siCO
siU7
phase contrast
Myogenin/nuclei
C.
PM
DM (48 h)
DMSO
HBX
DMSO
HBX
Usp7
Pax7
Myogenin
GAPDH
Mgn/Gapdh (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
*
DMSO
HBX
DMSO
HBX
PM
DM (48 h)

Figure 3:

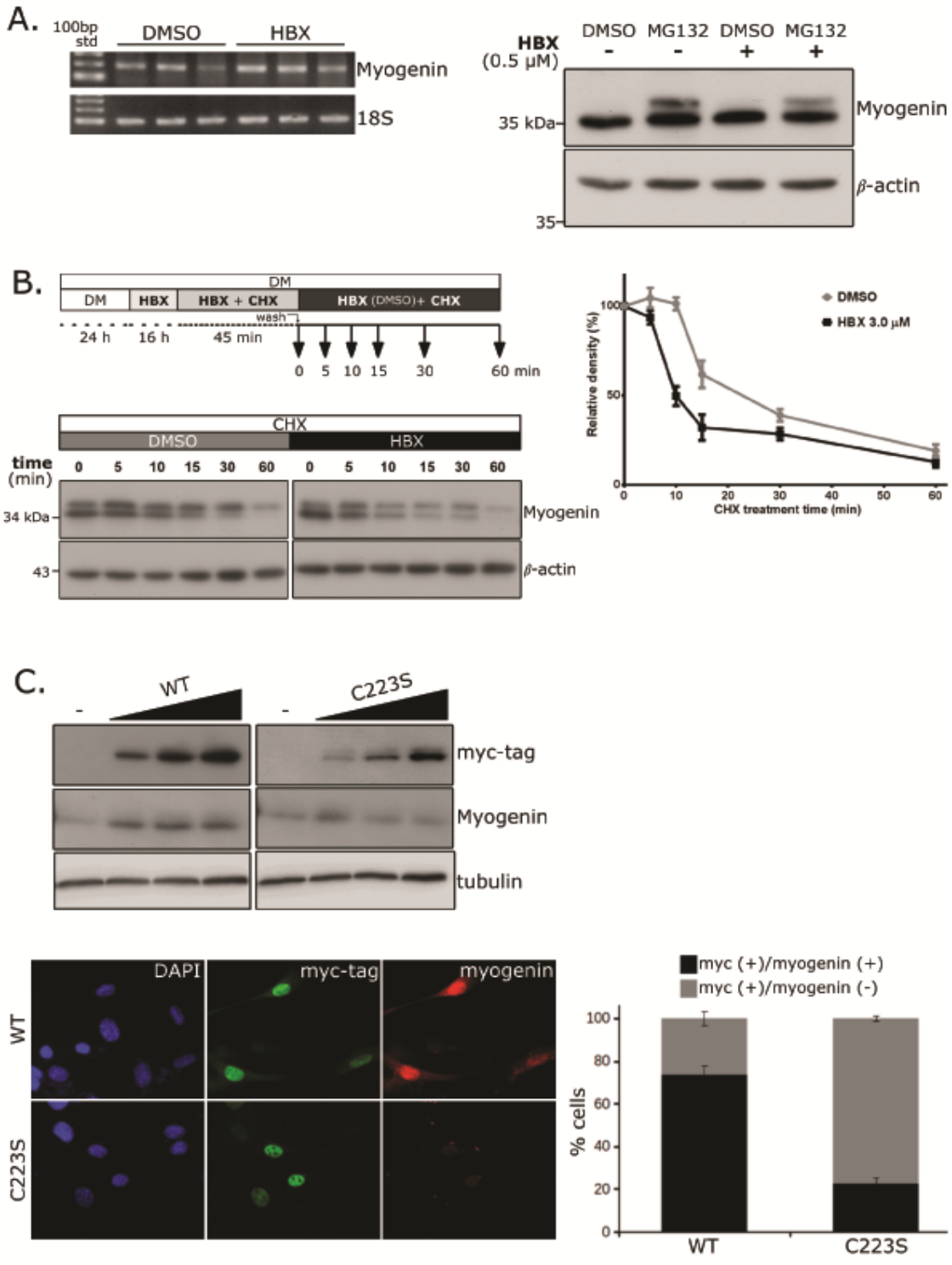

A.
100bp std
DMSO
HBX
Myogenin
18S
HBX (0.5 μM)
DMSO MG132 DMSO MG132
- - + +
35 kDa
Myogenin
β-actin
35
B.
DM
DM
HBX
HBX + CHX
HBX (DMSO)+ CHX
wash
24 h
16 h
45 min
0 5 10 15 30 60 min
CHX
DMSO
HBX
time (min)
0 5 10 15 30 60 0 5 10 15 30 60
34 kDa
43
Myogenin
β-actin
Relative density (%)
DMSO
HBX 3.0 μM
CHX treatment time (min)
C.
WT
C223S
myc-tag
Myogenin
tubulin
DAPI
myc-tag
myogenin
WT
C223S
myc (+)/myogenin (+)
myc (+)/myogenin (-)
% cells
WT
C223S

Figure 4:

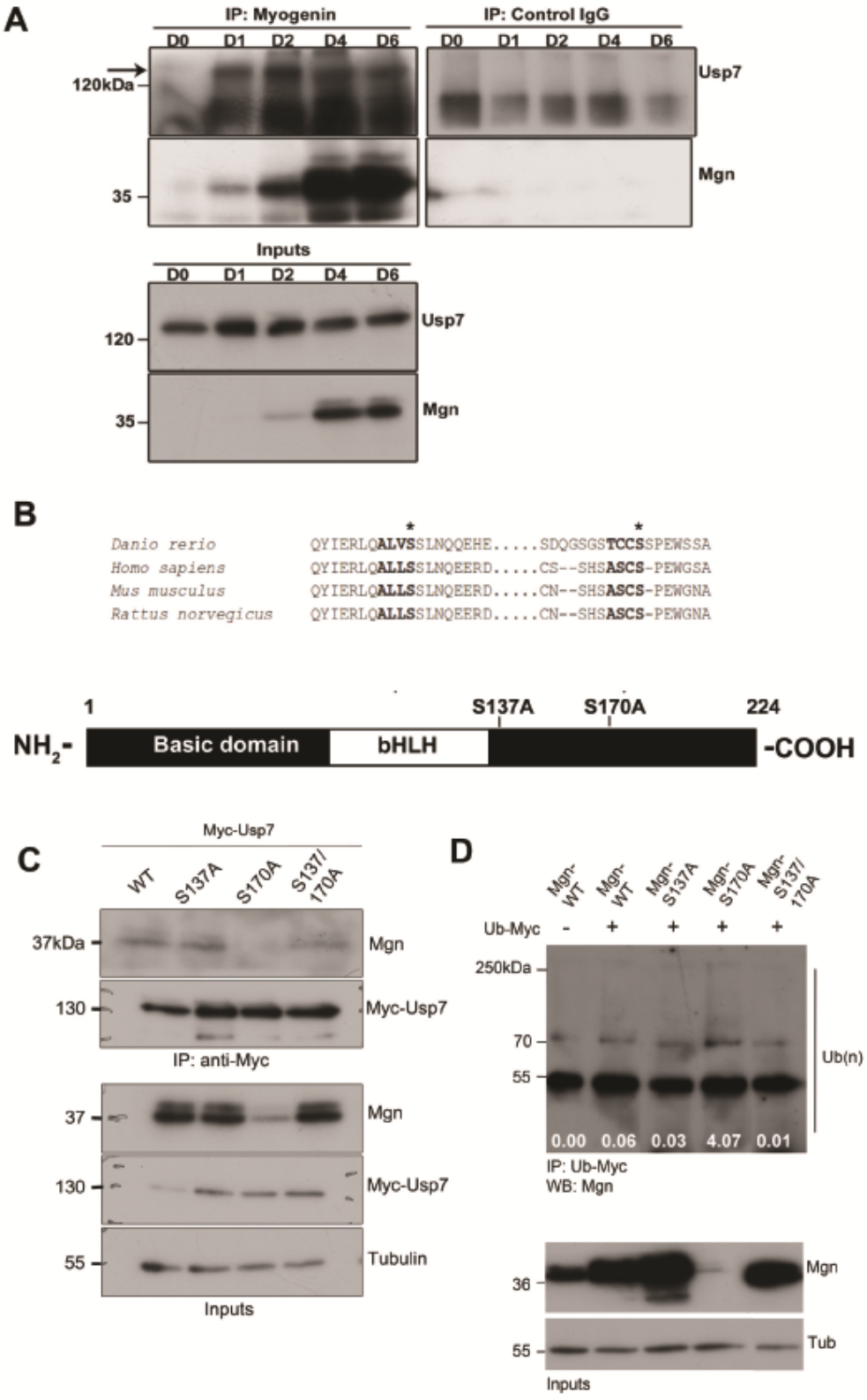
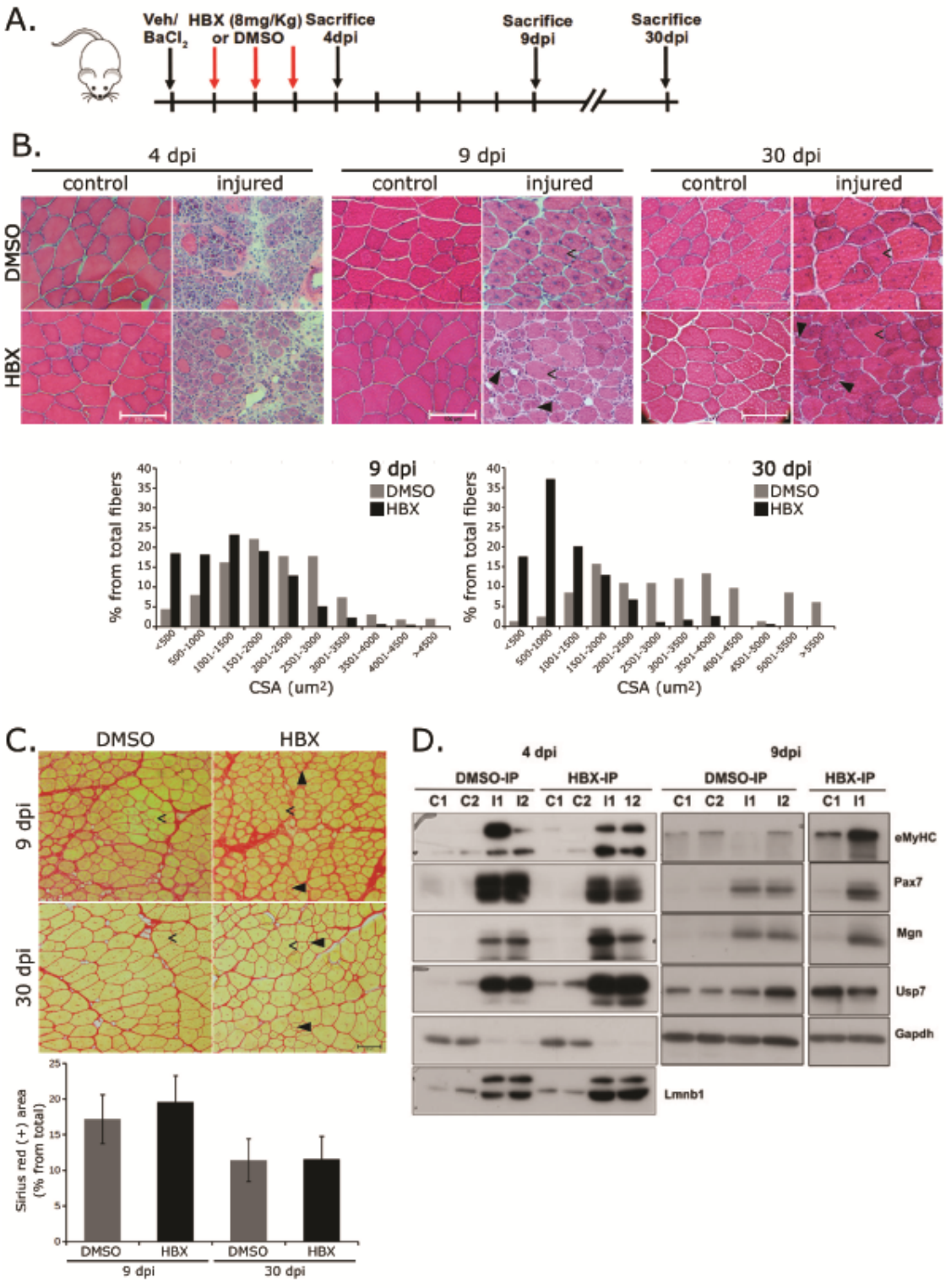
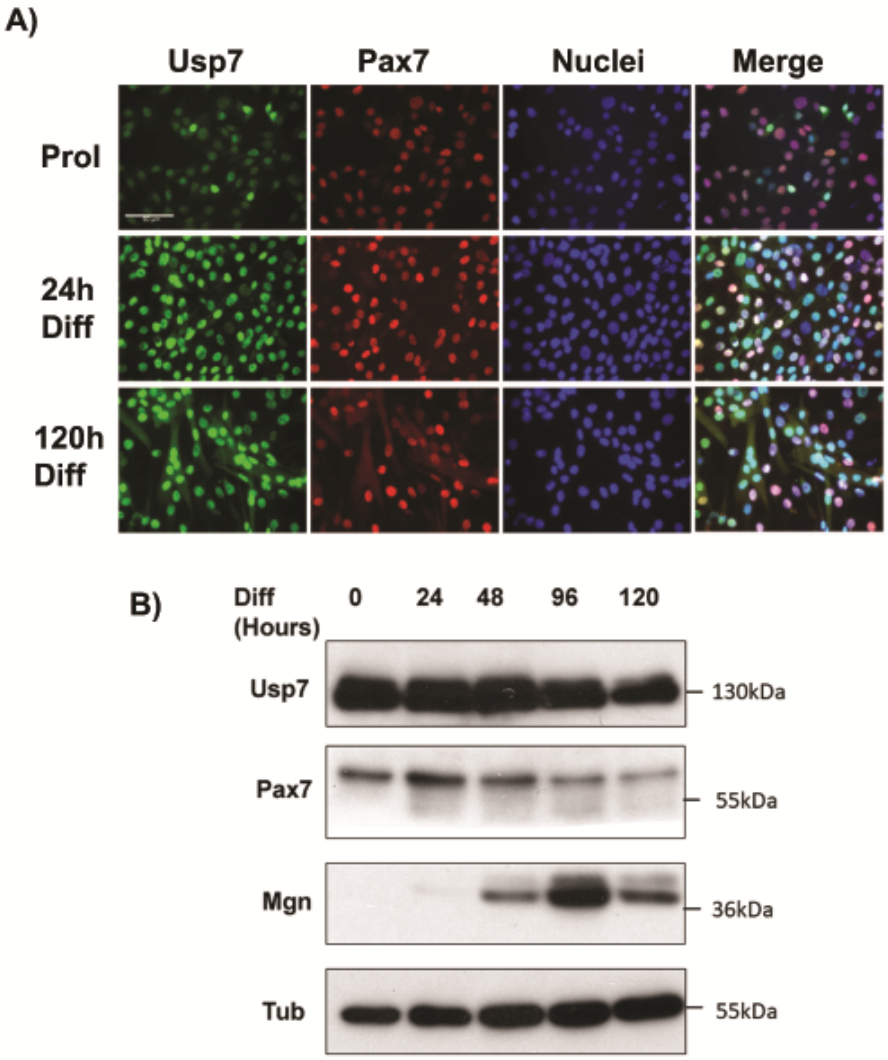

A
IP: Myogenin
IP: Control IgG
D0 D1 D2 D4 D6
120kDa
Usp7
Mgn
35
Inputs
120
B
Danio rerio QYIERLQALVSSLNQQEHE.....SDQGSGSTCCSSPEWSSA
Homo sapiens QYIERLQALLSSLNQEERD.....CS--SHSASCS-PEWGSA
Mus musculus QYIERLQALLSSLNQEERD.....CN--SHSASCS-PEWGNA
Rattus norvegicus QYIERLQALLSSLNQEERD.....CN--SHSASCS-PEWGNA
1
S137A
S170A
224
NH2-
Basic domain
bHLH
-COOH
C
Myc-Usp7
WT
S137A
S170A
S137/ 170A
37kDa
Mgn
130
Myc-Usp7
IP: anti-Myc
37
Tubulin
55
Inputs
D
Mgn- WT
Mgn- S137A
Mgn- S170A
Mgn- S137/ 170A
Ub-Myc
250kDa
70
55
Ub(n)
0.00 0.06 0.03 4.07 0.01
IP: Ub-Myc
WB: Mgn
36
Tub

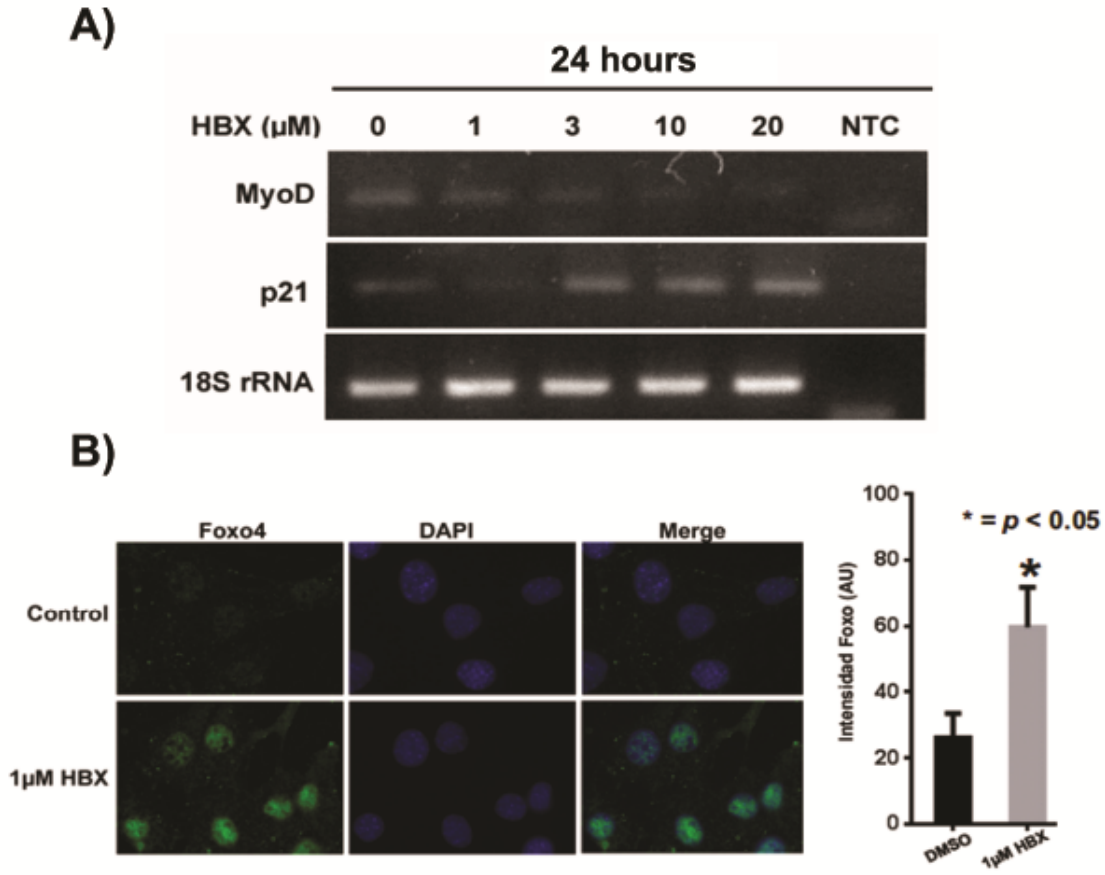
Figure 5:


A.
Veh/ BaCl2
HBX (8mg/Kg) or DMSO
Sacrifice 4dpi
Sacrifice 9dpi
Sacrifice 30dpi
B.
4 dpi
9 dpi
30 dpi
control
injured
DMSO
HBX
9 dpi
30 dpi
% from total fibers
CSA (um2)
C.
DMSO
HBX
9 dpi
30 dpi
Sirius red (+) area (% from total)
D.
4 dpi
9dpi
DMSO-IP
HBX-IP
C1 C2 I1 I2 C1 C2 I1 I2
C1 C2 I1 I2 C1 I1
eMyHC
Pax7
Mgn
Usp7
Gapdh
Lmnb1

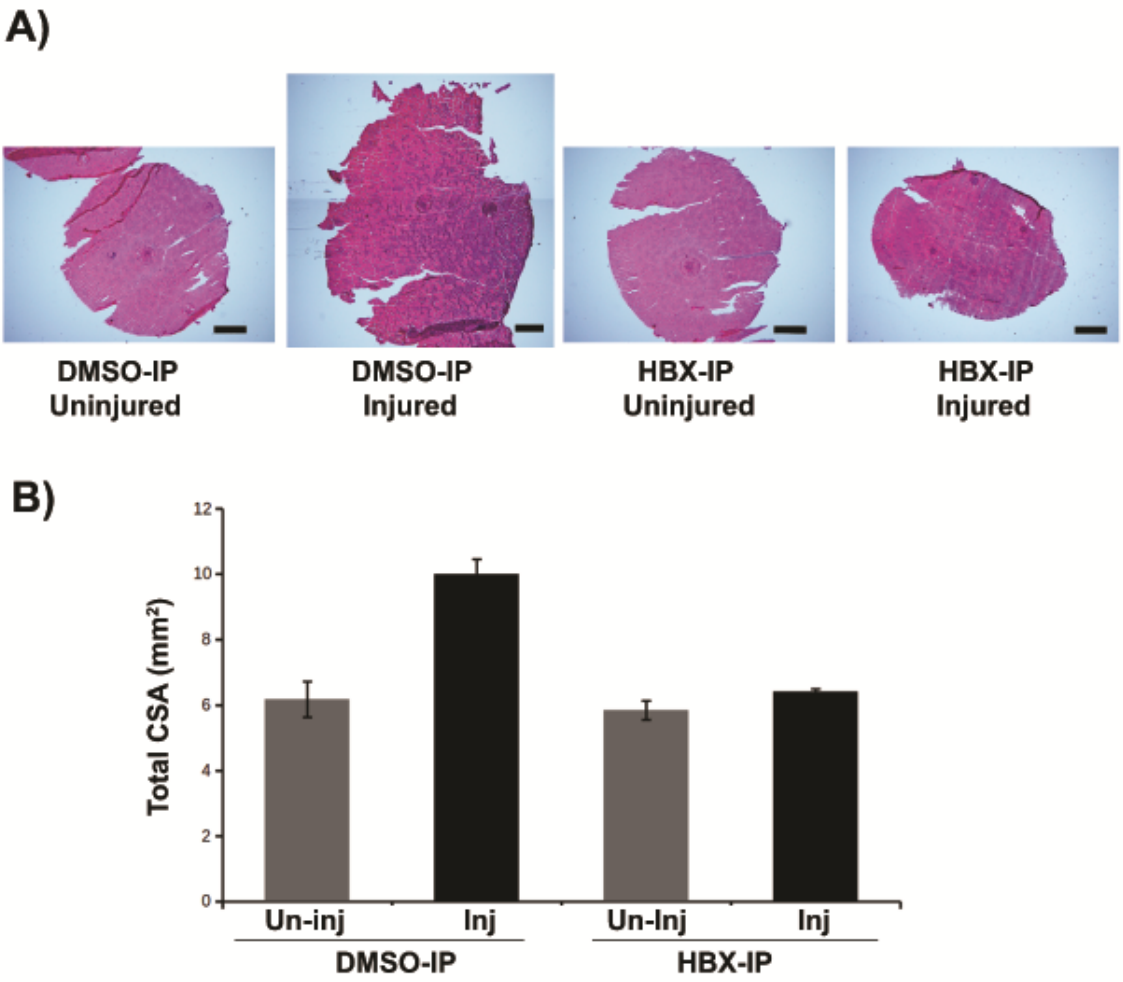
Supplementary figure 1:



Supplementary figure 2:

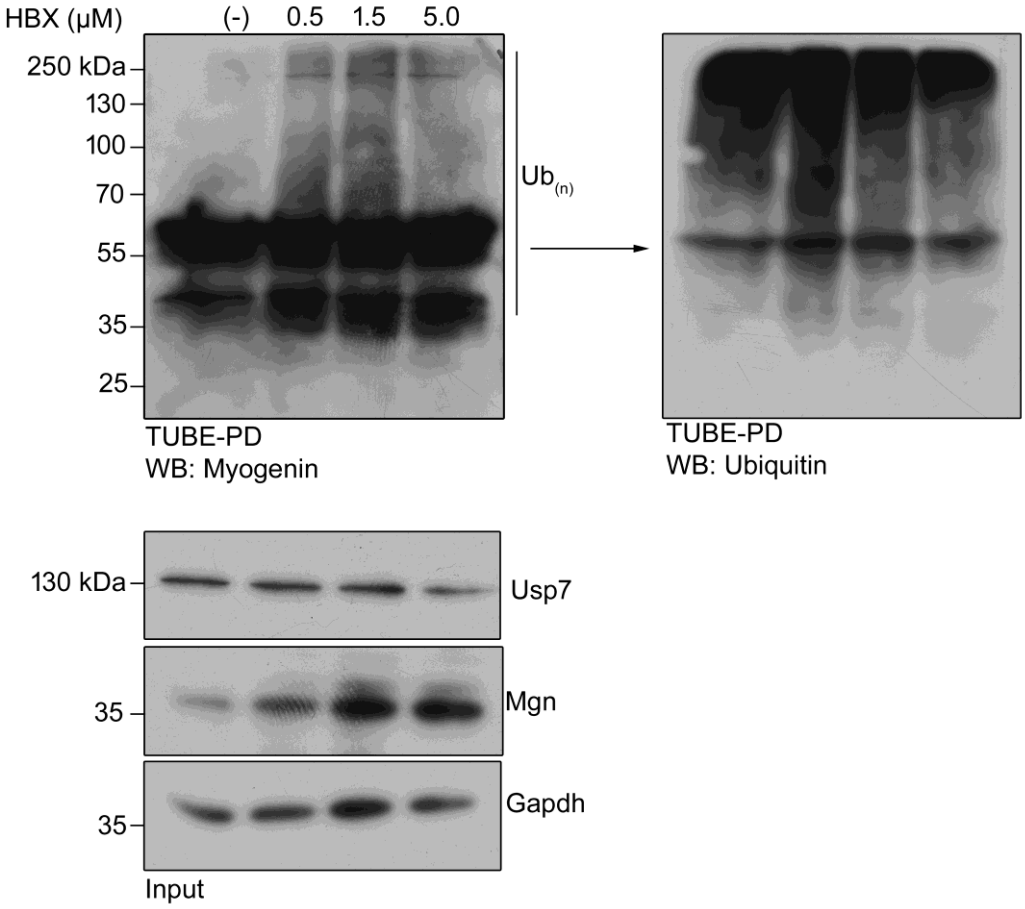


Supplementary figure 3:



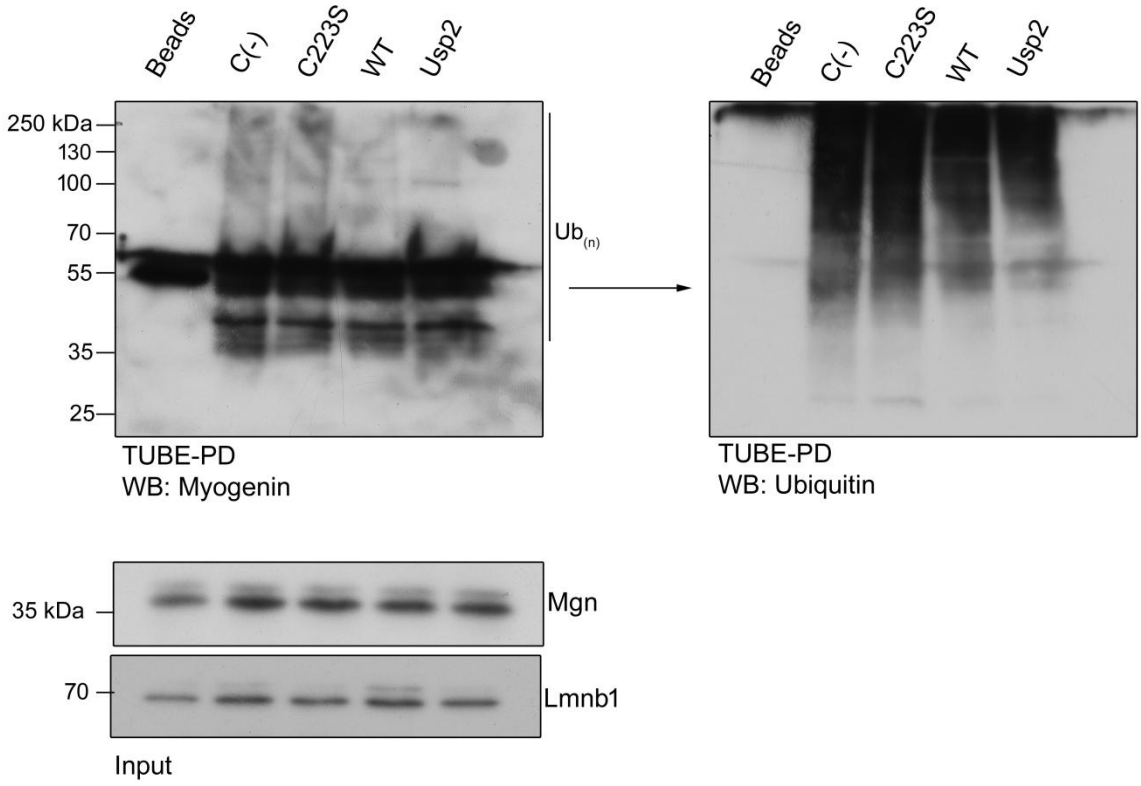
Manuscript annexed figures

Manuscript annexed figure 1:



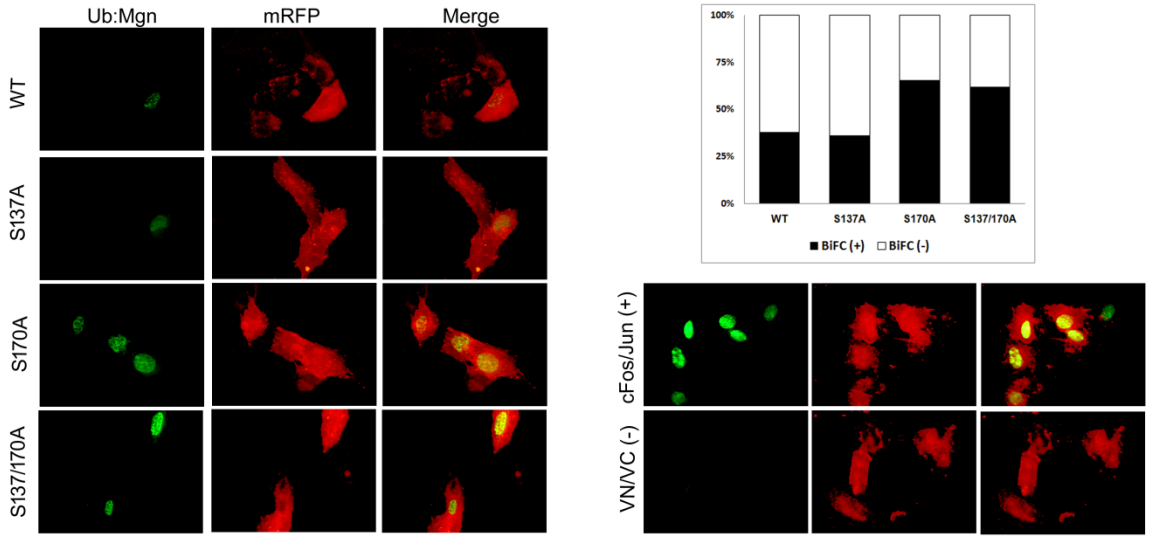
Dose reponse of ubiquitinated myogenin to HBX 41.108: 48 hours differentiated C2C12 were trteted with vehicle or 0.5, 1.5 and 5.0 μM of HBX and 12.5 MG-132 μM for 6 hours prior to lysis**.** Ubiquitinated species rescued with TUBE, were blooted against myogenin. In cells treted with MG-132 only, a barely visible signal of poliubiquitinated myogenin was observed, unless cells were treated with Usp7 inhibitor. At 0.5 and 1.5 μM cells reponse in a dose responde manner to HBX 41.108 treatment. Of note, at 5.0 μM is observed a dcreases in poliubiquitinated myogenin. Interestingly, USP7 at 5.0 μM treatment is also diminished, explaining the fall in ubiquitinated myogenin.

Manuscript annexed figure 2:



Usp7 deubiquitinates myogenin *in vitro*: Accumulated myogenin with HBX 1.5 uM and MG-132 12 uM co-treatment were lysed and separated on 5 different tubes. TUBE pull downed ubiquitinated specied were treated with USP7 WT, USP7 C223S or USP2 when indicated. There are a drastic decrease in polyubiquitinated myogenin on USP7 WT treatment. Also, this depend of catalytic activity of USP7 since catalytic dead mutant (C223S) did not remove polyubiquitin. As control, USP2 -a promiscuous DUB- removes poliubiquitin chains in similar fashion as USP7 WT, demostrating that the signal detected is ubiquitinated myogenin.

Mnuscript annexed figure 3:



Serine 170 is important for myogenin deubiquitination. We identified two putative sites of USP7 interaction with myogenin protein (S137 and S170), corresponding to a conserved P/AXXS region. Myogenin ubiquitination was analyzed as fluorescence complementation of Ubiquitin fused to VC155 venus protein and Ubiquitin fused to VNI152L half of fluorescent Venus protein. If myogenin is ubiquitinated, the Venus protein and fluorescence is restored. In the myogenin S170A and double (S137/170A) mutant increses the fluorescent signal in a ~30%, suggesting that this residue is important for myogenin deubiquitination. Also, the S137A mutant is ubiquitinated smilar to myogenin WT.

Manuscript annexed figures: Methods

Ubiquitin HALO-TUBE pulldown: C2C12 cells were plated at 10,000 cell/cm^2 and induced to differentiation with 5% HS (as described above), during 48 hours. Cells were treted prior to lysis with 12.5µM MG-132 for 6 hours and when indicated 1.5 µM HBX-41.108. Lysis was carried out with TUBE-LB (50mM Tris-Cl: pH 7.5; 1mM EDTA; 1% v/v Triton X-100; 0.27M sucrose; 1X protease and phosphatase inhibitor cocktail, 10mM freshly prepared Iodoacetamide). Lysate was clarified for centrifugation at 14.000 rpm for 15 minutes and transferred to a clean tube. HALO-TUBE were added at 20µL per 1 mg of extract and incubated ON in rotation at 4ºC. Next day, samples were clarify at 4000 rpm for 5 min 4ºC and washed 3 times with TUBE-LB supplemented with 500mM NaCl. When indicated captured ubiquitin were resuspended on DUB-reaction buffer (40mM Tris-Cl: pH 7.6; 5mM DTT; 0.005% v/v BSA) and treated with 25nM of recombinant USP7 WT, USP7 C223S or USP2 for 1.5 hours at 30ºC in waterbath and eluted in 3X Laemmli buffer.

Ubiquitin-mediated Bimolecular Fluorescence Complementation (BiFC): General protocol was performed as described previously (Fang and Kerppola, 2004) with minor modifications. Briefly, cells were transfected with plasmids encoding C-terminus fragment of Venus Fluorescent protein (VC155) fused to myogenin (WT, S137A, S170A and S137/170), cloned from pEMS-rMgn - or bFos- and N-terminus fragment of Venus (VN155-I152L) fused to ubiquitin -or bJun- as specified. Gap43-mRFP was included as a transfection marker. 24 h post transfection, fluorescence was evaluated in living cells, using Motic microscope BA410 Elite trinocular coupled to a refrigerated Moticam pro 252B camera and acquired with Motic

Images Plus 3.0 software. All cells were treated with 12.5 μM MG132 during 6 h prior fixation in 4% PFA.

GENERAL DISCUSSION AND PROJECTIONS:

A novel way to look an old mechanism of post-translational regulation of myogenin.

This doctoral dissertation proposes USP7 as a new regulator of the differentiation of adult muscle progenitors through the stabilization of the MRF myogenin. By using a variety of cellular models we demonstrated that USP7 regulates myogenin deubiquitination during the transition from activated myoblast to committed myocyte (Figure 4D).

In recent years, post-translational regulation of major transcription factors in development/regeneration has gained significant importance. Mostly because rapid regulation of protein levels could function as a rheostat that finely regulates their activity, adding an extra layer of control to the system. Myogenin post-translational regulation has been studied mostly in the context of i) phosphorylation (Li et al., 1992; Tang et al., 2004; Zhou and Olson, 1994) and ii) ubiquitination by the SCF complex (Jogo et al., 2009). Although it is well understood that phosphorylation and dephosphorylation are "two sides of the same regulatory mechanism", the mechanism by which myogenin is rescued from proteasomal degradation has not been described. In this work, we present strong evidence that the deubiquitinase USP7 counterregulate myogenin ubiquitination.

The transcriptional expression of myogenin is tightly regulated through an elegant mechanism involving several chromatin modifications and recruitment of transcriptional factors. Myogenin promoter is structured for two E-Box, a CCGG repeat, a TATA box and binding sites for Pbxb1, Mef2, Mef3. On Satellite Stem cells, myogenin repressive environment is maintained for DNA and Histone methylation. Once SCs are activated, KMT

(lysine methyltransferase) is recruited mediating the dimethylation of histone H3 on K9, which serves as a signal for the recruitment of DNA acetyltransferases and allowing the binding of MyoD and Mef2 into the myogenin promoter (at this point, myogenin promoter is in a poised state). Of note, SWI/SNF complex plays a crucial role in activation of Myogenin promoter by the switch of regulatory subunits of this chromatin remodeling complex. Under permissive conditions to terminal differentiation, myogenin gene is activated by Six and Mef2 binding that allows myogenin promoter demethylation and reinforcing the gene transcription by positive feed-forward loop by myogenin itself (Faralli and Dilworth, 2012). In this context, it is known that Myod is sufficient to trigger the myogenic program, but at late expressed genes, MyoD initiates regional histone modification but is not sufficient to induce gene expression. Based on this statement, myogenin was suggested as a potentiator of MyoD activity (which decays after early differentiation begins), supporting the irreversible nature of the differentiation process enhancing the expression of late differentiation genes (Cao et al., 2006). According to what we know about transcriptional mechanisms and formation/interaction of/with stable chromatin-binding complexes, it is reasonable to speculate that a myogenin critical threshold must be reached before to exert its activity on E-Box promoters. However, myogenin is rapidly degraded by the proteasome when ubiquitinated. A rescuing mechanism is its binding to DNA, in fact, formally shown for MyoD. This raises the question: How does myogenin reach supra-threshold levels in order to perform its function if it is efficiently degraded? As an approximation to this question, we proposed that myogenin ubiquitination should be counter-regulated via deubiquitination. Interestingly, we identified the deubiquitinase enzyme USP7, previously undescribed in satellite cells, which possesses an expression pattern similar to myogenin, being observed its colocalization along the myogenic

differentiation (Figure 1 and 4A). Additionally, by using USP7 knock-down experiments with siRNA, we observed morphological changes on C2C12 myoblasts which did not differentiate to myotubes and molecularly, Myogenin protein levels decreased drastically

Under this model, proteasome-mediated ubiquitination and degradation of myogenin would increase the MyoD/myogenin ratio, leading to terminal differentiation being disturbed in the absence of Usp7. Within this line, Cao et al propose that MyoD and myogenin are located in common gene promoter sites that lead to myogenic differentiation, but showing differential roles on the same gene set. (Cao et al., 2006). Thus, by reducing myogenin levels, their transcriptional targets, or myogenin-regulated proteins such as muscle enhancer factor 2 (MEF2), will be affected. (Ridgeway et al., 2000). MEF2 alone has no myogenic activity, but in combination with bHLH transcription factors, leads to the amplification of the myogenic differentiation program by a positive feed-forward loop mechanism. (Molkentin et al., 1995; Wang et al., 2001). On myogenin promoter, MEF2 allows its self-regulation by increasing myogenin transcriptional rate. In the context of our model, this positive regulation loop of MEF2 activation by myogenin, and the subsequent positive feedback of MEF2 on the transcriptional activity of myogenin, would be drastically affected, if myogenin mass is not sufficient to exert its activity over the activation of genes related to terminal differentiation. This self-regulatory mechanism proposed, dependent on the protein stability of myogenin may partly explain the effects observed in our differentiation results when Usp7 is depleted/inhibited.

Transcriptional and post-transcriptional regulation of Usp7. Delineations of a regulatory mechanism for the expression of Usp7 in satellite cells.

In the context of myogenic differentiation cells subject to myogenic conversion (by forced expression of MyoD), using a TAP-Pax7 protein as bait. Subsequently, Pax7 interacting proteins were identified by Multidimensional Protein Identification Technology (MudPIT). (Bustos et al., 2016) (Annexed Figure 1). Unexpectedly, when validating these results, the direct interaction between purified Usp7 and Pax7 proteins, could not be verified (results not shown). Additionally, in vivo expression of both proteins is inversely correlated (Figure 1C). This observation leaves open the question of how the expression of Usp7 is regulated in this context. By *in silico* analysis of the 5'UTR sequence and upstream regions of the Usp7 gene, we found putative promoter sites for MyoD binding in C2C12 cells. Through MyoD and Pax7 silencing in C2C12 cells, we did not observe a decline in Usp7 levels, as analyzed by total protein (Annexed Figure 2). To date, only one article has explored aspects of transcriptional activation of Usp7. By cloning 1 kb upstream of the Usp7 ORF in a luciferase reporter vector, the authors suggest FoxO6 as the transcription factor that controls expression from Usp7 to FoxO6 on lung tumor cells. (Hu et al., 2015). In satellite cells, although the expression pattern of FoxO6 is unknown, it is possible to infer its function from experiments carried out in C2C12 myoblasts, which propose that FoxO6 works as part of a repressive loop of PCG-1α mutually antagonizing each other, boosting the oxidative metabolism (Hu et al., 2015). Differentiation of myoblasts, amongst other aspects, implies a metabolic change to an oxidative metabolism during early differentiation, including mitochondrial biogenesis and

turnover. (Sin et al., 2016); events temporarily coincident with the expression of Usp7 that we observed (doctoral thesis Jeremy Salas).

On pMBs culture, we observed the expression of Usp7 mRNA correlates directly with the expression of Usp7 protein detected by IFI (Figure 1B). However, in late differentiation conditions (6 days in differentiation medium), this correlation is missed, resulting in a rapid decline in Usp7 protein levels. On page 57 we discussed a possible CK-II-mediated phosphorylation in S18 of USP7 that affects its stability (Khoronenkova et al., 2012), which could explain the rapid drop we observed in its levels by IFI in pMBs. Another possible explanation is a micro RNA regulation (miRNA) over the USP7 mRNA. Although this mechanism of post-transcriptional repression mediated by miRNA is associated with degradation of mRNA by deadenylation (Djuranovic et al., 2014), a few scenarios have been described in which mRNA is translationally repressed with minimal decay of mRNA levels (Fabian et al., 2010), thus maintaining the miRNA mediated translation inhibition. Post-transcriptional regulation of USP7 has been described in tumor cells that mir-205 negatively regulates the translation of USP7 mRNA (Zhu et al., 2015), however, this system has not been demonstrated in myogenic lineage cells.

Myogenin is post-translational modified during muscle differentiation. What is known about the protein turnover of myogenin?

Myogenin is a key MRF in the myogenic differentiation process. Whose function and expression indicate the onset of myogenic commitment of activated myoblasts. Since this important function is required, myogenin must be carefully regulated both at the level of

mRNA expression and at the protein level. Our working model proposes that myogenin must reach a supra-umbral expression/accumulation level to exercise its activity on gene regions with E-box promoters, reinforcing the transcriptional activity initiated by MyoD. (Cao et al., 2006). It is a well-established fact that many transcription factors involved in dynamic cell processes such as cell cycle regulation, checkpoints, apoptosis, exogenous stimulus-response, and differentiation are rapidly and efficiently degraded by the UPS. (Ee and Lehming, 2012; Fuqiang Geng et al., 2013). The stability of myogenin is finely regulated at the level of its stability by ubiquitination by the Skp1/Cullin 1/F-box protein (SCF) complex (Shiraishi et al., 2007), and increased its degradation rate by E3 ligase muscle atrophy F-box (MAFbx) (Jogo et al., 2009), which has been well characterized in atrophy models. Interestingly, TIP120B counter-regulates SCF activity, increasing the stability of myogenin in C2C12 cells committed to the myogenic lineage, without affecting the half-life of MRF MyoD, demonstrating the specificity of the system. (Shiraishi et al., 2007). In our system, the overexpression of Usp7 directly affects the half-life of myogenin, while a reduction of approximately 50% is noted when the catalytic activity is inhibited. In agreement with these results, we observe that under Usp7 knockdown with siRNA and cycloheximide treatment in the presence of HBX 41,108, MyoD total protein levels are not significantly affected. (Figure 2A and 2B and Annexed figure 3). Additionally, and similar to the previously described system (SCF/TIP120B), it has been reported that myogenin is polyubiquitinated by E3 ligase von Hippel-Lindeau (VHL) and destinated to proteasomal degradation; in this work it was demonstrated that prolyl hydroxylase EGLN3 overexpression has a positive effect on myogenin stability, although the exact mechanism is not established. It is believed that the interaction of myogenin during early

differentiation with EGLN3 (or protein 4.1), is a steric impediment for the interaction with VHL (Fu et al., 2007; Huang et al., 2016).

In this doctoral thesis, we show evidence that USP7 directly regulates the stability of myogenin, through several approximations. As part of our results, the accumulation of myogenin protein is dramatically reduced when one of P/AXXS interaction residues with USP7 is mutated in the myogenin sequence, reinforcing our model of proteasomal rescuing degradation by USP7 (Figure 4C y 4D). Taken together, these findings do not exclude the existence of synergistic mechanisms that may operate on the regulation of myogenin stability in the context of muscle regeneration, which makes USP7 an interesting candidate to continue dissecting its involvement in detail during myogenic progression.

Other possible regulations by USP7 in the myogenic context: What options remain to be explored?

USP7 is a DUB with a wide variety of substrates, not only participating in the rescue of proteins subject to proteasomal degradation. Ciechanover's group described that the transcription polycomb repressor complex is directly regulated by USP7 by de-ubiquitinating its catalytic subunit RING1B (de Bie et al., 2010), facilitating the transfer of ubiquitin in the K119 residue of histone H2A. (Wang et al., 2004). RING1B is activated by ubiquitination via heterotypic K6, K27 and K48 chains in the C-terminal region of the RING domain.USP7 activity specifically targets the homotypic K48 chains (typical mark of destination to proteasomal degradation) on the same region of RING1B, increasing the stability of this subunit (de Bie et al., 2010). Moreover, using ChIP-Seq technology, it was shown that USP7 and RING1B are located to sites in the genome which strongly correlate with gene silencing

elements (Lecona et al., 2015). On the other hand, USP7 is also associated with the direct modification of histone H2B, thereby removing this activating mark for transcription (Van Der Knaap et al., 2005), supporting the rationale that USP7 could play a direct role in gene silencing. Preliminary results from our laboratory support this idea, in which the pharmacological inhibition of USP7 in C2C12 cells under proliferation conditions during 48 hours showed an increased accumulation of total mRNA (Annexed Figure 4). In the context of muscle regeneration, has been established that Pax7 locus exhibits a bivalent chromatin mark of H3-K27^{3me} (repressive) and H4-K4^{3me} (activator). Upon activation of the TNF-α/p38 proinflammatory signaling pathway, the polycomb subunit RING1B is activated mediating the transcriptional repression of Pax7, thus allowing the myogenic progression to an activated satellite cell (Mozzetta et al., 2011; Palacios et al., 2010). Considering these antecedents, it seems plausible to study USP7 and its regulation/occupation with the polycomb complex during myogenic progression and its implications in the transition from activated myoblast to compromised myoblast. These mechanisms seem to be critical in stem cell biology in general: differentiation genes need to be repressed in order to maintain the stem cell character, but upon differentiation cues, stem cell-genes, need to be silenced in order to promote entry to cell cycle and differentiation progression.

In other contexts, it has been observed that USP7 is not only restricted to its nuclear location but that activity of this DUB has been described in the cytoplasm and associated with membrane proteins. Thus, USP7 has been described as a positive regulator of insulin/insulin-like growth factor (IGF-1) signaling by stabilizing insulin receptor 2 (IRS-2) in several cell lines, including L6 myoblasts, with an increase in downstream signaling by PI3K (Yoshihara et al., 2012) and in pro-apoptotic signaling pathways mediated by TNF-α, through the

stabilization of RIP1 (receptor interacting protein 1) by the TRIM27/USP7 protein complex. (Zaman et al., 2013). Finally, USP7 is also described as a negative regulator of NF-κB signaling triggered by TNF-α, through interaction with HSCARG that mediates NEMO deubiquitination reducing the proteasomal degradation of the inhibitor IκBα on this pathway. (Li et al., 2014). In the myogenic context, NF-κB was described as an important regulator downstream of TWEAK (Tumor necrosis factor (TNF)-like weak inducer of apoptosis) extrinsic signaling in satellite cells, showing that TWEAK depletion has a beneficial effect in maintaining the PAX7 (+) cells (Ogura et al., 2013), constituting an important approach to increase the satellite cell pool in several muscle pathologies, by pharmacological inhibition of TWEAK.

In our work, we have considered USP7 as a nuclear location protein for all our analyses, given its predominant nuclear location. This does not exclude that in the myogenic context it could fulfill important regulatory functions in the context of myogenesis, in particular in the metabolic shift associated with the mitochondrial fraction (*preliminary results thesis J. Salas*) and in the myogenic progression regulating membrane proteins. A plausible way to tackle these unknown regulations is to perform a mass spectrometry-based proteome-wide analysis of ubiquitinome and interacting proteins in order to reveal new USP7 substrates in the myogenic context, likewise showed recently for USP14 (Liu et al., 2018).

From our results, we observe that USP7 also strongly colocalizes with the mononuclear infiltrate during days 2 and 3 following damage induction (Figure 1D). This observation leaves open the question of: What is the regulatory function of USP7 in this infiltrate? What cell types in the myogenic niche express USP7 and what is their contribution during *in vivo* regeneration? One of the proposals is that USP7 is differentially expressed in M1/M2

macrophages during regeneration, given that based on our results, we found that the detection of USP7 is restricted to the inflammatory phase of regeneration and is not detected in other cells that are not associated with myofibres. Our methodology to inhibit the catalytic activity of USP7 in vivo was through intraperitoneal injection of HBX 41,108. This method, although not specific on satellite cells, orients us to USP7 plays a functionally relevant role in the regenerative process (Figure 5). In the laboratory, we now have a specific conditional knock-out model in satellite cells and a floxed USP7 strain, which would allow us to investigate in depth the function/role of USP7 specifically in the satellite cell population during muscle regeneration, avoiding off-target effects on other cell types (e.g. macrophages M1 and/or M2). Combined with the pharmacological inhibition strategy, it would be possible to delineate the USP7 requirement for different cell population during the regenerative process.

Biomedical projections of USP7 function in adult muscle progenitors:

The understanding of the mechanisms that regulate the transition from myoblasts to myocytes (activated satellite cell to cell committed to myogenic lineage), is of vital importance in the comprehension of the phenomena involved in differentiation, growth and muscle disease. Therefore, with the knowledge generated in this doctoral thesis, considering the current challenges in the study of satellite cell biology, our results deliver important projections with implications in biomedical practice and regenerative medicine.

The main muscle groups represent about 30-40% of the total weight of an adult person and are defined during embryonic development and perinatal growth. Likewise, the skeletal muscle has the capacity to increase its mass by physiological processes of hyperplasia and

hypertrophy (Bentzinger et al., 2012; Guttridge, 2004). Conversely, muscle mass decreases during the physiological course of aging and in a wide variety of myodegenerative diseases, sharing as a common characteristic the loss of the net balance in the synthesis and degradation of muscle proteins.

USP7 has been extensively studied in cancer mainly due to its role in regulating the stability of the MDM2/p53 complex (Hu et al., 2006; Kon et al., 2010; Li et al., 2004; Sheng et al., 2006) which has led to the development and rational design of new and specific drugs directed against its catalytic activity. (Desroses and Altun, 2017; Zhou et al., 2018). As one of the most promising the XL188 drug, which demonstrates in vitro inhibition of only USP7 (compared to 40 other DUBs) at concentrations of 0.09μM and presenting minimal interaction with USP47 (Lamberto et al., 2017). This emergent pharmacological development places USP7 as a promising druggable target with great potential for the treatment of musculoskeletal pathologies (discussed below).

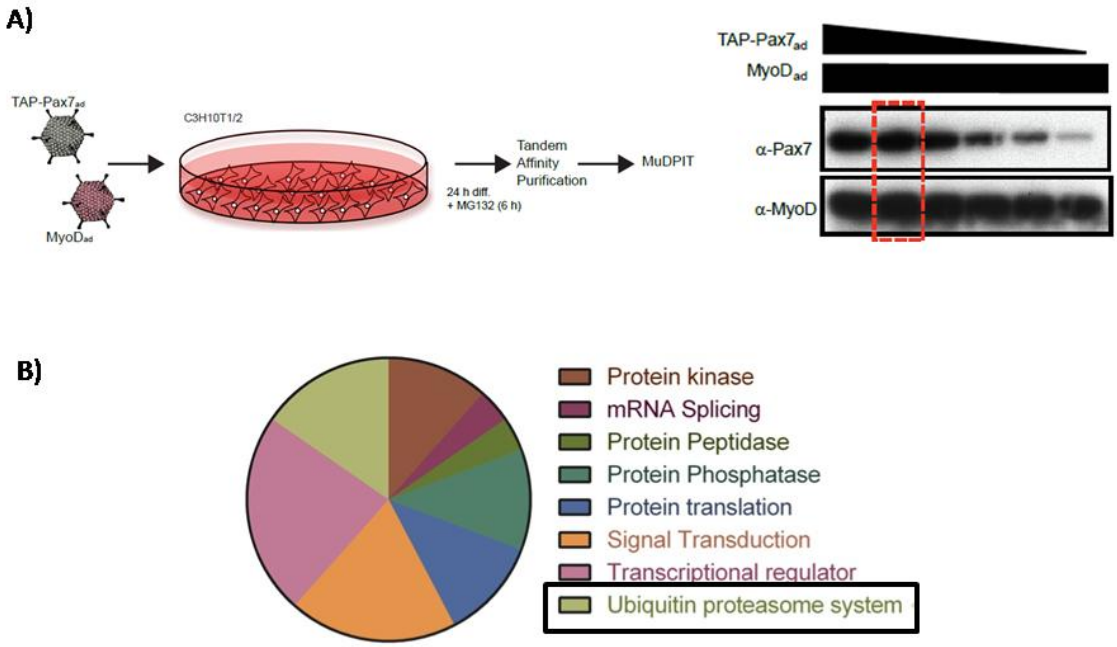
On the other hand, it is well known that imbalances in the UPS in muscle tissue leads to the presentation of muscular atrophy, caused by an increase in the activity of E3 ligases such as MURF, Atrogin and/or MAFbx. (Lecker, 2003; Wing, 2013). In our model, we established that USP7 rescues myogenin from proteasomal degradation. MAFbx is one of the E3 ligases overactivated in muscular atrophy that catalyzes the transfer of ubiquitin into myogenin, enhancing its degradation and preventing adequate myogenic differentiation. (Jogo et al., 2009). From this standpoint, the overexpression of USP7 rescues this effect on myogenin, suggesting the development of possible drugs that stimulate the catalytic activity of USP7. The most challenging aspect of this proposal is the ubiquitous expression of USP7 at the tissue level and its great promiscuity in substrate recognition, which can signify a great

difficulty when systemically administering a drug. Recent advances in drug delivery with specificcally targeted nanoparticles in skeletal muscle could improve and bypass this drawback problem (Bibee et al., 2014; Nimbalkar et al., 2018; Ungerleider et al., 2018).

Currently, satellite cell-based transplantation therapy is one of the most promising theoretical approaches for the treatment of degenerative skeletal muscle diseases (Bentzinger et al., 2013). From this perspective, the pipeline that is being developed is aimed at the *ex vivo* expansion of muscle progenitors (or derived cells), further editing of the defect if necessary and its subsequent reincorporation into the damaged tissue in the satellite cell niche, allowing a new source of satellite cells that will repair muscle tissue. One of the challenges is to maintain the cells *ex vivo* from differentiating. From This perspective, USP7 inhibition could be used to maintain the population as progenitors, extending their myogenic potential and successful incorporation into the satellite cell niche upon transplantation.

Annexed figures and legends:

Annexed Figure 1

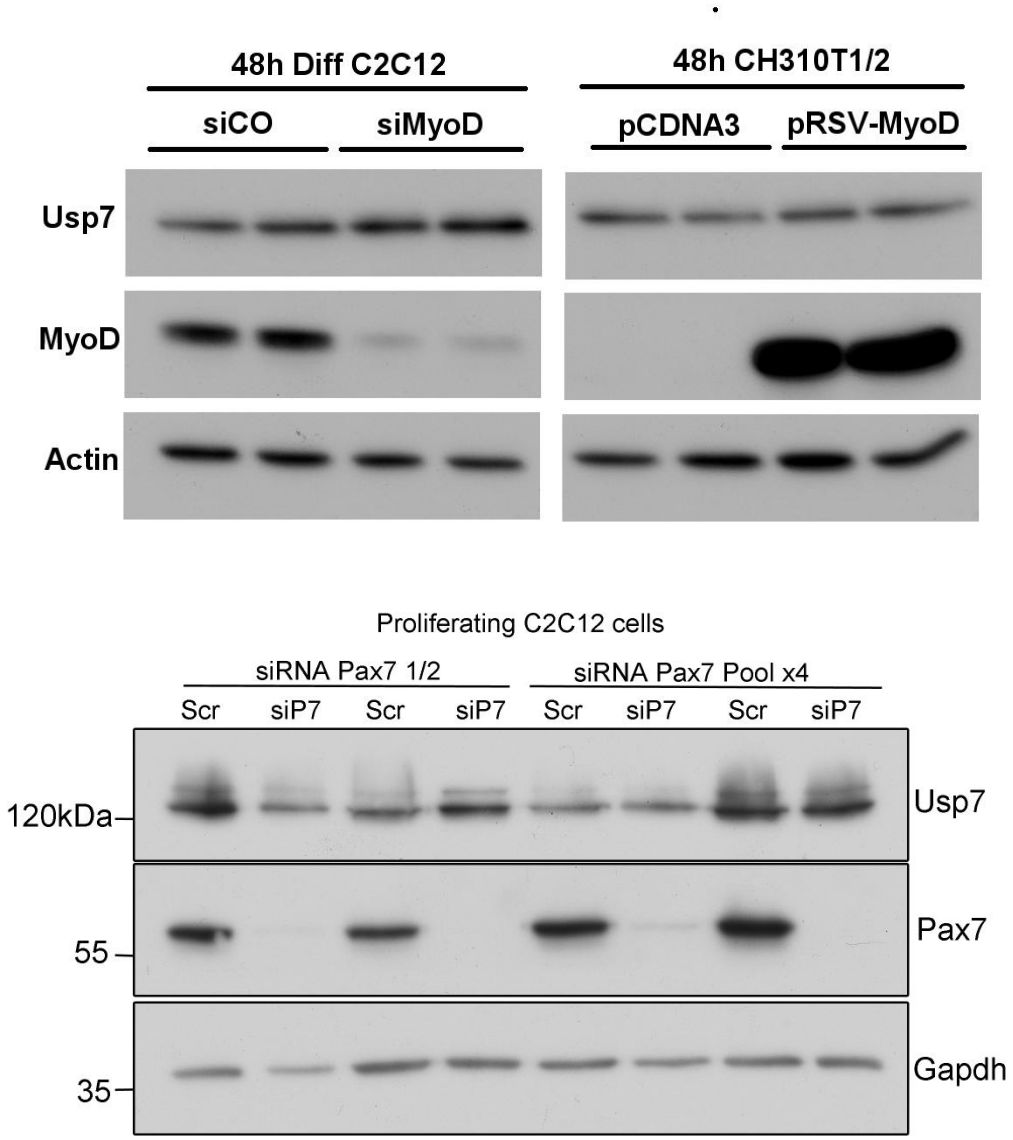


Selected Interacting Pax7 proteins identified by MuDPIT

Abbreviature	Protein name	Gene ID
CTNNd1	Catenin delta	12388
NPM1	Nucleophosmin	18148
NCOR2/SMRT	Nuclear receptor corepressor 2	20602
TBL1XR1	F-box-like/WD-repeat protein	81004
HMBG1	high mobility group box 1	15289
ARID4B	AT rich interactive domain 4B (RBP1-like)	94246
YWHAZ	14-3-3 protein zeta/delta	22631
YWHAB	14-3-3 protein beta/alpha	54401
YWHAG	14-3-3 protein gamma	22628
YWHAE	14-3-3 protein epsilon	22627
MyoD*	Myoblast determination factor1	17927
Uba1	Ubiquitin-like modifier activating enzyme 1	22201
Usp7	Ubiquitin specific peptidase 7	252870
Psma3	Proteasome (prosome, macropain) subunit, alpha type 3	19167
Nedd4	Nedd4 neural precursor cell expressed, developmentally down-regulated 4	17999

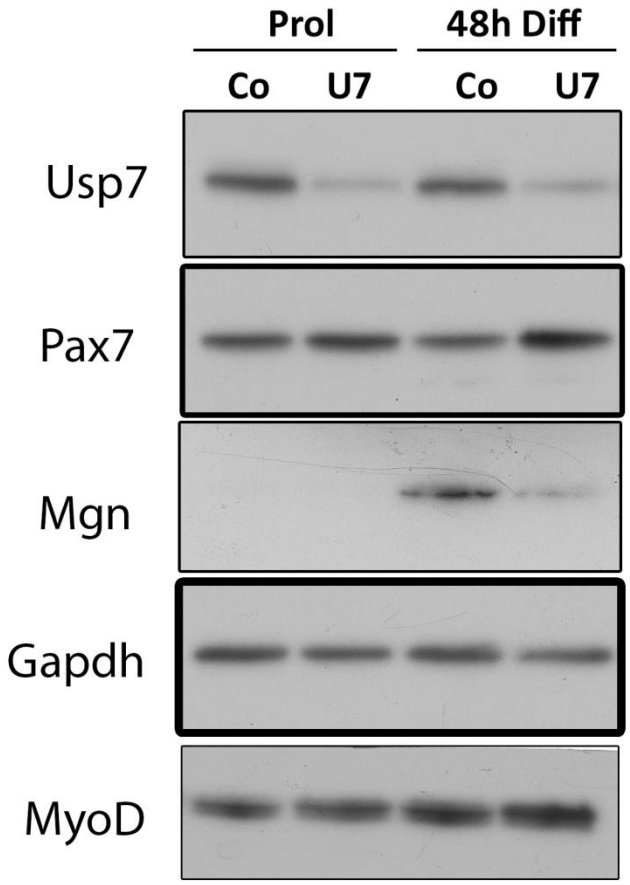
Pax7 interacts with UPS-related proteins during myogenesis.
(A) Overall strategy to identify proteins that interact with Pax7 by mass spectrometry. Approximate equimolar levels of Pax7 and MyoD were checked by Western Blot (right panel, highlighted box).
(B) The classification according to function of the proteins interacting with Pax7 is shown. In the table below, the candidate proteins with the best score are detailed. Note that as already reported, the interaction of Pax7 with MyoD was detected in this analysis (*). In the highlighted box are noted the proteins benlonging as components of the UPS. Note that Usp7 is detected.

Annexed figure 2:



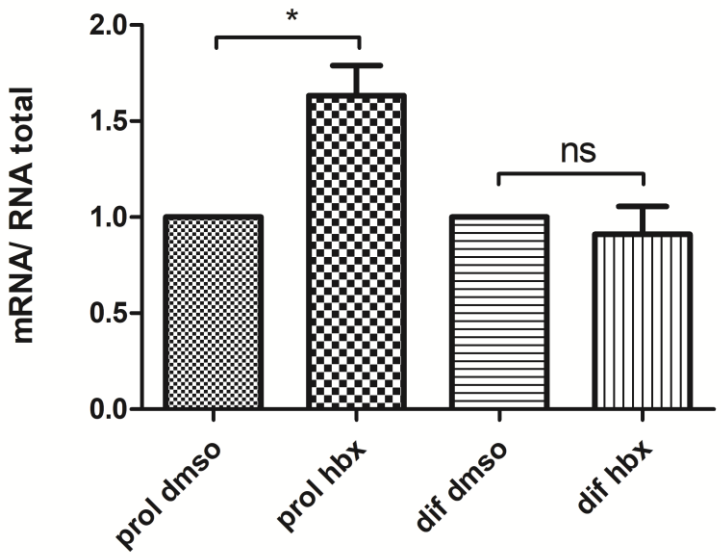
Effect of Pax7 and MyoD silencing on USP7 expression. *In silico* analysis of the USP7 promoter sequence revealed putative binding sites in DNA for MyoD. MyoD gain and loss of function experiment was performed and USP7 protein levels were analyzed, remaining unchanged (Top Panel). On the other hand, we decided to check if the expression of Pax7 is necessary for the expression of USP7, to do so we silenced Pax7 with siRNA in C2C12 cells. There were no changes in the relative protein levels of USP7. (Lower Panel).

Annexed Figure 3:



Loss of function of USP7 does not affect MyoD protein levels. C2C12 cells were subjected to silencing with siRNA directed against USP7. The protein levels of myogenin, Pax7 and MyoD were analyzed. Under these conditions a drastic decline in myogenin protein levels is observed. Whereas when observing the levels of MyoD and Pax7, a slight increase in protein levels can be observed. These results, together with those presented above, support the idea of delay/arrest in differentiation that we observe both *in vitro* and *in vivo*.

Annexed figure 4:



Total mRNA increses when USP7 is inhibited in proliferating cells: C2C12 cells were treated for 48 hours with the USP7 inhibitor HBX 41.108 on proliferating and differentiating conditions. Total mRNAs were notably incresed on prliferating conditions compared to vehicle control. Interestingly, in differentiation the total mRNA (D.O. quantified) did not change compared to vehicle. Of note, the identity of differentiation genes was modified, with a decrease on myogenin target genes (data not shown, Natasha Blanco thesis).

Concluding remarks:

- USP7 desubiquitinase enzyme is expressed in muscle satellite cells and interacts with myogenin during early differentiation of adult muscle precursors.
- USP7 stabilizes protein levels of myogenin through its catalytic activity in C2C12 myoblasts during early differentiation.
- Serine 170 (within motif 166-ASCS-171) of myogenin appears to be important for its protein level stability and a potential site of interaction with USP7.
- USP7 deubiquitinates myogenin *in vitro* and *in vivo*.
- USP7 is necessary for muscle differentiation of C2C12 myoblasts *in vitro* and for muscle regeneration *in vivo*.
- The function of USP7 as a regulator of myogenin levels promotes muscle differentiation of adult muscle progenitors.

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